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TRANSLOCATION AND BINDING OF ADENINE NUCLEOTIDES BY RAT LIVER MITOCHONDRIA PARTIALLY DEPLETED OF PHOSPHOLIPIDS

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SUMMARY

1. Rat liver mitochondria were partially depleted of their phospholipids using phospholipase A prepared from porcine pancreas (substrate specificity, cardiolipin > phosphatidylethanolamine > phosphatidylcholine) or from *Crotalus adamanteus* venom (substrate specificity, phosphatidylethanolamine = phosphatidylcholine ≫ cardiolipin).

2. Removal of only about 1 % of the mitochondrial phospholipid with the pancreatic enzyme leads to 50 % and 25 % losses in ADP and ATP translocation, respectively. Concomitant with the loss in translocation is a decline in the ability of both carbonylcyanide *m*-chlorophenylhydrazone and Ca^{2+} to stimulate ATP translocation.

3. To achieve comparable losses in ADP and ATP translocation with the venom enzyme, it is necessary to remove about 8 % of the total mitochondrial phospholipid. Following such treatment, carbonylcyanide *m*-chlorophenylhydrazone and Ca^{2+} are still capable of stimulating ATP translocation.

4. Control experiments involving treatment of the mitochondria with the products of phospholipase digestion indicate that the effects observed on the translocase reflect a loss of phospholipid from the membrane.

5. Binding studies indicate that the loss in adenine nucleotide translocation following phospholipase treatment cannot be accounted for by an altered ability to bind adenine nucleotides to atractyloside-sensitive sites.

6. The data are interpreted in terms of a mechanism of adenine nucleotide translocation involving a lipoprotein carrier system, consisting of the translocator protein and phospholipids, possibly cardiolipin and phosphatidylethanolamine.

Abbreviations: EGTA, ethyleneglycol bis(α-aminoethylether)-*N,N'*-tetraacetic acid.

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INTRODUCTION

In previous reports we have advocated that phospholipids play a major role in the translocation of adenine nucleotides across the inner membrane of liver mitochondria [1–6]. Evidence substantiating this claim emanates from the specific stimulation by Ca^{2+} and La^{3+} [1–5] and from the specific inhibition by local anaesthetics especially butacaine [5] of adenine nucleotide translocation. More recent evidence from studies with butacaine [7] indicates that it is the translocation rather than the binding step that has a particular requirement for phospholipids.

In extending our approach to this problem we have examined the effect of controlled lipid depletion on the activity of the translocator. Using purified phospholipases A from porcine pancreas (specificity, cardiolipin > phosphatidylethanolamine > phosphatidylcholine [8]) and *Crotalus adamanteus* venom (specificity, phosphatidylcholine = phosphatidylethanolamine ≫ cardiolipin [9]) we have been able to obtain data on the specific phospholipid requirements for the translocation of ADP and ATP by rat liver mitochondria, as well as for the stimulation by Ca^{2+} and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) of ATP translocation. Our data show also that binding of adenine nucleotides to the translocator is less susceptible to lipid depletion than the translocation of adenine nucleotides across the mitochondrial membrane.

EXPERIMENTAL PROCEDURES

Liver mitochondria from Wistar rats were prepared [10] in a medium of 250 mM sucrose, 2 mM Tris, 0.5 mM EGTA (pH 7.4) and washed in the same medium but without the EGTA.

Porcine pancreatic phospholipase A was prepared by the method of de Haas et al. [8] and *Crotalus adamanteus* venom phospholipase by the method of Wells and Hanahan [9]. The purified phospholipases were lyophilised and stored at −20 °C. Stock suspensions of 5 mg/ml were prepared on the day of use in distilled water.

Bovine serum albumin (fraction 5) obtained from Sigma Chemical Co., St. Louis, Mo., was defatted with acidified charcoal [11].

Treatment of the mitochondria with phospholipase was carried out at 25 °C for the times indicated in the figure legends in the following medium: 200 mM sucrose, 20 mM Hepes-KOH (pH 7.4), 1 mM $CaCl_2$, 1 % defatted bovine serum albumin and mitochondrial protein at a concentration of 10 mg per ml. The presence of the bovine serum albumin ensured that the products of phospholipase digestion were removed from the mitochondria and subsequently could be washed away (see also refs. 12, 13). After a 1 min. preincubation period, phospholipase was added (20 μg per mg protein i.e. approx. 6 units per mg protein [8, 9]) and at the required times the reaction terminated by the addition of 10 vol. of ice-cold medium (200 mM sucrose, 2 mM Hepes-KOH (pH 7.4), 0.5 % defatted bovine serum albumin and 2.0 mM EGTA). This suspension was left on ice for 5 min. Control experiments showed that the addition of EGTA in this way completely prevented any further action of the phospholipases on the mitochondrial phospholipids. The mitochondria were then sedimented by centrifuging for 10 min. at 12500 rev./min in a Sorvall centrifuge (SS 34 rotor). The pellet was resuspended after one wash in the above

medium but without bovine serum albumin and EGTA, to a protein concentration of 14 mg/ml.

Endogenous mitochondrial adenine nucleotides were labelled [14] and initial rates of adenine nucleotide translocase activity measured [2] using the back-exchange technique [14]. In calculating the rates of translocation, account was always taken of the extent to which endogenous adenine nucleotides had leaked from the mitochondria during treatment with phospholipase. Control experiments showed that little leakage occurred in the initial 3–4 min. of phospholipase treatment especially when 1 % bovine serum albumin was also present in the reaction medium.

Depletion of endogenous adenine nucleotides from the mitochondria was carried out exactly as described by Erdelt et al. [15]. After washing, the depleted mitochondria were finally suspended in 250 mM sucrose, 5 mM Hepes-KOH (pH 7.0) at a concentration of 40 mg/ml.

Binding of adenine nucleotides to atractyloside-sensitive sites on depleted mitochondria was carried out exactly as described elsewhere [7, 16]. For each determination of the extent of adenine nucleotide binding, the mean value and the standard deviation of the mean were determined from the measured radioactivity in the three parallel binding samples [7]. Adenine nucleotides were assayed as described elsewhere [17, 18]; they were separated by paper chromatography [19].

$^{32}P_i$-labelled mitochondrial membranes were prepared by injecting rats intraperitoneally with 0.5 mC of $^{32}P_i$ (Australian Atomic Energy, Lucas Heights, N.S.W.). After 40 h the liver mitochondria were isolated and phospholipase digestions performed as described above. Lipids were extracted [20], washed overnight with 0.9 % NaCl [21] and chromatographed in a two-dimensional system [22] on Whatman SG 81 silica gel paper. Phospholipids were detected with 0.0012 % rhodamine G, identified using known R_F values and standards, cut out and counted in a Packard Tricarb scintillation counter.

RESULTS

Specificity of phospholipases for mitochondrial phospholipids

Data in Fig. 1 show that in the present experimental conditions, during the initial 5 min the pancreatic phospholipase hydrolyses some 40 % of the cardiolipin and about 30 % of the phosphatidylethanolamine. However only a very small proportion of the phosphatidylcholine (about 5 %) is hydrolysed in the same time. The venom phospholipase on the other hand, hydrolyses in the initial 5 min about 30 % of each of phosphatidylethanolamine and phosphatidylcholine but is unable to hydrolyse the cardiolipin. These findings confirm the substrate specificity for these two phospholipases as described [8, 9].

Ability of phospholipase-treated mitochondria to translocate ADP and ATP in the presence and absence of CCCP

A. Pancreatic phospholipase. The ability of rat liver mitochondria to translocate ADP and ATP after increasing times of incubation with the pancreatic phospholipase was examined (Fig. 2A). It is seen first that in the absence of added CCCP, the rate of translocation of both nucleotides is considerably reduced after treatment of the mitochondria with the phospholipase; ADP translocation is particularly

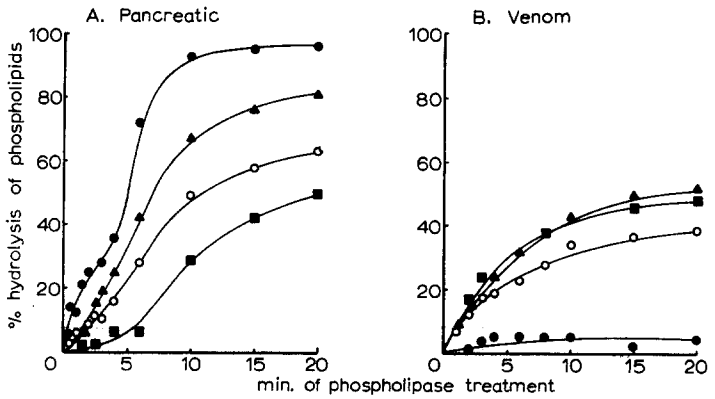

Fig. 1. Ability of phospholipase A to hydrolyse individual mitochondrial phospholipids. Mitochondria prelabelled in vivo with ^{32}P-labelled inorganic phosphate were incubated with phospholipase as described in Experimental in a total volume of 8.0 ml. Samples (0.5 ml) were taken for phospholipid analysis at the times indicated. (a) Pancreatic phospholipase A; (b) Venom phospholipase A. ●–●, cardiolipin; ▲–▲, phosphatidylethanolamine; ■–■, phosphatidylcholine; ○–○, total phospholipid.

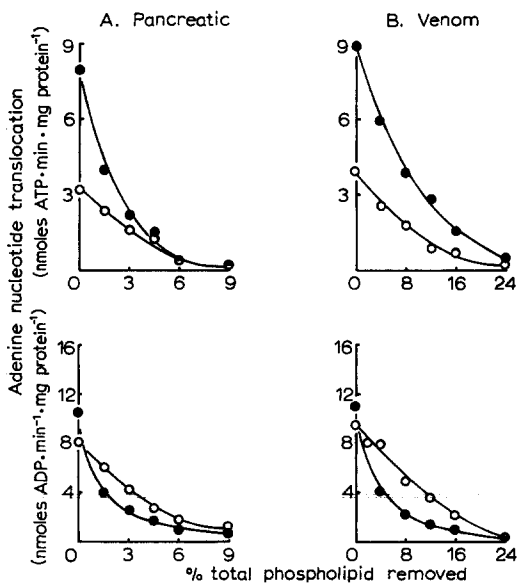

Fig. 2. Effect of phospholipase treatment on the activity of the adenine nucleotide translocase. Mitochondria were treated with phospholipase from pancreas (Fig. A) or venom (Fig. B) as described in Experimental for the times indicated. Initial rates of translocase activity were then determined in a medium consisting of 200 mM sucrose, 2 mM HEPES · KOH (pH 7.4) and 0.4 mg mitochondrial protein in a final volume of 0.25 ml. The reaction was initiated with 200 μM adenine nucleotide with (●–●) or without (○–○) 5 μM CCCP, allowed to proceed for 10 or 20 s and terminated by the addition of 60 μM atractyloside. For further details see ref. 2.

sensitive to such treatment. When only 3 % of the total mitochondrial phospholipid is removed for example, ADP is translocated at about 30 % of the control rate while ATP is still translocated at about 50 % of the control rate. The second point is that the ability of CCCP to stimulate ATP translocation (see ref. 23) is preferentially diminished after removal of only small amounts of phospholipid; when approx. 6 % of the phospholipid has been removed, CCCP no longer is able to stimulate ATP translocation. Thirdly, the response of ADP translocation to added CCCP as phospholipid is removed, differs from that observed with ATP translocation (Fig. 2A, bottom trace).

B. Venom phospholipase. Treatment of rat liver mitochondria with venom phospholipase also leads to diminished rates of translocation of both ADP and ATP (Fig. 2B). As with pancreatic phospholipase treatment, the translocation of ADP is more susceptible to phospholipid hydrolysis than is ATP translocation. To produce a corresponding decline in translocation of adenine nucleotides in these experiments, a greater proportion of the total mitochondrial phospholipid needs to be removed. Thus a 50 % loss in ATP translocation is achieved when approx. 8 % of the phospholipid is removed; ADP translocation is reduced some 50 % when approx. 4 % of the phospholipid is removed. An important point of difference between the action of the two phospholipases is that stimulation of ATP translocation by CCCP is not preferentially affected by venom phospholipase treatment.

The experiments described in Fig. 2 were repeated using Ca^{2+} in place of CCCP. Essentially similar results were obtained viz. pancreatic phospholipase treatment of rat liver mitochondria reduced the ability of Ca^{2+} to stimulate ATP translocation; this was not seen when the mitochondria had been treated with the venom phospholipase. As well ADP translocation was more vulnerable than ATP translocation to phospholipid depletion.

Binding of adenine nucleotides to rat liver mitochondria treated with pancreatic phospholipase

It was important to determine whether the diminution in rates of adenine nucleotide translocation seen in the previous experiments could be attributed to an

TABLE I

ADENINE NUCLEOTIDE POOL SIZES IN NATIVE MITOCHONDRIA AND IN THOSE DEPLETED OF THEIR ENDOGENOUS NUCLEOTIDES

The pool sizes were measured as described in Experimental. The exchangeable pool was determined by measuring the amount of exogenous (^{14}C) ADP that had exchanged after 15 min.

	Mitochondria adenine nucleotide content (nmol · mg protein^{-1})	
	Native	Depleted*
AMP	4.5– 5.7	0.50
ADP	5.8– 6.2	1.06
ATP	4.9– 5.1	0.95
Total pool size	15.2–17.0	2.41
Exchangeable pool size	10.8–11.1	2.05

* Phospholipase treatment of the depleted mitochondria did not significantly alter the adenine nucleotide content of the depleted mitochondria.

TABLE II

EFFECT OF PANCREATIC PHOSPHOLIPASE ON BINDING OF ADP TO DEPLETED RAT LIVER MITOCHONDRIA

The incubation, carried out at 0 °C, contained in a final vol. of 0.4 ml., 250 mM sucrose, 5 mM HEPES, 5 mM Mes (pH 7.0), 2 mM EDTA, 1 mM adenosine 3′-phosphate, 1.5 to 2.0 mg of mitochondria per ml. and 5 μM ADP, The amount of ADP non-specifically bound, bound to atractyloside-sensitive sites or translocated, were determined as described in Experimental. The data are from two of several experiments which gave similar results.

	Experiment	Control (depleted) mitochondria	Phospholipase-treated (depleted) mitochondria
		(pmol/mg protein)	
Atractyloside-removable binding	A	150	122
	B	200	165
Exchange	A	820	200
	B	790	230
Non-specific binding	A	90	160
	B	120	200

altered ability of the mitochondria to bind adenine nucleotides. Precise measurement of such binding to atractyloside-sensitive sites can be made only when the endogenous adenine nucleotide content is low [15, 16]. Thus the mitochondria were first partially depleted of their endogenous adenine nucleotides using potassium phosphate (see Experimental). Direct measurements (see Table I) indicate that the total and exchangeable adenine nucleotide pool sizes in the depleted mitochondria were in the range 2.5 and 2.0 nmol/mg protein, respectively (see also Table I in ref. 7).

The depleted mitochondria were then treated with pancreatic phospholipase in the same way as were intact mitochondria. In a number of separate experiments essentially similar data were obtained using either ADP or ATP. Table II shows results of two experiments of the effect of phospholipase treatment on the amount of ADP bound to atractyloside-sensitive sites, to non-specific sites and on that translocated (exchanged) at 2 min. Consistent with the data presented earlier (Fig. 2), translocation of ADP is reduced by approx. 70 % after removal of approx. 3 % of the mitochondrial phospholipid. By contrast, the binding of ADP to atractyloside-sensitive sites is reduced by only about 15–20 %. Binding of ADP to non-specific sites in these experiments increases by approx. 30 %. This increase is atypical and was not usually observed. These data thus indicate that the diminution in translocase activity following phospholipase treatment cannot be accounted for entirely by an altered ability to bind adenine nucleotides.

Restoration of adenine nucleotide translocase activity in phospholipid-depleted mitochondria

Attempts to reconstitute adenine nucleotide translocase activity in phospholipid-depleted mitochondria have been made repeatedly but to date have met with only partial success. The experiments have involved the addition back of both complete phospholipid extracts and individual phospholipids prepared therefrom to

phospholipase-treated mitochondria. The complicated nature of this transport system, which involves an obligatory exchange of endogenous and exogenous adenine nucleotides [23], makes this task all the more difficult.

Influence of oleic acid and lysophospholipids on adenine nucleotide translocation in intact rat liver mitochondria

In control experiments the effect of the products of phospholipase A hydrolysis, ie. fatty acid and lysophospholipids, on the translocation of ADP and ATP was examined. The concentration of the products used was the same as would expected to be formed after the action of the venom phospholipase on the mitochondria for 6 min. The following observations were made (data not presented): First, treatment of mitochondria with the lyso-compounds produced only slight inhibition of adenine nucleotide translocation, particularly when assayed in the presence of Ca^{2+}. Second, oleic acid inhibited ADP and ATP translocation by some 25 and 65 %, respectively (cf. refs 24–26). Moreover following such treatment, ADP (and to a lesser extent, ATP) translocation in the presence of Ca^{2+} occurred at a rate only slightly less than that of the control. Third, ADP translocation was not stimulated by CCCP after the addition of either fatty acid or the lyso-compounds whereas ATP translocation was stimulated by the uncoupler. Each of these events is quite different to those which occur after phospholipase treatment of the mitochondria (see above). Together they provide good evidence that the primary effect of the phospholipases on translocase activity is mediated through a loss in essential phospholipids rather than a direct effect of the hydrolysis products.

DISCUSSION

This study provides new insights into the mechanism of adenine nucleotide translocation in rat liver mitochondria. The experiments demonstrate that a marked diminution of translocase activity occurs when the mitochondria are partially depleted of their phospholipids. The use of phospholipases to bring about this depletion proved to be most advantageous since it was possible to effectively control the extent and specificity of this depletion. The loss in ability of mitochondria to translocate adenine nucleotides could then be correlated directly with the loss in specific membrane phospholipids. Moreover, it was possible to obtain information on the way in which effectors such as Ca^{2+} and uncouplers influence translocation of adenine nucleotides in intact mitochondria.

The first point revealed in this report is that mitochondria need lose only a very small fraction of their phospholipid complement before incurring a considerable loss in ability to translocate ADP and ATP. Thus treatment of the mitochondria with pancreatic phospholipase so as to remove only about 1 % of the total mitochondrial phospholipid reduced by some 50 % the initial rate of ADP translocation. With venom-treated mitochondria a 50 % decrease in ADP translocation was achieved after only 8 % of the total phospholipid had been removed.

The second point to be noted is that ADP translocation is considerably more susceptible to phospholipid depletion than is ATP translocation. Treatment of the mitochondria with sufficient phospholipase to cause for example, a 50 % decline in ADP translocation, resulted in a loss of only 25 % of ATP translocation (see Figs. 1 and 2).

The hydrolytic action of pancreatic phospholipase was always more effective than that of the venom phospholipase in reducing the ability of the mitochondria to translocate adenine nucleotides. With the pancreatic enzyme, when ADP and ATP-translocation were diminished by 50 % and 25 %, respectively, cardiolipin, phosphatidylethanolamine and phosphatidylcholine were hydrolysed to extents of 8 %, 2 % and 0 %, respectively. By contrast, with the venom enzyme, when mitochondria had lost 50 and 25 % of their ability to translocate ADP and ATP, respectively, phosphatidylethanolamine and phosphatidylcholine were hydrolysed equally to an extent of about 10 %. No cardiolipin is hydrolysed in the same period. These findings provide evidence that phosphatidylethanolamine and cardiolipin but possibly not phosphatidylcholine are obligatory components in the overall translocation system. Confirmation of this conclusion must await the results of appropriate reconstitution experiments.

The ability of both Ca^{2+} and CCCP to stimulate translocation of ATP was progressively reduced with increasing times of treatment of the mitochondria with the pancreatic phospholipase. As this change in pattern was not seen after venom phospholipase treatment, it would seem that cardiolipin is involved in the Ca^{2+} and CCCP stimulation of ATP translocation in intact mitochondria. Consistent with this conclusion is the well-known ability of cardiolipin to bind Ca^{2+} as observed in phospholipid bilayers [27].

The final point is that the action of the phospholipases and by implication, the involvement of the specific phospholipids in the overall adenine nucleotide transport system, is primarily at the translocation step rather than the binding step. This is consistent with the conclusion we have drawn regarding the mode of action of butacaine on adenine nucleotide transport by rat liver mitochondria. We showed that this compound, which is thought to preferentially interact with phospholipids (for review see ref. 28), inhibits not the binding but the actual translocation of adenine nucleotides across the inner mitochondrial membrane [7].

What emerges then from this and other work we have undertaken on this problem is a mechanism of adenine nucleotide translocation involving interactions between both a specific protein component (the atractyloside-sensitive adenine nucleotide translocase or exchange protein; reviews in ref. 23, 29, 30) and a specific "boundary" phospholipid component. The two components are located in the inner membrane in such a way that the phospholipids provide both the necessary microenvironment to permit mobility of the carrier protein [31, 32] and as well specific sites at which effector molecules like Ca^{2+} can interact so as to modify such mobility [1–7]. While this "lipoprotein" concept of a metabolite transport system is not new, until now there has been little direct evidence to support it.

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REFERENCES

1 Spencer, T. and Bygrave, F. L. (1971) Biochem. Biophys. Res. Commun. 43, 1290–1295
2 Spencer, T. and Bygrave, F. L. (1972) Biochem. J. 129, 355–365
3 Spencer, T. and Bygrave, F. L. (1972) FEBS Lett. 26, 225–227
4 Spencer, T. and Bygrave, F. L. (1973) Bioenergetics 4, 347–362
5 Spencer, T. and Bygrave, F. L. (1974) Biochem. J. 140, 413–422
6 Doy, F. A., Daday, A. A. and Bygrave, F. L. (1975) FEBS Lett. 54, 245–248
7 Fayle, D. R. H., Barritt, G. J. and Bygrave, F. L. (1975) Biochem. J. 148, 527–531.
8 de Haas, G. H., Postema, N. M., Nieuwenhuizen, W. and van Deenen, L. L. M. (1968) Biochim. Biophys. Acta 159, 103–117
9 Wells, M. A. and Hanahan, D. J. (1969) Biochemistry 8, 414–424
10 Reed, K. C. and Bygrave, F. L. (1974) Biochem. J. 138, 239–252
11 Chen, R. F. (1967) J. Biol. Chem. 242, 173–181
12 Gul, S. and Smith, A. D. (1972) Biochim. Biophys. Acta 288, 237–240
13 Gul, S. and Smith, A. D. (1974) Biochim. Biophys. Acta 367, 271–281
14 Pfaff, E. and Klingenberg, M. (1968) Eur. J. Biochem. 6, 66–79
15 Erdelt, H., Weidemann, M. J., Buchholz, M. and Klingenberg, M. (1972) Eur. J. Biochem. 30, 107–122
16 Weidemann, M. J., Erdelt, H. and Klingenberg, M. (1970) Eur. J. Biochem. 16, 313–335
17 Adam, H. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) pp. 573–577, Academic Press, New York
18 Lamprecht, W. and Trautschold, I. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 543–551, Academic Press, New York
19 Morrison, J. F. (1968) Anal. Biochem. 24, 106–111
20 Dawson, R. M. C., Hemington, N. and Lindsay, D. B. (1960) Biochem. J. 77, 226–230
21 Folch, J., Lees, M. and Sloana-Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509
22 Fang, M. and Marinetti, G. V. (1969) in Methods in Enzymology (Lowenstein, J. W. ed.), Vol. XIV, pp. 598–612, Academic Press, New York
23 Klingenberg, M. and Pfaff, E. (1968) Biochem. Soc. Symp. 27, 105–122
24 Wojtczak, L. and Zaluska, H. (1967) Biochem. Biophys. Res. Commun. 28, 76–81
25 Shug, A., Lerner, E., Elson, C. and Shrago, E. (1971) Biochem. Biophys. Res. Commun. 43, 557–563
26 Vaartjes, W. J., Kemp, A., Jr., Souverign, J. H. M. and van den Bergh, S. G. (1972) FEBS Lett. 23, 303–308
27 Papahadjopoulos, D. and Ohki, S. (1970) in Ordered Fluids and Liquid Crystals (Johnson, J. F. and Porter, R. S., eds.) pp. 13–32, Plenum Press, New York
28 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655
29 Vignais, P. V., Vignais, P. M., Lauquin, G. and Morel, F. (1973) Biochemie 55, 763–778
30 Klingenberg, M., Wulf, R., Heldt, H. W. and Pfaff, E. (1969) FEBS Symposium 17, 59–77
31 Scherer, B. and Klingenberg, M. (1974) Biochemistry 13, 161–170
32 Lauquin, G. and Vignais, P. V. (1973) Biochim. Biophys. Acta 305, 534–556