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LOCALIZATION OF THE ATRACTYLOSIDE-SENSITIVE NUCLEOTIDE BINDING SITES IN RAT LIVER MITOCHONDRIA

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SUMMARY

The inner membrane of the mitochondrion has been confirmed as the locus of atractyloside-sensitive adenine nucleotide binding. These binding sites were found in both inner and outer membrane fractions prepared by the digitonin fractionation procedure. However, binding by the outer membrane fraction was shown to be due to contamination by inner membrane. This was demonstrated by the effect of excess digitonin on the outer membrane fraction: the outer membrane marker-enzymes and phospholipid were solubilized while the ADP binding sites were not. The properties of the binding sites of both fractions were also shown to be identical. An inhomogeneous distribution of atractyloside-sensitive binding sites on the inner membrane was indicated by the observation that the ratio of ADP binding sites to cytochrome oxidase activity of the fragments of inner membrane contaminating the outer membrane fraction was greater than the ratio in the inner membrane fraction.

INTRODUCTION

Mitochondria possess a highly specific transport system for the translocation of ADP and ATP[1–9]. Studies of intact mitochondria have shown that this transport system involves an obligatory exchange process and that concentrations of the plant glycoside, atractyloside, as low as 1 μM inhibit both the influx and the efflux of ADP and ATP. Characterization of these atractyloside-sensitive binding sites was begun using submitochondrial vesicles derived by the action of the non-ionic detergent Lubrol-WX on rat liver mitochondria[10]. In the present study the distribution of the atractyloside-sensitive ADP–ATP binding sites was investigated in inner and outer membrane fractions[11]. This study, using direct methods to determine the binding to membrane fractions, supports the concept of the inner membrane being the locus of atractyloside-sensitive adenine nucleotide transport and also suggests an inhomogeneous distribution of these binding sites on the inner membrane. This inhomogeneity can account for contamination of the outer membrane fraction by inner membrane fragments rich in atractyloside-sensitive adenine nucleotide binding sites relative to cytochrome oxidase activity.

EXPERIMENTAL PROCEDURE

Preparation of membrane fractions

Mitochondria were isolated from the livers of male albino rats by the 0.25 M sucrose procedure of SCHNEIDER[12] as modified by GEAR[13]. Outer membrane and inner membrane-matrix fractions were prepared essentially by the procedure of SCHNAITMAN *et al.*[11]. To prepare the inner membrane fraction, the inner membrane-matrix fraction was resuspended in 0.25 M sucrose buffered with 0.01 M Tris–HCl, pH 7.4, *plus* 0.001 M EDTA (hereafter referred to as Tris–sucrose–EDTA medium). Lubrol-WX was then added at 95 μg per mg of original mitochondrial protein. The suspension was incubated for 30 min at 0° and then centrifuged at 144000 $\times$ *g* for 1 h. The resultant pellet was designated inner membrane fraction. When the outer membrane fraction was to be treated with Lubrol, Lubrol-WX was added prior to the 144000 $\times$ *g* centrifugation at a concentration of 95 μg per mg original mitochondrial protein. The terms "inner membrane" and "outer membrane" will be used throughout to indicate these membranes *per se*. Whereas "inner membrane fraction" and "outer membrane fraction" will be used to refer to the experimental preparation.

Binding of ADP

To determine ADP binding to the outer and inner membrane fractions, the membrane pellets were resuspended in Tris–sucrose–EDTA at approx. 0.5 mg protein/ml. Five ml of this suspension were added to 0.5 ml of [^{3}H]ADP (45 nmoles ADP/ml, 1 μC/ml) and incubated for 30 min at 0°. The suspension was then centrifuged for 1 h at 144000 $\times$ *g*. Atractyloside when present was 10 μM in concentration. The supernatant fluid was decanted, the tubes were wiped free of adhering medium, and the pellets dried and dissolved in 85 % formic acid. An aliquot of the formic acid solution was counted in a liquid scintillation system as previously described[10].

Enzyme assays

Cytochrome oxidase was assayed polarographically as described by SCHNAITMAN *et al.*[11]. Monoamine oxidase was measured by monitoring the oxidation of benzylamine[11]. Rotenone-insensitive NADH–cytochrome C reductase was determined as described by SOTTOCASA *et al.*[14] with rotenone present at 5 μM. ATPase was assayed as previously described[10]. Phospholipid was extracted essentially by the method of FOLCH *et al.*[15] with 20 vol. of chloroform–methanol. The extract was washed with 0.2 vol. 0.9 % NaCl and then with 0.33 vol. of synthetic upper phase. The total phosphate of the extract was determined after ashing with $Mg(NO_3)_2$ by the method of AMES AND DUBIN[16].

Chemicals

Lubrol-WX was a gift from ICI Chemicals, Providence, R.I. Atractyloside was a gift from Professor Santi and Dr. Bruni, University of Padova, Italy. Non-radioactive nucleotides were purchased from P–L Biochemicals, Milwaukee. Radioactive nucleotides were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Binding to the outer and inner membrane fractions

Rat liver mitochondria were fractionated into an inner membrane fraction and an outer membrane fraction by a combination of the digitonin method of SCHNAITMAN *et al.*[11] and Lubrol treatment[10] of the resulting inner membrane-matrix fraction. After Lubrol treatment, the soluble proteins have been removed but the membrane proteins and phospholipids as well as the atractyloside-sensitive adenine nucleotide binding sites are retained in the particulate fraction[10]. The enzymatic markers monoamine oxidase, cytochrome oxidase and malate dehydrogenase were routinely used to monitor the outer membrane, inner membrane and soluble matrix fractions, respectively (refs. 11, 17 and 18).

The total and atractyloside-sensitive ADP binding to the inner and outer membrane fraction were determined and are summarized in Table I. Three main points may be noted. First, there was significant ADP binding to both inner and outer membrane fraction and the specific activity of both the total and atractyloside-sensitive binding of ADP was greater in the outer membrane fraction than in the inner. Second, the portion of the total binding which was atractyloside-insensitive and hence not representative of the binding sites involved in transport was much less in the outer than in the inner membrane fraction. Third, the inner membrane fraction con-

TABLE I

ADP BINDING IN INNER AND OUTER MEMBRANES

Membrane fractions were prepared and their cytochrome oxidase activity and ability to bind ADP determined as described in the text. The values shown are the averages of 14 determinations.

Membrane fraction	*Cytochrome oxidase (μatoms O_2/min per mg protein)*	*ADP binding sites (% of total binding in the mitochondrion)*	*ADP bound (nmoles/mg protein)*		*Ratio of ADP binding sites to cytochrome oxidase activity*	
			Total	*Atractyloside-sensitive*	*Total*	*Atractyloside-sensitive*
Inner	4.4	80	0.87	0.56	0.20	0.13
Outer	1.9	20	1.06	0.88	0.56	0.46

TABLE II

ADP BINDING IN MICROSOMAL FRACTION

The supernatant fluid after a 15-min 15000 × *g* centrifugation of the microsome containing fraction of the usual mitochondrial preparation was treated with the indicated concentration of digitonin for 15 min at 0° and centrifuged for 1 h at 144000 × *g*. The sedimented microsomes were resuspended in Tris–sucrose–EDTA and assayed for ADP binding in the usual fashion.

Digitonin added (mg/mg protein)	*ADP binding (nmoles/mg protein)*	
	Total	*Atractyloside-sensitive*
0	0.093	0.016
0.1	0.090	0.022
1.0	0.094	0.020

tained the majority of the atractyloside-sensitive ADP binding sites of the mitochondrion inspite of its lower specific activity by virtue of its 5-fold greater abundance in the mitochondria.

The possibility that the binding of ADP in the outer membrane fraction was due to contamination by microsomes was investigated by determining ADP binding and its atractyloside-sensitivity in a rat liver microsomal fraction. As shown in Table II, the specific activity of ADP binding to microsomes is of a much smaller magnitude and could not be responsible for the binding observed in the mitochondrial fractions. Digitonin was employed at various concentrations in the microsomal assay to show that there was no activation of ADP binding by the digitonin treatment. Similar experiments demonstrated that the addition of Lubrol to microsomes caused no activation of ADP binding sites.

The problem of contamination of the outer membrane fraction with fragments of inner membrane was examined using cytochrome oxidase as a marker for inner membrane. The ratio of the ADP binding sites to cytochrome oxidase activity was determined for both fractions. The fractions were handled in a similar fashion to avoid artefacts due to denaturation of enzyme activity. As shown in Table I, the outer membrane fraction has three times more ADP binding sites per unit of cytochrome oxidase activity than could be accounted for by contaminating inner membrane. These data are, however, insufficient evidence to indicate whether the outer membrane has atractyloside-sensitive binding sites or whether these sites reside in a fragment of an inhomogeneous inner membrane.

Identity of the properties of the binding sites in outer and inner membrane fractions

The specificity of the atractyloside-sensitive binding sites in the outer and inner membrane fractions was determined. As shown in Table III, when the binding of ADP was measured in the presence of other nucleotides as possible competitive inhibitors, the specificity displayed by the inner and outer membrane fractions was the same, *i.e.*, specific for ADP and ATP. Entirely similar results were obtained whether or not the outer membrane fraction was treated with Lubrol. The K_i for atractyloside is the same for inner and outer membrane fractions and is identical to that previously

TABLE III

INHIBITION OF ADP BINDING

Membrane fractions were prepared as described in the text. Nucleotides to be tested for their inhibitory effect were present at 20 nmoles/ml and [^{3}H]ADP was 3 nmoles/ml. The usual binding assay was employed.

Addition	*Atractyloside-sensitive ADP bound (nmoles/mg protein)*	
	Inner membrane fraction	*Outer membrane fraction*
None	0.51	0.63
ATP	0.16	0.01
AMP	0.48	0.61
IDP	0.46	—
CDP	0.56	0.68
GDP	—	0.64
UDP	—	0.65

measured for unfractionated Lubrol membranes from whole mitochondria[10] (Fig. 1). The lower level of atractyloside-insensitive binding in the outer membrane fraction is again observed (Fig. 1). The ADP binding of Lubrol-treated inner membranes was markedly inhibited by KCl (Table IV), as was that of the Lubrol membranes derived from whole mitochondria[10]. The binding of ADP in the outer membrane fraction was only slightly inhibited by KCl. However, treatment of the outer membrane fraction with Lubrol, while having no effect on the control ADP binding, rendered the binding of ADP in this fraction sensitive to the presence of KCl. Lubrol, then, appears to alter the native structure or constitution of the membrane in such a way that the atractyloside-sensitive binding site can be inhibited by high salt concentrations without marked effect on ADP binding in the absence of salt. Thus, the binding sites of the

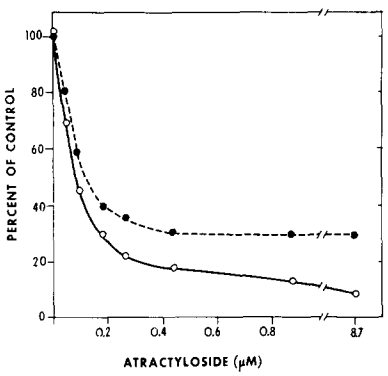

Fig. 1. Atractyloside inhibition of ADP binding. Inner and outer membrane (Lubrol treated) fractions were assayed for their ability to bind ADP in the presence of the indicated concentrations of atractyloside. ●---●, inner membrane fraction; ○—○, outer membrane fraction.

TABLE IV

EFFECT OF KCl ON ADP BINDING

Membrane fractions were prepared using the indicated amounts of Lubrol. Lubrol was added to the inner membrane material sedimented by the 12000 × *g* centrifugations to prepare inner membrane fraction and to the supernatant fluid after the 40000 × *g* centrifugation to prepare outer membrane fraction. After a 30-min incubation at 0° with Lubrol, the suspensions were centrifuged for 1 h at 144000 × *g*. The sedimented membranes were resuspended in Tris–sucrose–EDTA medium and the standard binding assay employed with and without the addition of 1 M KCl (final concentration).

Membrane fraction	*Lubrol added (mg Lubrol per mg membrane protein)*	*ADP bound (nmoles/mg membrane protein)*	
		Control	*KCl*
Inner	0.19*	1.1	0.3
Inner	0.38**	1.1	0.1
Outer	0.0	1.2	0.9
Outer	0.35*	1.3	0.5
Outer	3.5**	1.3	0.3

* Equivalent to 95 μg Lubrol/mg total protein present during the Lubrol treatment.
** Equivalent to 95 μg Lubrol/mg original whole mitochondria from which treated fraction was derived.

outer and inner membrane fraction are essentially identical with respect to their nucleotide specificity, atractyloside-sensitivity and salt-sensitivity when assayed under the same conditions.

Digitonin solubilization of the outer membrane

Careful titration of the outer membrane with digitonin provides a means for selectively breaking this membrane and is thereby a tool for the isolation of inner and outer membrane fractions. Excessive digitonin levels, on the other hand, lead to the solubilization of the outer membrane. Release of monoamine oxidase from the outer membrane as the digitonin concentration is raised has been noted in rat liver mitochondria[11] and digitonin has been used to purify monoamine oxidase from kidney mitochondria[19]. In the present study it was of interest to determine if, as the outer membrane was solubilized with excess digitonin, the atractyloside-sensitive ADP binding sites would be solubilized in parallel with the outer membrane markers, monoamine oxidase and rotenone-insensitive NADH–cytochrome C reductase. If the outer membrane fraction binding sites were not solubilized by excess digitonin in parallel with the outer membrane, this would suggest that the outer membrane fraction binding sites were located on insoluble membrane fragments derived from the inner membrane. As shown in Table V, incubation of the outer membrane fraction with excess digitonin resulted in the solubilization of both monoamine oxidase and rotenone-insensitive NADH–cytochrome C reductase. More than 90 % of the monoamine oxidase activity after digitonin treatment was no longer sedimentable and could be recovered in the supernatant solution. The monoamine oxidase was solubilized whether it occurred in the outer membrane fraction or in the inner membrane fraction as an outer membrane contaminant. Likewise the rotenone-insensitive NADH–cytochrome C reductase activity was largely

TABLE V

EFFECT OF SOLUBILIZATION BY DIGITONIN ON INNER AND OUTER MEMBRANE

Inner membranes and outer membranes were prepared in the usual fashion as described above. The membranes (about 8 mg outer membrane and 14 mg inner membrane fraction) were resuspended directly in 5 ml of either Tris–sucrose–EDTA or 1.5 % digitonin in Tris–sucrose–EDTA. The mg digitonin per mg protein ranged from 4 to 20 for the outer membrane fraction and from 3 to 13 for the inner. The membranes were incubated 40 min at 0° and then diluted with an equal volume of Tris–sucrose–EDTA. The membrane suspensions were then incubated with [^{3}H]ADP to determine total and atractyloside-sensitive binding in the usual fashion. After centrifugation to obtain a 144000 × *g* 1 h pellet and supernatant fluid, enzyme assays were performed on the original suspensions and on the pellet or supernatant or both. Values are given as percentages of the total activity present in the unfractionated suspension which was not sedimented by centrifugation, except for ADP binding where the value represents the percentage of the binding by the pellet lost after digitonin treatment as compared to the control without digitonin. The range of values is shown in parentheses and the number of experiments in brackets.

Membrane fraction	*Activity solubilized* (%)				
	ADP binding [8]	*Monoamine oxidase* [6]	*Cytochrome reductase* [2]*	*Cytochrome oxidase* [5]	*ATPase* [2]
Outer, no digitonin	—	9 (0–26)	10 (5–14)	9 (0–20)	5 (1–10)
Outer, *plus* digitonin	16 (0–34)	93 (78–100)	80 (76–85)	12 (0–42)	18 (0–36)
Inner, no digitonin	—	0 (0)	24 (10–38)	13 (0–22)	14 (10–18)
Inner, *plus* digitonin	25 (12–43)	93 (73–100)	57 (46–67)	36 (17–57)	20 (0–41)

* Rotenone-insensitive NADH–cytochrome C reductase.

solubilized by digitonin treatment. The smaller percentages of rotenone-insensitive NADH–cytochrome C reductase activity solubilized, especially in the inner membrane fraction, might reflect the contribution to the rotenone-insensitive NADH–cytochrome C reductase activity by microsomal contamination. If some of the rotenone-sensitive activity of the inner membrane were rendered rotenone-insensitive by these treatments, this could also account for some insoluble activity. The inner membrane enzymes, ATPase and cytochrome oxidase, were solubilized to a minor extent by the digitonin treatment, although large amounts of protein were released from both the outer and inner membrane fraction.

TABLE VI

EFFECT OF SOLUBILIZATION BY DIGITONIN ON MEMBRANE PHOSPHOLIPID

Membranes were prepared and treated with excess digitonin as described in the legend to Table V. Phospholipid phosphate was then determined on the supernatant solution and the sedimentable material (pellet) after centrifugation as well as on an aliquot of the suspension before centrifugation (unfractionated).

Membrane fraction	*Phospholipid solubilized* (%)	*Phospholipid to protein ratio* (*μmoles phospholipid per mg protein*)
Inner, unfractioned	—	0.33
Inner, pellet, no digitonin	7	0.54
Inner, pellet, after digitonin	43	0.43
Outer, unfractioned	—	0.70
Outer, pellet, no digitonin	15	0.93
Outer, pellet, after digitonin	77	0.54

As demonstrated by the phospholipid release shown in Table VI, the solubilization of monoamine oxidase and rotenone-insensitive NADH–cytochrome C reductase does not represent simply removal of enzyme from the membrane matrix, but rather a solubilization of the outer membrane *in toto*. While the amount of protein release into the supernatant solution is similar in both the inner and outer membrane fraction (55 and 62 %, respectively), almost twice as much phospholipid is solubilized by digitonin in the outer as in the inner membrane fraction. After treatment of the outer membrane fraction with excess digitonin the initial high phospholipid to protein ratio of this fraction was reduced to a lower value. This would be typical of inner membrane fragments remaining after solubilization of the relatively protein-poor outer membrane.

The key point in these experiments is that while the outer membrane was extensively solubilized as judged by both enzyme and phospholipid release, the majority of the ADP binding originally present in the outer membrane fraction was still sedimentable after solubilization of the outer membrane (Table V). Furthermore, the ADP binding present in the membranes treated with excess digitonin remained atractyloside-sensitive as shown in Table VII. In summary, it is apparent that the ADP binding sites in the outer membrane fraction were present neither in the outer membrane nor in the microsomal membrane and the ratio of ADP binding sites to cytochrome oxidase activity was too high to represent a fragment derived from a homogeneous inner membrane. Our conclusion is that the inner membrane is inhomogeneous and that the binding sites in the outer membrane fraction are derived from

TABLE VII

EFFECT OF SOLUBILIZATION BY DIGITONIN ON ADP BINDING

Membranes were prepared, treated with digitonin when indicated and assayed for ADP binding as described in the legend to Table V. The range of values is shown in parentheses, the number of assays in brackets. The breadth of the range of values was due to experimental variations which could not be controlled from preparation to preparation. Duplication within a given preparation was quite satisfactory.

Membrane fraction	*Digitonin treated*	*ADP bound (nmoles/mg sedimented membrane protein)*	
		Total	*Atractyloside-insensitive*
Inner	−	1.2 (0.9–1.8) [6]	0.45 (0.40–0.49) [5]
Inner	+	1.2 (0.7–1.9) [6]	0.39 (0.36–0.46) [5]
Outer	−	2.1 (1.9–2.4) [5]	0.27 (0.25–0.30) [3]
Outer	+	2.6 (2.2–3.3) [5]	0.43 (0.35–0.47) [3]

fragments of inner membrane which are enriched in ADP binding relative to cytochrome oxidase.

DISCUSSION

The presence of atractyloside-sensitive binding sites for ADP and ATP in the inner membrane fraction was anticipated since after removal of the outer membrane from an intact mitochondrion the resultant inner membrane-matrix particle retained its ability to transport ADP and ATP in an atractyloside-sensitive fashion[1]. This observation, as well as the original evidence of KLINGENBERG AND PFAFF[7] and PFAFF *et al.*[30] while showing the presence of the binding sites on the inner membrane, did not demand that the inner membrane be the exclusive locus of these sites and the binding to outer membrane had not been previously investigated. However, if binding sites were present on the outer membrane their presence there must be gratuitous since the outer membrane does not constitute an atractyloside-sensitive barrier to the passage of nucleotide. This "porous sieve" property of the outer membrane has been demonstrated in two ways. First, enzymes utilizing adenine nucleotides which are located in the space between the inner and outer membrane, are not inhibited by atractyloside[3,4,6,8,20]. Second, the filling by ADP of the compartment between the inner and outer membranes is a process characteristic of simple passive diffusion[7].

The identity of the fractions derived by the digitonin treatment of intact mitochondria has been exhaustively investigated in order to demonstrate the authenticity of the fractions obtained[11,17,18,20,21]. Two other methods for the separation of outer and inner membranes have given similar results and thereby support the digitonin fraction[11,14,22,23]. Another method, that of GREEN *et al.*[24–28], has given essentially the opposite localization of various enzyme markers but the ultrastructural and biochemical support for the assignment of these markers is largely lacking.

The finding that the outer membrane fraction had atractyloside-sensitive ADP binding sites was examined in light of possible contamination of this fraction by either fragments of inner membrane or microsomes. Microsomal membranes were investigated and possessed no appreciable atractyloside-sensitive ADP binding. The use

of the ADP binding to cytochrome oxidase ratio as an index of inner membrane contamination of the outer membrane fraction is valid only if the stoichiometry of these two activities is constant throughout the entire inner membrane. If some regions exist in the inner membrane which have more ADP binding sites than molecules of cytochrome oxidase compared with other regions of the same membrane, the ratio cannot be used as a criterion of contamination. Therefore, the ability of excess digitonin to solubilize the outer membrane was used to determine the true locus of the ADP binding sites in the outer membrane fraction. After solubilization of the outer membrane within the outer membrane fraction, the ADP binding sites remained sedimentable. These data lead to the hypothesis that the binding of the outer membrane fraction was due to contamination by fragments of an inhomogeneous inner membrane.

The rationalization of the inhomogeneous distribution of ADP binding sites in the inner membrane and the occurrence, after fractionation of the membranes, of these ADP-binding-site rich fragments derived from the inner membrane in the outer membrane fraction is not difficult. Since these atractyloside-sensitive sites function in the mitochondrion to transport ADP and ATP between the cytoplasm and the matrix compartment, these sites would be ideally located at those points in the inner membrane most available to the environment, such as the region of the inner membrane is in closest proximity to the outer membrane and would serve no useful function if buried deep within the crypt of a crista (Fig. 2). After the mild digitonin treat-

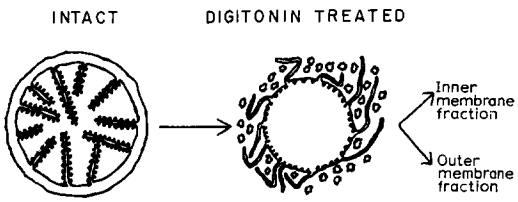

Fig. 2. Schematic for the preparation of inner and outer membrane fractions. The intact mitochondrion is shown with the outer membrane and the inner membrane with its infoldings to form crista. Inner membrane subunits (elementary particles) are shown on the cristal portion of the inner membrane. Treatment with digitonin results in the disruption of the outer membrane and the fragments of this membrane are represented by small vesicles. Although remaining largely intact, the inner membrane undergoes pronounced morphological change with the formation of slender pseudopods. These pseudopods are assumed to be formed by a spreading apart of the crista and an outfolding of the membrane between crista as opposed to an evertion of the crista. This results in the presence of ADP binding site-enriched portions of the membrane on the pseudopod.

ment used to remove the outer membrane, slender pseudopodal processes are formed on the inner membrane-matrix structure[18] in which, based on the above argument, the ADP binding sites could be located. The shearing forces involved in the fractionation procedure could break off some of these processes and the vesicles derived from them would be recovered in the outer membrane fraction. Recently, HACKENBROCK[29] has described points of contact between the inner and outer membrane and suggests that these could be the site of ADP–ATP translocation. This suggestion is in accord with the present study and presents a possible model to account for both their physiological locus and behavior during fractionation.

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