THE RESPONSE OF CATIONIC DYES TO ENERGY-LINKED CHANGES OF RECONSTITUTED MITOCHONDRIAL MEMBRANE

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1. Introduction

One approach for studying the molecular organization of mitochondrial membranes has been that of testing the capacity to carry out energy-linked reactions in reconstituted preparations [1]. Recently [2] a new parameter for membrane energization has been reported, i.e. the stacking of acridine and aminophenazine dyes in submitochondrial particles. The purpose of the present paper is that of introducing the stacking effect as a tool for studying the reconstitution of energy transducing membranes. This has been obtained by investigating the correlation between the stacking effect and other energy-linked reactions in various reconstituted preparations. It will be shown: a) that the stacking effect is present in preparations which are capable of P_i–ATP exchange but not in those capable only of oligomycin-sensitive ATPase; and b) that acidic phospholipids are required for inducing the reconstitution of membranes capable of energy-linked reactions.

2. Methods

The preparations used were obtained as follows: beef heart mitochondria [3]; submitochondrial particles [4]; phospholipid dependent, oligomycin-sensitive ATPase [5] slightly modified; F_1 [6]. The membrane protein was extracted from the submitochondrial particles following the cholate treatment suggested by Kagawa and Racker [1]. The reconstitution of P_i–ATP exchange activity was obtained through prolonged dialysis in the presence of phospholipids also as described by Kagawa and Racker [1].

The binding of acridine dyes was measured as described by Dell'Antone et al. [3]. The procedure for measuring the P_i–ATP exchange is described in table 1. Inorganic phosphate was determined according to Fiske and Subbarow. Protein was measured according to Lowry et al. [7] and phospholipid phosphorus after ashing according to Ames and Dubin [8].

3. Results

3.1. *Oligomycin sensitivity, P_i–ATP exchange and neutral red stacking*

Neither P_i–ATP exchange nor neutral red stacking were observed in ATPase preparations showing a high sensitivity to oligomycin. The oligomycin-sensitive ATPase was induced by incubating sonicated phospholipids and cholate-extracted proteins from submitochondrial particles for 5 min at 37%. This treatment has been observed to induce a high rate of oligomycin-sensitive ATPase activity (about 2 μmoles ATP split/mg protein per min).

Both the P_i–ATP exchange and the stacking effect were on the other hand present in preparations reconstituted through prolonged dialysis in the presence of phospholipids as described by Kagawa and Racker [1]. Fig. 1 shows that addition of ATP to those preparations incubated in the presence of 10 μM NR resulted in a large decrease of absorbance identified from the spectra as a metachromatic shift. The presence of 2 μg oligomycin resulted in a large

Table 1
Absorbance change of neutral red and P_i–ATP exchange in submitochondrial particles and reconstituted preparations.

Preparation	Absorbance change of neutral red as % of SMP activity (A/mg protein)	P_i–ATP exchange (nmoles/mg/min)
a) Submitochondrial particles	100	230
b) Preparation reconstituted with soya-bean phospholipids	38	12
c) (b) + F_1	62	32

The absorbance change of neutral red was estimated as described in fig. 2. P_i–ATP exchange was determined at 37° in 50 mM Tris-HCl, pH 7.4; 10 mM KH_2PO_4, pH 7.4; 10 mM ATP, pH 7.4, 10 mM $MgCl_2$, 20 mM KCl, 0.1% bovine serum albumin, 900,000 cpm $^{32}P_i$, 50 mM sucrose, 0.1 mM EDTA, 0.5 mg protein enzyme preparations. Final volume, 1.0 ml; incubation, 5 min. The reaction was determined with trichloroacetic acid. The final concentration of inorganic phosphate was determined by Fiske and Subbarow procedure. ATP was separated by paper chromatography [9]. Paper discs corresponding to ATP were placed on scintillation vials containing 5 ml of toluene, 0.4% POP, 0.01% POPOP and counted. F_1 (80 μg/mg protein) was added to the preparation 10 min before the assay.

inhibition (the inhibition was complete at higher oligomycin concentrations) while that of FCCP in a restoration of the initial absorbance. An enhancement of ANS fluorescence was also observed in these preparations.

Fig. 2 shows the relationship between extent of absorbance change and amount of mitochondrial protein. The change of absorbance increased proportionally to the amount of protein. After a maximum a further increase of protein caused a decrease of absorbance. The extent of maximal absorbance change was higher in the case of the submitochondrial fragments and lower for the two phospholipids-reconstituted membrane preparations. The absorbance change was markedly increased by the addition of F_1. The end-point titration of fig. 2 may be used for the spectrophotometric determination of the number of

Table 2
Effect of individual phospholipids on energy-dependent absorbance change of neutral red.

Phospholipid	Energy-linked changes of absorbance ($\Delta A \times 10^3$)	% of effect
Soya bean	65.66	100
Egg phosphatidylcholine	0	0
+ 10% cardiolipin	21.65	33
+ 10% phosphatidylserine	13.51	20.5
+ 30% phosphatidyl ethanolamine	16.03	24.4

The absorbance change of neutral red was measured as described in figs. 1 and 2. The reconstitution was performed as described by Kagawa and Racker [1] to yield the 33–50 P fraction, with the phospholipids or mixtures of phospholipids indicated (5 μmoles phospholipid-phosphorus/mg protein).

binding sites/per gram protein [3], and this value may be taken as a quantitative description of the degree of energization of the membrane. However, in the titration of fig. 2 there were two parameters changing, the maximal extent of absorbance change and the extent of absorbance change per mg protein. We take the parameter ΔA/mg protein as a measure of the degree of energization of the membrane.

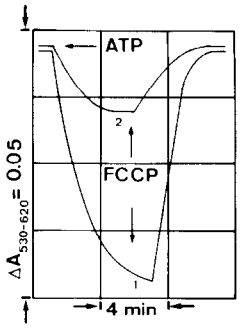

Fig. 1. Energy-linked changes of absorbance of neutral red in reconstituted preparation. The reconstituted preparation was the 33–50 P fraction obtained as described by Kagawa and Racker [1]. Binding of neutral red was estimated at room temp. in a medium containing 10 μM neutral red, 80 mM KCl, 20 mM Hepes (pH 6.5), 5 mM $MgCl_2$, 0.25 mg protein. Final volume, 2.0 ml. Where indicated, 0.5 mM ATP or 2 μM FCCP were added. The absorbance changes were measured at 530–620 nm [3]. Curve 1, no oligomycin; curve 2, 2 μg oligomycin.

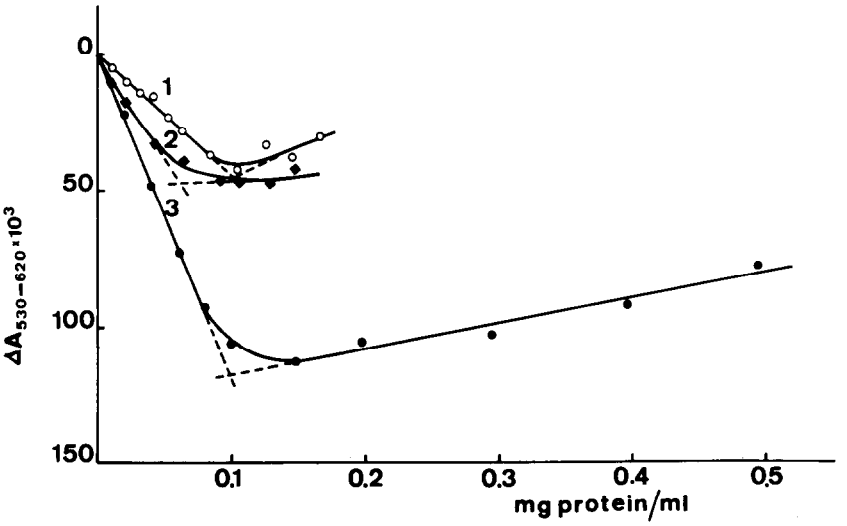

Fig. 2. Spectrophotometric titration of binding sites for neutral red in submitochondrial particles and reconstituted preparations. Binding of neutral red was determined by addition of increasing amount of protein as described in fig. 1. Curve 1, 33–50 P fraction; curve 2, 33–50 P fraction plus F_1 (80 μg/mg protein) added 10 min before the assay; curve 3, submitochondrial particles.

Table 1 shows a comparison of P_i–ATP exchange and stacking effects. The rate of exchange activity was about 5% in the reconstituted preparation in respect to the original particles and was increased to about 15% by the addition of F_1. In comparison the stacking effects were about 38% and 62% in the reconstituted and F_1 supplemented preparations, respectively.

3.2. *The role of phospholipids*

Table 2 shows that in order to re-establish the stacking effect it was necessary to dialyse the membrane protein in the presence of mixtures containing acidic phospholipids. Phosphatidyl choline alone was ineffective while it became effective in the presence of small amounts of cardiolipin or of phosphatidyl serine. In the case of phosphatidyl ethanolamine larger amounts were required to observe stacking. This latter result is in accordance with the observation of Racker [10] with respect to the P_i–ATP exchange. The more marked effect of the acidic phospholipids in reconstituting coupled membrane preparations may be related with their notable capacity in reactivating the mitochondrial ATPase [11].

Kagawa and Racker [1] have also reported that the extent of P_i–ATP exchange in reconstituted preparations is dependent on the phospholipid–protein ratio. Fig. 3B shows that the metachromatic effect was enhanced up to a ratio of about 5 μmole phospholipid/mg protein and then was inhibited. In the case

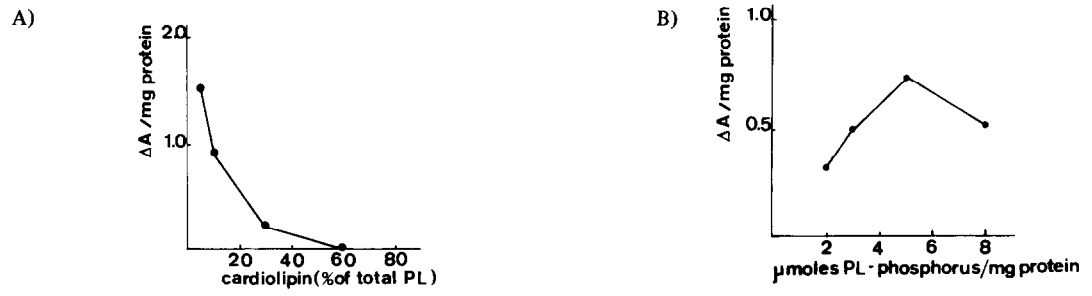

Fig. 3. Dependence of absorbance change on the amount of phospholipids (PL). Experimental conditions as described in fig. 1. A) Reconstitution was performed with phosphatidylcholine containing the indicated amount of cardiolipin. B) Total phospholipids from soya bean were used.

of cardiolipin, fig. 3A, the increase of the amount of phospholipid resulted in an inhibition of the metachromatic effect. This may be related to the capacity of cardiolipin to deplete the submitochondrial fragments of F_1 [12].

4. Discussion

The utilization of the stacking effect as a tool for evaluating the extent of energy transfer in reconstituted membranes requires a consideration of the quantitative aspects of the reaction. It should be recalled that the change of absorbance is not due primarily to binding but to the interaction between two dyes occupying nearest neighbor sites [3]. If we assume that: a) the degree of energization is given by the number of binding sites per mg protein involved in the metachromatic shift and b) there is a proportionality between change of absorbance and binding of the dye to the sites, it is possible to calculate the number of active sites (and therefore the degree of energization) from equation (1):

$$\Delta A = (\epsilon_{bound} - \epsilon_{free})\, \Delta[Dye]_{bound} \qquad (1)$$

where free and bound are the absorbance coefficients of the two forms of the dye, free and bound [3]. It should be noted that the use of equation (1) implies that there are only two absorbance coefficients for the dye. The experiment shown in fig. 2 however, indicates that the ΔA_{max}, obtained by extrapolating the change of absorbance at infinite protein concentration, is not constant among the various preparations. This result is not compatible with a straightforward application of equation (1). Two alternatives may be considered: i) that the absorbance coefficient of the dye, ϵ_{bound}, varies among the various preparations. This is not unlikely since the stacking may be affected by several structural parameters; ii) that part of the dye be bound unspecifically, that is to sites which are not involved in the energy linked changes. To overcome these difficulties we have introduced the term ΔA/mg protein which means the evaluation of the degree of energization from the slope of the plot ΔA *vs* mg protein. The use of this term is largely based on the acceptance of the second alternative. However, it may immediately be seen, that if the first alternative is the correct one, then the term ΔA/mg protein will provide a lower limit for the degree of energization of the preparation.

The comparison between stacking and P_i–ATP exchange in reconstituted membrane preparations indicates that the former was present to a much larger extent that the latter. This may be interpreted in two ways: i) that the P_i–ATP exchange requires a higher energy level than the stacking effect or b) that the P_i–ATP exchange competes less efficiently than the binding of the dye for energy utilization and thus implies a larger leak. In both cases the stacking effect is more sensitive than the P_i–ATP exchange for detecting the degree of energization in reconstituted membrane preparations. Simplicity and rapidity represent further advantages in relation to other tests.

The mechanism by which acidic phospholipids act in determining the reconstitution of these preparations is still not understood. A question to be answered is also whether their effectiveness is related mainly to chemical or physical factors. In the latter case for example it is possible that the more polar character of these phospholipids permits a more intimate interaction with the protein components and possibly also a better removal of the cholate used in the extraction procedure.

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