ENHANCEMENT OF RECONSTITUTED ADP,ATP EXCHANGE ACTIVITY BY PHOSPHATIDYLETHANOLAMINE AND BY ANIONIC PHOSPHOLIPIDS

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1. Introduction

The transport activity of the purified ADP,ATP carrier from beef heart mitochondria has been reconstituted in liposomes from egg yolk phospholipids, as was previously reported. The substrate specificity [1], interaction with inhibitors and sidedness of incorporated carrier [2,3] and the regulation of exchange activity by membrane potential [4], have been characterized in the reconstituted system. These studies concerned properties of the incorporated carrier whereas the influence of the composition of the phospholipid membrane was only briefly discussed [1,5].

In the present report the phospholipid composition of the liposomes used to reconstitute the purified ADP,ATP carrier protein is varied. The experiments demonstrate that special compositions of the phospholipid membrane are required for a high activity of the reconstituted carrier.

2. Materials and methods

Egg yolk phospholipids were isolated according to [6], PC and PE was purified by silicic acid chromatography [7]. PS and PA were prepared by headgroup exchange [8] with phospholipase D from egg yolk PC and also purified on silicic acid columns. DPG was purchased from Koch-Light Laboratories. The phospholipid composition of the reconstituted liposomes was determined after the corresponding exchange experiment by thin-layer chromatography and estimation of phosphorus [9]. Isolation of the adenine nucleotide carrier for reconstitution followed the shortcut procedure as previously described [4].

For the preparation of liposomes, phospholipid solutions in chloroform were dried under a stream of nitrogen and sonicated with buffer [4] for 10–15 min in intervals. Reconstitution, adjustment of membrane potential and exchange assay were essentially as described in [4]. Only for the inhibitor stop 20 μM CAT and 0.5 μM BKA was added simultaneously. The amount of bound [^{3}H]CAT was measured by a gel filtration method [2]. All exchange velocities were determined as initial uptake velocities.

Abbreviations: BKA, bongkrekic acid; CAT, carboxyatractylate; DPG, diphosphatidylglycerol (cardiolipin); PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine

3. Results and discussion

A critical parameter found to influence the activity of the reconstituted adenine nucleotide carrier is the PE to PC ratio (fig.1). With purified PC the exchange activity is very low. Increasing amounts of added PE dramatically enhance the reconstituted exchange. The results obtained with ratios of PE/PC > 1 show extensive scatter. It is known that at compositions of PE/PC $\gg 1$, phospholipids form very unstable liposomes [10] and possibly also hexagonal structures [11], the contribution of which to the reconstituted exchange is not clearly defined. In fact, stable liposomes with PE/(PC + PE) > 0.8 could not be formed with phospholipids isolated from egg yolk. Therefore, the observed exchange optimum with a ratio of PE/(PE + PE) $\simeq 0.65$ results from the superposition of the activity enhancement by increasing amounts of PE and the parallel suppression of exchange due to nonvesicular structures and unstability of the liposomes. Results with these high contents of PE, which have been used also in reconstitution studies by other groups, should be treated with caution.

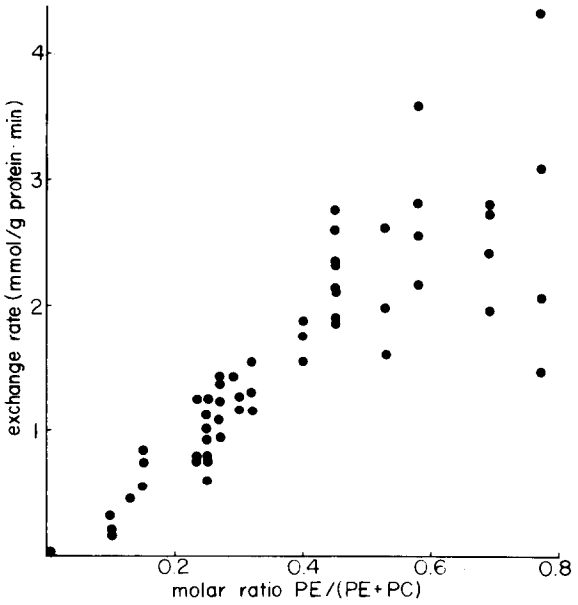

Fig.1. Influence of the PC and PE content of liposomes on the reconstituted adenine nucleotide exchange. All rates are measured on initial velocities.

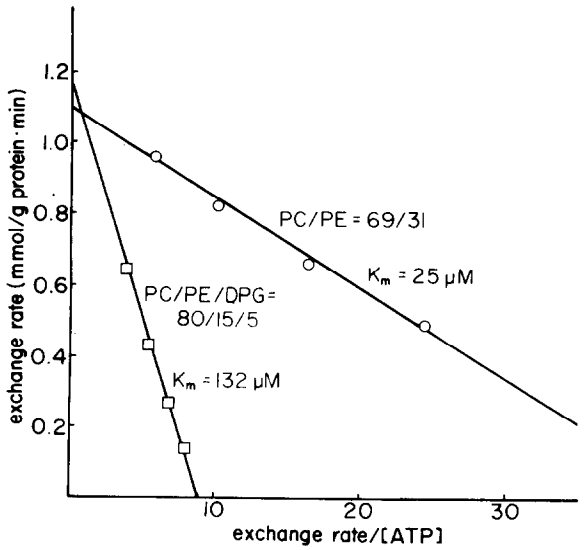

Fig.2. Dependence of ATP uptake on nucleotide concentration and on liposome composition. For both types of liposomes the initial uptake rates are measured at 20, 40, 80 and 160 μmol external ATP.

The inner mitochondrial membrane is characterized by its high amount of negatively charged phospholipids, especially cardiolipin. The influence of this type of phospholipids has also been tested in the reconstituted system. This causes the following experimental difficulty: under physiological conditions the carrier is fully saturated with ADP and ATP since the nucleotide concentrations in the cell largely exceed the dissociation constants at the carrier protein. Saturation cannot be achieved for external nucleotides in the reconstituted system due to experimental restrictions. Exchange velocities are therefore measured in this work as a function of the external nucleotide concentration in order to extrapolate the corresponding maximum velocities. The importance of this procedure is shown in fig.2. When negatively charged DPG is incorporated into the liposomes, the K_m increases markedly as compared to that with liposomes composed of neutral PC and PE only. For that reason only the extrapolated V_{max}-values can be used to describe the exchange activity of the carrier in phospholipid membranes under conditions of substrate saturation which are the intracellular working conditions.

Following this procedure, K_m and V_{max} have been measured with different phospholipid compositions. The data are summarized in table 1. Not only PE, but also negatively charged phospholipids, such as PS and PA increase the maximum uptake velocity when added to PC. The stimulation is even somewhat stronger than that by PE if one compares the values of low amounts (10%) of added phospholipids. When negatively charged phospholipids are added to PC/PE mixtures, the exchange activity increases further. With high amounts of PS and especially DPG, the nucleotide uptake rate is considerably stimulated over that of the corresponding PC/PE basis, given in the second column of table 1. The highest velocities referred to protein content (last line of table 1) are equivalent to about 50% of mitochondrial exchange activity. This statement, however, will be qualified by the results of molecular activity determination shown later (cf. table 2). The enhancement of exchange activity by negatively charged phospholipids added either to PC or to egg yolk phospholipids was already observed earlier [1]. The relatively low exchange rates obtained with egg yolk phospholipids were due to a low (<10%) content of PE.

Negatively charged phospholipids have an ambivalent influence; they increase the V_{max} but also decrease the affinity for ATP as already demonstrated in fig.2. This can be explained by the electrostatic repulsion between the negative charges both of the phospholipids and the nucleotides since the affinity can again

Table 1
Adenine nucleotide exchange with liposomes of different phospholipid composition

Phospholipid composition (molar ratio)		PE / (PE + PC)	Uptake rate (μmol/g · min)	K_m (μM)
PC		<0.01	57 ± 33	65 ± 15
PC/PE	= 90/10	0.10	230 ± 85	54
	75/25	0.25	915 ± 220	39 ± 4
	69/31	0.31	1280 ± 260	27
	55/45	0.45	2260 ± 315	22 ± 8
	42/58	0.58	2750 ± 510	15 ± 4
PC/PA	= 89/11	–	905	77
	68/32	–	935	155
PC/PS/PA	= 87/ 7/ 6	–	970	88
	67/20/13	–	940	295
PC/PE/PA	= 38/42/20	0.52	2630	45
PC/PE/PS	= 68/23/ 9	0.25	955 ± 140	142 ± 37
	46/44/10	0.49	4630 ± 1260	29
	36/42/22	0.54	5060 ± 1450	26 ± 7
PC/PE/DPG	= 80/15/ 5[a]	0.16	1245 ± 185	151 ± 47
	54/38/ 8[a]	0.41	3940 ± 520	110 ± 22
	58/31/11[a]	0.35	7080 ± 1660	51 ± 21

All uptake rates and K_m-values are for external ATP. The molar ratios are calculated according to the phosphate content of the individual thin layer spots.
[a] The weight ratio of DPG would be twice as high since DPG contains two phosphate groups

be raised on saturating the negative surface charges with divalent cations (unpublished results). With higher amounts of PS and DPG added to PC/PE mixture the K_m-values are also decreased. Possibly an asymmetric distribution in the liposomes of the negatively charged phospholipids [12] is responsible. When PS or DPG are located mainly on the inside they should not repel the external nucleotides.

In view of the widely varying exchange activities of the carrier protein when incorporated into different phospholipid membranes the question arises: Is this dependence due to a varying amount of incorporated and reconstituted carrier protein or does the intrinsic exchange activity of the ADP,ATP carrier change? This question can be elegantly answered by titration with the tightly bound inhibitor CAT as was previously shown [3]. The residual exchange activity and simultaneously the amount of protein-bound inhibitor is determined. By correlating both values, the molecular activity of the reconstituted adenine nucleotide carrier can be calculated (table 2). The amount of incorporated active carrier molecules, titrable with CAT, is

Table 2
Correlation of exchange rate with inhibitor binding

Phospholipid composition PE/(PC + PE)	Uptake rate (μmol/min)	CAT bound (nmol)	Molecular activity (21°C) (l/min)	Protein concentration (μg/ml)	% active carrier
0	0.17	10.8	16	11	5.9
0.10	3.3	21.2	155	13.7	9.2
0.27	15.4	17.3	890	12.5	8.2
0.47	31.7	16.1	1970	12.2	7.9
0.58	35.4	14.7	2410	11.6	7.7

For the calculation of the amount of active carriers a molecular weight of 60 000 (active dimer) for the adenine nucleotide carrier was assumed

Table 3
Influence of membrane potential on the ratio of ADP uptake/ATP uptake in liposomes with different phospholipid composition

Phospholipid composition		v_0(ATP)/v_0(ADP) at a membrane potential of			v_0(ADP) (μmol/g · min) 0 mV
		0 mV	+60 mV	+180 mV	
PC		1.38	0.64	–	118
PC/PE	= 90/10	1.15	0.52	0.17	365
PC/PE	= 55/45	1.1	–	0.145	1820
PC/PE/DPG	= 75/19/6	0.78	–	0.21	1960

The external nucleotide concentration was 50 μmol

not markedly increased. Mainly the activity of the reconstituted carrier molecules, i.e., the molecular activity in the exchange kinetics, is influenced by different membrane compositions.

Furthermore, it can be shown that the regulation mechanism of the reconstituted ADP,ATP carrier [4] remains constant over the wide range of measured exchange activities (table 3). Although the overall activities are very different in liposomes with different PC/PE ratios, the influence of the applied membrane potential and thus the mechanism of energy transduction in the reconstituted system seems not to be significantly changed.

The reported observations demonstrate that the exchange activity of the reconstituted ADP,ATP carrier is dependent on the composition of the liposomal phospholipids. A specific requirement of one single phospholipid, however, i.e., of a specific type of headgroups, cannot be discerned in the reconstituted system. Only the fact, that the isolated adenine nucleotide carrier contains some tightly bound PE (H. Hackenberg, unpublished) might indicate such a specific interaction. However, at least three results of the reconstituted system are not in agreement with a headgroup specificity: (i) the inability to 'saturate' the exchange activity by PE even with high amounts of PE, (ii) the possibility to replace PE with negatively charged phospholipids which enhance the activity to a comparable extent, and (iii) the possibility to substitute different kinds of negatively charged phospholipids, namely DPG, PS and even the unphysiological PA to some extent.

Instead, one has to consider that the membrane structure can be changed by varying the phospholipid composition and thus the translocator activity of the incorporated carrier be modulated. Structural changes in the membrane due to different phospholipid compositions have been discussed by Cullis and deKruijff [11,13]. We like to propose that the observed phospholipid requirement of the reconstituted ADP,ATP carrier is caused by such phenomena.

Acknowledgements

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