THE ISOLATION AND PROPERTIES OF A PEPTIDE IONOPHORE FROM BEEF HEART MITOCHONDRIA[1]

G.A. Blondin[2], A.F. DeCastro[3], and A.E. Senior

Institute for Enzyme Research
University of Wisconsin
Madison, Wisconsin 53706

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SUMMARY: An ionophoretic peptide has been obtained from beef heart mitochondria by extraction with organic solvents. The purified peptide interacts with both sodium and potassium ions and facilitates the transport of these ions across the mitochondrial inner membrane. The similarity in behavior between this endogenous ion carrier and certain depsipeptide antibiotics (e.g., gramicidin-D and valinomycin) suggests that the mechanism of monovalent cation permeation in mitochondrial membrane systems is accountable in terms of endogenous ion carriers analogous to known, microbiologically derived ionophores.

In 1964, the macrolide antibiotic valinomycin, was shown by Moore and Pressman (1) to have the property of inducing the active transport of monovalent cations (K^+, Rb^+, Cs^+) in mitochondria. Since that time, an extensive list of analogous agents, of both microbiological and synthetic origin, has been compiled (2,3). These substances, collectively called "ionophores," share the common property of forming complexes with alkali metal ions and facilitating their transmembrane movement. Interest in these compounds up to now has centered about their use as exogenous reagents for the modulation and study of ion transport in biological and synthetic membrane systems (4-7). However, it seemed to us possible that these exogenous agents are in effect models for analogous ionophores which are endogenous components of membrane systems in general. Since we were familiar with the permeability and ion transport properties of beef heart mitochondria we investigated the possibility of isolating sodium and potassium ionophores from this organelle. In this communication we will describe the isolation of one of two monovalent

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[2]Established Investigator of the American Heart Association.

[3]Present address: Elkhart General Hospital, Research Laboratory, Elkhart, Indiana 46514.

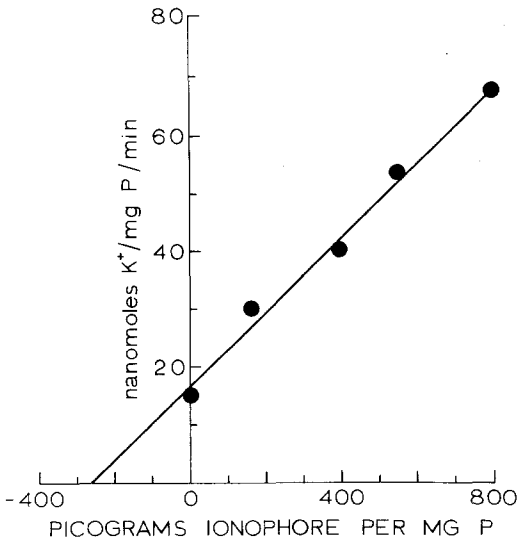
ion carriers which we have postulated to be endogenous to beef heart mitochondria (8).

Beef heart mitochondria are capable of an energy-linked translocation of both sodium and potassium ions from media decimolar in salts of weak acid anions in absence of added ionophore (9). However, the addition of a neutral exogenous ionophore is required for the stimulation of ion transport when the salt concentration is decreased (5-10 mM) and sucrose or mannitol is added for osmotic support (9). If it is assumed that these observations relate to the involvement of an endogenous ionophoretic principle, then certain clear facts emerge concerning the properties of such an ionophore. Since only neutral ionophores have been shown to be active in the induction of alkali metal ion transport in intact mitochondria (5), the endogenous ionophore would probably be a neutral molecule. Furthermore, since both sodium and potassium ions are actively transported into mitochondria from decimolar salt media, the endogenous ionophore must possess little specificity with respect to one or the other of these ions. Finally, since an emergence of a requirement for neutral exogenous ionophore in active transport parallels a decrease in external salt concentration, the endogenous ionophore must normally be present at concentrations less than those used for stimulation of active transport by ionophore in low salt media.

The assay of the endogenous ionophore was based on the ability of added ionophore to stimulate mitochondrial ion translocation in media of low salt concentration. The rate of energized potassium ion translocation in 4.0 mM potassium acetate is increased in a progressive manner by the addition of an exogenous ionophore such as valinomycin (Figure 1). The assay measurements are made with a Beckman cationic electrode and the sensitivity of the assay allowed us to accurately detect as little as 20 nanograms of added valinomycin.

The ionophoretic agent was first extracted from the starting material (lyophilized heavy beef heart mitochondria) with n-pentane or ethyl acetate.

An alternate procedure involved the extraction of mitochondrial suspensions (protein concentration adjusted to 50 mg per ml in 10 mM Tris-HCl, pH 7.4) with 2:1 chloroform/methanol according to the Folch procedure for lipid extraction (10). The purification procedures consisted of three stages of adsorption chromatography which were chosen on the basis of the chromatographic properties of a model neutral ionophore (valinomycin) in identical systems. In the initial step the lipid extract (18.2 gms from 100 gms mitochondria) was submitted to column chromatography on silicic acid (Mallincrodt CC-4, 365 gms). The ionophoretic material was retained by the column during elution with chloroform (1250 ml), but emerged during elution with 6:1 chloroform/methanol (1250 ml). At this stage the ionophoretic fractions contained free fatty acids, cardiolipin and phosphatidyl ethanolamine as shown by thin layer chromatography. Since ionophoretic activity is seldom even observed at this stage, the entire chloroform/methanol eluate (6.33 gms) was submitted to column chromatography on neutral alumina (Woelm W200, 600 gms, deactivated with 4% H_2O). The ionophoretic activity was retained by the



Experimental details are identical to those in the legend of Table 1. Ethanolic solutions of valinomycin of various concentrations were added to induce the transport of potassium ions which were monitored continuously in the external medium.

column during elution with benzene, but emerged during elution with 25% ethanol in benzene. The latter procedure produced either an emergence or a considerable enhancement of activity, probably due to removal of contaminating free fatty acids which are known to uncouple ion transport in intact mitochondria. Still a greater resolution can be obtained by recycling the entire 25% ethanol in benzene eluate (452 mg) through a smaller (70 gms) alumina column using a stepwise gradient of (1) benzene; (2) 10% ethanol in benzene; and (3) 25% ethanol in benzene. The most reproducible results are obtained when ionophoretic activity is assayed only in fractions derived from the latter chromatographic procedure.

The average yield of ionophoretic material was 20 to 100 nanograms per gram of mitochondrial protein. (This calculation assumes that the endogenous mitochondrial ionophore has a molecular weight and specific activity equal to those of valinomycin.) If the rate of potassium translocation in the absence of added ionophore is proportional to the level of endogenous ionophore, then by extrapolation we would not normally expect a yield in excess of 260 nanograms per gram mitochondrial protein (see Figure 1).

We encountered two batches of mitochondria from which unusually high yields of ionophore were obtained (35 and 47 μgrams ionophore per gram mitochondrial protein). This material was submitted to an additional purification process by chromatography on silicic acid. The active principle remained on the column during elution with ethyl acetate but emerged during elution with 8% ethanol in ethyl acetate. The latter procedure did not enhance the total activity but did remove a considerable amount of inactive impurities. The active residue (1.4 $\pm$ 0.2 mg by weight and 1.1 mg by calculation from specific activity as described above) was examined as follows.

When submitted to thin layer chromatography on silica gel in the solvent system $CHCl_3$:MeOH:H_2O (65:25:4), the ionophoretic material cochromatographed with authentic valinomycin. The material gave a negative ninhydrin test but a positive chlorine-starch-iodide test (11), indicating the presence of a non-

TABLE I

Ionophore Induced Active Transport of Sodium or Potassium in Beef Heart Mitochondria

Ionophore	Sodium		Potassium	
	nmoles/mg protein/min	Total nmoles/mg protein	nmoles/mg protein min	Total nmoles/mg protein
None	24.7	49.4	15.5	49.7
Endogenous	210	680	752	1565
Valinomycin	18.5	98.9	325	610
Gramicidin	260	766	660	1646

The incubation medium contained Tris-acetate, pH 7.4 (20 mM), Mannitol (175 mM), potassium or sodium acetate (4.0 mM), Tris-glutamate (13.0 mM) and Tris-malate (3.0 mM) all in a total volume of 9.5 ml at 25 C. Heavy beef heart mitochondria (0.5 ml of suspension containing 50 mg per ml) were added at zero time and the sodium or potassium ion concentration of the external medium was monitored continuously with the appropriate Beckman cation electrode. After preincubation for 30 seconds 20 µl of an ethanolic solution of either gramicidin or valinomycin (both at 5 µg/ml) or of purified endogenous ionophore was added and the ionic activity was monitored until the achievement of steady state.

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terminal N-H containing compound. It possessed no significant U.V. absorption above 240 mµ and no detectable phospholipid phosphorus. After digestion in 6N HCl for 46 hours at 110° the resulting material gave a positive ninhydrin test. Thin layer and paper chromatography of the hydrolysate indicated the presence of (1) valine, (2) leucine, or one of its isomers, (3) proline

TABLE II

Effect of Endogenous Ionophore on Pseudoenergized Swelling in 0.15 M Alkali Metal Chloride Media

Medium	Change in sbsorbance at 520 mμ after 5 minutes (x 10^3)	
	Without Ionophore	With Ionophore
NaCl	35	120
KCl	31	131

Heavy beef heart mitochondria (0.1 ml of a suspension containing 5.0 mg per ml) was added to 2.9 ml of 0.15 M sodium or potassium chloride containing Tris-Cl (10 mM) pH 7.4 and antimycin and rotenone (2.5 μg/mg protein). Absorbance at 520 mμ was monitored in a Beckman DU before and after the addition of 20 μl of an ethanolic solution of purified endogenous ionophore.

or hydroxyproline and (4) serine.[4] The ionophoretic material had an elution volume identical to that of authentic valinomycin during gel filtration on LH-20 Sephadex in 95% ethanol, indicating a molecular weight in the range of 1100, which is consistent with a dodecapeptide.

Investigation of the cationic specificity of the purified ionophoretic material revealed (Table 1) that it differed from authentic valinomycin in

[4]We feel that the proline or hydroxy proline content of the endogenous ionophore is especially significant in terms of molecular architecture. If it is assumed that the four amino acids observed are present in equimolar amounts, then a dodecapeptide consisting of three repeating sequences of Pro-Leu-Val-Ser would yield not only a molecular weight consistent with our gel filtration studies but also a space filling model capable of forming a cyclic structure similar to valinomycin. The unusual property of proline which compels a peptide chain to turn a corner appears to be essential for such a cyclic structure. In addition, the presence of serine allows for the incorporation of 6 internal hydrogen bonds as in the case of valinomycin.

its ability to induce transport of sodium as well as potassium ions thus confirming our original prediction regarding specificity. In terms of biological effects therefore the isolated ionophore resembled gramicidin D more closely than it did valinomycin. The lack of ionic specificity was also revealed in studies on the induction of pseudo-energized swelling (12) (Table 2) in decimolar alkali metal salts. The purified ionophore potentiated swelling in both sodium and potassium chloride media.

DISCUSSION

The isolation of a peptide ionophore from beef heart mitochondria is clearly indicated from the above results. This endogenous ion carrier shares several features in common with other neutral ionophores such as gramicidin and valinomycin. The gel filtration studies suggest that it is a small molecular weight compound probably in the range of 1000 to 1200 versus 1110 for valinomycin and 2000 for gramicidin. The ninhydrin and chlorine-starch-iodide tests suggest that it is a peptide without a free terminal amino group. Valinomycin behaves identically to the above reagents. On the basis of the biological activity of neutral ionophores as compared to that of negative ionophores, the endogenous ionophore may be assumed to be a neutral species. It cochromatographs with valinomycin and gramicidin on thin layer chromatography, and appears to possess similar solubility properties. Nevertheless, the endogenous ionophore is clearly different from valinomycin and gramicidin. The lack of discrimination between sodium and potassium clearly distinguishes it from valinomycin, while the lack of absorption in the 280 mμ range due to tryptophane as well as the molecular weight clearly distinguishes it from gramicidin.

We would have obviously preferred to present a more detailed account of the structural features of the neutral endogenous ionophore at this time; however, the vanishingly small yields of active principle normally encountered has made more detailed structural studies impractical. Because of this factor, one obvious criticism presents itself. While the yield of active principle

normally observed is consistent with the data of Figure 1, we do not have an explanation for the excessively high yields which were twice observed. Since the chemical features were determined on material obtained in the latter two isolations, it may be argued that the active principle normally encountered is distinct from that which was submitted to more rigorous structural investigation. We feel that this interpretation is unlikely since (1) both active principles exhibit identical chromatographic properties on silicic acid and alumina columns; (2) both yield identical molecular weight characteristics based on gel filtration through LH-20; and (3) both exhibit identical sodium-potassium specificities as described in Table I. Because of these observations, we would suggest that the excessively high yields are probably related to a specific biochemical imbalance in the animal prior to sacrifice. Work is underway in our laboratory to test this interpretation through the use of laboratory animals.

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