Induction of Transmembrane Proton Transfer by Mercurials in Mitochondria

II. RELEASE OF A NA⁺/K⁺ IONOPHORE*

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SUMMARY

The induction of energized proton release by mercurials in beef heart mitochondria appears to be mediated through the “activation” of an endogenous mitochondrial Na⁺/K⁺ ionophore. The effects of mercurials on mitochondria can be duplicated by the addition in their place of either purified mitochondrial Na⁺/K⁺ ionophore or a classical ionophorous species (e.g., gramicidin). Mitochondria which are submitted in turn to each of these three experimentally defined conditions acquire the capacity not only for energized proton release, but also for nonenergized proton uptake, nonenergized swelling in the presence of 0.10 M potassium or sodium chloride or nitrate in a sucrose-free medium, active transport of sodium or potassium acetate, and increased succinate-dependent respiration. While such an extensive spectrum of near identical agent-activity relationships has led us to adopt the present thesis, more directly in support of the latter is the observation that the yield of ethanol-extractable Na⁺/K⁺ ionophore from mitochondria is consistently increased upon pretreatment of mitochondria with mercurials such as fluorescein mercuric acetate.

EXPERIMENTAL PROCEDURE

The details for the following preparative and analytical procedures have already been described by Southard et al. (1): preparation of beef heart mitochondria, changes in the concentration of protons and potassium ions, and composition of the reaction media used for the measurement of proton and potassium ion concentrations. Mitochondrial volume changes were measured by monitoring the absorbancy change at 520 nm of a suspension of mitochondria (0.17 mg of protein per ml) in a Beckman DK-2 spectrophotometer. This method is similar to that described by Blondin and Green (2), and the conditions of the experiment are given in the legends to the figures. The rate of oxygen utilization was measured with a Beckman oxygen electrode and oxygen analyzer in the same reaction media used to study changes in either proton or potassium ion concentration; rate studies were carried out at 30° except where otherwise indicated. A peptide ionophore specific for monovalent cations was isolated from beef heart mitochondria by the method of Blondin et al. (3) or by a modification of the original procedure (4). The mitochondrial Na⁺/K⁺ ionophore was dissolved and concentrated in ethanol to a level which, when added to mitochondrial suspensions (as 20- to 40-μl aliquots), would induce ion movements similar in magnitude to those observed upon addition of gramicidin at concentrations indicated in the legends to tables.

Studies aimed at describing the effect of mercurials on the yield of ethanol-extractable Na⁺/K⁺ ionophore from mitochondria were carried out as follows. Freshly prepared heavy beef heart mitochondria (200 ml at a mitochondrial protein concentration of 50 mg per ml) in 20 mM Tris-Cl (pH 7.4) were incubated for 15 min at 25° in the presence or absence of 0.48 mM potassium fluorescein mercuric acetate. The suspensions were then freeze-dried and the residues extracted twice for 90 min with 300-ml portions of absolute ethanol. The combined extracts derived after filtration through a medium porosity glass filter were evaporated to dryness, and the chloroform-soluble residues were charged onto columns (29 × 3.0 cm outer diameter) containing 40 g of oven-dried (120° for 12 hours) silicic acid (Bio-Sil A) layered over 40 g of neutral alumina (Woelm W200 containing 4% by weight of water) in chloroform. Fractions (10 to 15 ml) were collected during elution with (a) 150 ml of chloroform; (b) 150 ml of chloroform-meth-
Table I

Summary of the enzymic capabilities induced by gramicidin, fluorescein mercuric acetate, and the mitochondrial Na+/K+ ionophore

Ion movements (proton or potassium), optical density change (after 5 min), and respiration rates were measured as described under "Experimental Procedure." Gramicidin D was added to a level of 1 µg per ml except for the swelling experiments where it was added to a level of 10 µg per ml. Fluorescein mercuric acetate was added to a level of 6 to 10 nmoles per mg of protein, and 20 to 40 µl of an ethanolic solution of the mitochondrial Na+/K+ ionophore was added as described under "Experimental Procedure." The abbreviations used are: O.D., optical density; TMPD, tetramethylphenylenediamine.

<table>
<thead>
<tr>
<th></th>
<th>Energized proton release</th>
<th>Energized potassium uptake</th>
<th>Swelling in potassium chloride</th>
<th>Swelling in sodium chloride</th>
<th>Respiration in presence of succinate + TMPD</th>
<th>Respiration in presence of ascorbate + MgATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.0-5.0</td>
<td>55-80</td>
<td>0.8-1.0</td>
<td>0.6-1.0</td>
<td>75-90</td>
<td>380-400</td>
</tr>
<tr>
<td>Gramicidin</td>
<td>31.0-36.2</td>
<td>850-1960</td>
<td>3.0-3.4</td>
<td>3.8-4.2</td>
<td>188-200</td>
<td>540-500</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>33.2-37.7</td>
<td>310-670</td>
<td>3.5-3.6</td>
<td>3.2-3.5</td>
<td>130-150</td>
<td>590-620</td>
</tr>
<tr>
<td>Na+/K+ ionophore</td>
<td>26.3-32.7</td>
<td>300-320</td>
<td>2.2-2.5</td>
<td>2.8-3.0</td>
<td>150-160</td>
<td>540-560</td>
</tr>
</tbody>
</table>

Fig. 1. Fluorescein mercuric acetate-induced nonenergized swelling in potassium chloride or sodium chloride. Mitochondria were suspended in 0.1 M potassium chloride or 0.1 M sodium chloride, buffered with 10 mM Tris-Cl (pH 7.4), and the absorbance was measured as described under "Experimental Procedure." Where indicated, Tris-fluorescein mercuric acetate (FMA) was added at a level of 10 nmoles per mg of protein. The results obtained in both sodium and potassium chloride media in the absence of fluorescein mercuric acetate are described by the CONTROL line.

TABLE II

<table>
<thead>
<tr>
<th></th>
<th>Energized proton release</th>
<th>Energized potassium uptake</th>
<th>Swelling in potassium chloride</th>
<th>Swelling in sodium chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles proton released/mg protein</td>
<td>nmoles potassium taken up/mg protein/min</td>
<td>O.D. changes during swelling X 100</td>
<td>O.D. changes during swelling X 100</td>
</tr>
<tr>
<td>Control</td>
<td>3.0-5.0</td>
<td>55-80</td>
<td>0.8-1.0</td>
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</tr>
</tbody>
</table>

Spectrum of Enzymic Capabilities Induced in Beef Heart Mitochondria by Fluorescein Mercuric Acetate

Mitochondria exposed to fluorescein mercuric acetate (6 to 10 nmoles per mg protein) exhibit several emergent enzymic capabilities: two of which have already been described, namely nonenergized potassium uptake or energized proton ejection, and active transport of potassium acetate (1), and three which are documented here, namely, nonenergized swelling in the presence of potassium or sodium chloride (see Table I and Fig. 1), active transport of sodium acetate (see Table II), and increased respiration (see Table I and Fig. 2). All of these capabilities require the presence of an appropriate monovalent cation. Table I contains a summary of the data documenting the induction by fluorescein mercuric acetate (and other reagents) of the following enzymic capabilities: energized proton ejection (first column), energized uptake of potassium ions (second column), nonenergized swelling in presence of either potassium chloride (third column) or sodium chloride (fourth column), and increased respiration in presence of either succinate (fifth column) or ascorbate plus tetramethylphenylenediamine (sixth column).

Duplication of Fluorescein Mercuric Acetate-induced Effects with Gramicidin D—Gramicidin D is an ionophore of bacterial origin specific for monovalent ions; notably sodium and potassium (7). At a concentration of 1 to 10 µg per ml, gramicidin can induce in beef heart mitochondria the same enzymic capabilities as does the mercurial (see Table I). The magnitude of...
of these capabilities is much the same whether induced by fluorescein mercuric acetate or by gramicidin except for the rate of active transport of potassium, which is greater in the presence of gramicidin. Since there is more cycling of potassium in the presence of the mercurial (1) than in the presence of gramicidin, this difference can be readily explained.

Other ionophores of bacterial origin specific for monovalent cations, such as valinomycin (8), can substitute for gramicidin in respect to inducing these enzymic capabilities (data not shown). This possibility of substitution suggests that the induction by gramicidin is not specific for a particular ionophore but rather is characteristic for ionophores generally that are specific for monovalent cations.

Each of the enzymic capabilities shown here to be induced by fluorescein mercuric acetate or by gramicidin except for the rate of active transport of potassium, which is greater in the presence of gramicidin. Since there is more cycling of potassium in the presence of the mercurial (1) than in the presence of gramicidin, this difference can be readily explained.

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Each of the enzymic capabilities shown here to be induced by gramicidin has already been reported in the literature by several investigators (9-11). Our intent in the context of the present study is not to represent these as novel effects but rather to compare the inducing patterns for fluorescein mercuric acetate and gramicidin, respectively. The data in Table I provide the evidence that the mitochondrial Na+/K+ ionophore shares with fluorescein mercuric acetate and gramicidin the same induction pattern. More significantly related to the present thesis are the observations shown in Table II that the sodium-potassium ionic specificities of the fluorescein mercuric acetate and mitochondrial Na+/K+ ionophore-induced active transport run closely parallel and are both characteristically different from that of either gramicidin D or valinomycin.

Effect of Fluorescein Mercuric Acetate on Yield of Mitochondrial Na+/K+ Ionophore—When freshly prepared, freeze-dried mitochondria are extracted with ethanol; the yield of Na+/K+ ionophore observed after purification is consistently larger than that observed by other investigators (1). The approximately 30-fold increase in sensitivity of our presently described assay system now allows us to detect the mitochondrial Na+/K+ ionophore only in approximately 10% of our experiments. The actual yield was, therefore, based solely on data obtained from successful experiments. The approximately 30-fold increase in sensitivity of our presently described assay system now allows us to detect the mitochondrial Na+/K+ ionophore with much greater regularity (>95%) in the normal lower range of its availability from freshly prepared beef heart mitochondria. We are indeed still unable to account for the relatively few instances (1 in 25) of unusually high yields of Na+/K+ ionophore obtained in absence of mercurial. However, since the latter yields represent such a significant departure from the norm in both quantity and frequency, we believe that the range of values quoted in Table III more accurately reflect the average yield of mitochondrial Na+/K+ ionophore under control conditions.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Sodium</th>
<th>Potassium</th>
<th>Na+/K+ ratio x 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein mercuric acetate</td>
<td>61</td>
<td>566</td>
<td>1.08</td>
</tr>
<tr>
<td>Na+/K+ ionophore</td>
<td>84</td>
<td>612</td>
<td>1.37</td>
</tr>
<tr>
<td>Gramicidin D</td>
<td>368</td>
<td>998</td>
<td>3.69</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>10</td>
<td>600</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Blondin et al. (3, 4) have described the isolation and purification of a peptide ionophore from beef heart mitochondria which is specific for monovalent cations. A purified sample of this peptide was tested for its capacity to induce in beef heart mitochondria the same enzymic capabilities as were induced by fluorescein mercuric acetate and gramicidin, respectively. The data in Table I provide the evidence that the mitochondrial Na+/K+ ionophore shares with fluorescein mercuric acetate and gramicidin the same induction pattern. More significantly related to the present thesis are the observations shown in Table II that the sodium-potassium ionic specificities of the fluorescein mercuric acetate and mitochondrial Na+/K+ ionophore-induced active transport run closely parallel and are both characteristically different from that of either gramicidin D or valinomycin.

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Table II

**Stimulation by fluorescein mercuric acetate, mitochondrial Na+/K+ ionophore, valinomycin, and gramicidin D of the rate of active accumulation of sodium and potassium acetate by mitochondria**

Ion sensitive electrodes were used to follow the rate of accumulation of potassium (Beckman cationic electrode No. 39137) or of sodium (Beckman sodium ion electrode No. 39278) ions as described previously (1). The reaction media have also been described (1) and contained either 4 mM potassium or sodium acetate. Fluorescein mercuric acetate was added as the Tris salt at a concentration of 10 nmoles per mg of protein, while valinomycin and gramicidin D were each added to a final concentration of 1 μg per mg of protein.
show, in addition, that the increase in yield is on the order of a 10-fold enhancement which is also the observed order of magnitude at which the mercurial is able to stimulate active transport of potassium acetate in intact mitochondria (Table III). It should also be recognized, in the context of the results shown in Table III, that efforts to identify either fluorescein mercurec acetate or bound mercury in the purified fractions of the isolated mercurial-induced ionophore demonstrated conclusively that contamination by either of these reagents was insignificant. Contamination of the isolated mercurial-induced ionophore by fluorescein mercurec acetate was equivalent to its addition at a level of less than 1.9 × 10⁻⁴ nmoles per mg of mitochondrial protein in the various experiments described in the text. Similarly, contamination by bound mercury was less than 1.0 × 10⁻³ nmoles per mg of mitochondrial protein. By contrast, mercurial levels of from 6 to 10 nmoles per mg of protein are required in order to elicit the described effects.

**DISCUSSION**

The pattern of ion movements and of increased respiration induced in beef heart mitochondria by fluorescein mercurec acetate can be duplicated by monovalent cation-specific ionophores of bacterial origin or by the corresponding Na⁺/K⁺ ionophore isolated from the mitochondrion. We deduce from this correspondence of effects that a monovalent cation-specific ionophore exists largely in a latent form in freshly prepared beef heart mitochondria isolated in 0.25 M sucrose, and that fluorescein mercurec acetate induces the conversion of this ionophore into a form which exhibits greater activity and is more easily extractable. Therefore, the induction of energized proton ejection by the mercurial can be correlated with the emergence of Na⁺/K⁺ ionophoretic activity. The important conclusion may be drawn that under certain conditions, proton movements in mitochondria are inextricably tied to cation movements which effectively prevent charge separation and since all such cation movements are ionophore-dependent, proton movements must necessarily show an absolute dependence on the presence of an ionophore.

Until recently, the problem of mitochondrial ionophores was wrapped in the ambiguity arising from the lack of consistency in their isolation (4). This difficulty has finally been partially resolved with the discovery that the mitochondrial Na⁺/K⁺ ionophore can exist in free and bound forms and that mercurials and heavy metals profoundly affect the availability of the bound form. Ordinarily, the spectrum of activities described in Table I is minimal in freshly prepared beef heart mitochondria by virtue of the apparent tight control of endogenous ionophores. These internal ionophores are minimally operative under conditions required for oxidative phosphorylation and maximally operative under conditions required for active transport. It is not surprising, therefore, that the same reagents which induce massive proton movements also uncouple oxidative phosphorylation (12, 13).

The physicochemical nature of the mercurial induced "bound" to "extractable" form, ionophore transformation is at present unknown. Mercurials are known to severely perturb biological membranes (14) and in some cases to release soluble protein (15) from membrane preparations. In this context, we have also found (4) that partial tryptic digest of lipid-free mitochondria as well as solubilization in cetyl pyridinium chloride are other tactics by which mitochondrial Na⁺/K⁺ ionophore can be "released" into organic solvents. Therefore, the picture which emerges from this and previous studies (4) is that the mitochondrial Na⁺/K⁺ ionophore does not normally exist as a classically monodispersed ionophore analogous to valinomycin. On the contrary, the evidence suggests that it is tightly associated with membrane protein subunits even to a large extent in its functionally liberated form. The latter view is derived from the finding (4) that yields of mitochondrial Na⁺/K⁺ ionophore approaching 400 ng of valinomycin equivalents per g have been observed by submitting ethanol-extracted mercurial-treated mitochondria to additional membrane percents (e.g. trypsin, heat, cetyl pyridinium chloride), followed by reextraction with organic solvents.

**Acknowledgments—** We are grateful to Mrs. Judith Yocum for her technical assistance. Mrs. Poonson Olson and Ms. Carol Sakowski performed with unstinting dedication during the course of the isolation phases of the present study.

**REFERENCES**

12. Liberman, E. A., Markova, E. N., Skulachev, V. P., and Topali, V. P. (1968) Biokhimiya 13, 188

**TABLE III**

Effect of fluorescein mercurec acetate on the yield of ethanol-extractable mitochondrial Na⁺/K⁺ ionophore

<table>
<thead>
<tr>
<th>Additions</th>
<th>ETOH-extractable Na⁺/K⁺ ionophore</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/g mitochondrial protein</td>
</tr>
<tr>
<td>Control</td>
<td>3.79 ± 2.07 (12)</td>
</tr>
<tr>
<td>Fluorescein mercurec acetate</td>
<td>22.2 ± 7.35 (7)</td>
</tr>
</tbody>
</table>

The yield was normalized to the specific activity of valinomycin when tested in the KNO₃ swelling assay as described under "Experimental Procedure." The ionophore derived from experiments with fluorescein mercurec acetate was, in addition, quantitated according to a previously published procedure (3) based on the rate of induced active transport of potassium acetate by intact mitochondria. Because the latter procedure is 30-fold less sensitive than the swelling assay, it was not suitable for quantitation of control experiments. The numbers in parentheses represent the number of experiments included in the calculation of average yield.

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