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Functional Properties of Purified and Reconstituted Mitochondrial Metabolite Carriers

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Eight mitochondrial carrier proteins were solubilized and purified in the authors' laboratories using variations of a general procedure based on hydroxyapatite and Celite chromatography. The molecular mass of all the carriers ranges between 28 and 34 kDa on SDS-PAGE. The purified carrier proteins were reconstituted into liposomes mainly by using a method of detergent removal by hydrophobic chromatography on polystyrene beads. The various carriers were identified in the reconstituted state by their kinetic properties. A complete set of basic kinetic data including substrate specificity, affinity, interaction with inhibitors, and activation energy was obtained. These data closely resemble those of intact mitochondria, as far as they are available from the intact organelle. Mainly on the basis of kinetic data, the asymmetric orientation of most of the reconstituted carrier proteins were established. Several of their functional properties are significantly affected by the type of phospholipids used for reconstitution. All carriers which have been investigated in proteoliposomes function according to a simultaneous (sequential) mechanism of transport; i.e., a ternary complex, made up of two substrates and the carrier protein, is involved in the catalytic cycle. The only exception was the carnitine carrier, where a ping-pong mechanism of transport was found. By reaction of particular cysteine residues with mercurial reagents, several carriers could be reversibly converted to a functional state different from the various physiological transport modes. This "unphysiological" transport mode is characterized by a combination of channel-type and carrier-type properties.

KEY WORDS: Mitochondria; transport; carrier proteins; reconstitution; kinetics; liposomes.

INTRODUCTION

In spite of the enormous impact of the techniques of molecular biology also in the field of carrier-mediated membrane transport, the detailed functional characterization of carrier proteins is still of utmost importance in understanding their mechanism, regulation, and physiological significance. In this paper, a summary of the methods used for successful solubilization, purification, and functional reconstitution of various mitochondrial carriers, as carried out in the authors' laboratories, will be given.

The work in this direction started around 1970, when several laboratories focused on the purification of mitochondrial carrier proteins. Actually the first carrier to be purified by chromatography on hydroxyapatite was the ADP/ATP carrier (Riccio *et al.*, 1975). This useful procedure has since been extensively varied and changed in many ways. However, it still represents the methodical basis for purification of most of the other mitochondrial carriers (Krämer and Palmieri, 1989; Krämer and Palmieri, 1992).

The most important tool for both functional identification and characterization of the different mitochondrial carriers has been the method of functional reconstitution. The first carrier to be reconstituted from the purified state was the AAC[3] (Krämer and Klingenberg, 1977). In recent years all

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major mitochondrial carriers have been reconstituted after successful purification (Krämer and Palmieri, 1989, 1992). Once these transporters were available in the reconstituted state, detailed kinetic studies of functional properties and of transport mechanism were carried out in proteoliposomes. By comparing the results obtained for the different carriers, a common functional family of the mitochondrial carrier proteins was suggested (Krämer and Palmieri, 1992). This finding corresponds to the known structural family of mitochondrial translocators as indicated by close relation on the basis of primary sequence and topology (Palmieri *et al.*, 1993; Walker and Runswick, 1993).

PURIFICATION AND RECONSTITUTION

Purification of carrier proteins still remains the main tool for their functional characterization as well as for important structural studies. The various methods applied for successful solubilization and purification of mitochondrial carriers have been extensively reviewed (Krämer and Palmieri, 1989, 1992). A selection of representative procedures for solubilization, purification, and identification of mitochondrial carriers is summarized in Table I. In this table, only the first publication reporting complete purification and/or functional reconstitution of a particular carrier is mentioned. Obviously, the apparent molecular mass of all carriers falls into a very narrow range between 28 and 34 kDa. Although the primary sequence of only four mitochondrial carriers is known so far (Aquila *et al.*, 1987; Runswick *et al.*, 1987, 1990), this observation suggests that also the other carriers of unknown sequence may be members of the same protein family. The general purification scheme applied for isolation of mitochondrial carriers involves (i) solubilization by non-ionic detergents, (ii) chromatography on hydroxyapatite, and (iii) final purification using various further chromatographic procedures. This scheme has been modified to a great extent for the different carrier proteins (for details see Krämer and Palmieri, 1992 and references therein). The following modifications were of major importance for successful isolation of functionally active mitochondrial carrier proteins: for step (i) preextraction of mitochondrial membranes with various non-ionic detergents, particular ionic conditions (high salt concentration), and addition of lipids during solubilization; for step (ii) extreme variation of the protein/hydroxyapatite ratio and particular pretreatment of hydroxyapatite (conditioning) or of the solubilized protein (e.g., removal of detergent) before chromatography; for step (iii) application of a variety of materials for additional chromatography procedures like Celite, silica gel, and affinity gels including both unspecific (Matrex or SH-reactive columns) and specific adsorption (2-cyano-4hydroxycinnamic acid).

For functional characterization in terms of transport activity, the purified carrier proteins have to be reconstituted into liposomal membranes. Although binding studies have successfully been carried out with carrier proteins in solubilized form (reviewed by Krämer and Palmieri, 1989), kinetic studies require the presence of two distinct compartments. Through the years, several methods have been applied for functional reconstitution of mitochondrial carrier proteins. The selection of methods is restricted by the fact that mitochondrial carriers in general are denatured by detergents with high critical micellar concentrations (e.g., ionic detergents or octylglucoside), thus dialysis and related methods cannot be applied. The first successful reconstitutions were carried out using freeze/thaw/sonication procedures (Krämer and Klingenberg, 1979; Wohlrab, 1980; Kolbe *et al.*, 1981; Stipani and Palmieri, 1983; Bisaccia and Palmieri, 1984), whereas later a method was developed which uses detergent removal by recycling hydrophobic chromatography (Ueno *et al.*, 1984; Krämer and Heberger, 1986). In this procedure, mixed micelles containing phospholipids, detergent, and protein are repeatedly passed through the same column filled with polystyrene beads. This method in general leads to much larger and more homogeneous liposomes as compared to the freeze/thaw/sonication procedure, and in most cases leads to incorporation of higher amounts of functionally active carrier protein into the vesicular membranes. The reconstitution conditions (e.g., ratios of lipid/protein/detergent, composition of lipid, column procedure, etc.) had to be carefully optimized

[3] Abbreviations: for the carrier proteins: AAC, adenine nucleotide carrier; AGC, aspartate/glutamate carrier, CAC, carnitine carrier: CIC, citrate (tricarboxylate) carrier; DIC, dicarboxylate carrier; OGC, oxoglutarate carrier; ORC, ornithine carrier; PiC, phosphate carrier; PYC, pyruvate (monocarboxylate) carrier; UCP, uncoupling protein, H^+ carrier; other abbreviations: DOPC, dioleylphosphatidylocholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearylphosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

Table I. Purification and Reconstitution of Mitochondrial Carriers[a]

Carrier	Source	Solubilization	Purified by	Mol. wt. (SPAGE), kDa	Identification by	References
1. ADP/ATP	BHM	TX-100	HA-chrom.	30	inhib. binding	Riccio *et al.* (1975)
					reconstitution	Krämer and Klingenberg (1977)
2. Uncoupling protein	BFM	TX-100	HA-chrom./ultracentr.	32	GTP-binding	Lin and Klingenberg (1980)
3. Phosphate	PHM	TX-100 + DPG	HA/affin. chrom.	33	reconstitution	De Pinto *et al.* (1982)
	BHM	TX-100	HA-chrom.	34	SPAGE	Kolbe *et al.* (1984)
	RLM	TX-100 + DPG	HA and other chrom.	33	reconstitution	Kaplan *et al.* (1986)
4. Oxoglutarate	PHM	TX-114	HA/Celite chrom.	31.5	reconstitution	Bisaccia *et al.* (1985)
	BHM	TX-114 + DPG	HA/Celite chrom.	31.5	reconstitution	Indiveri *et al.* (1987a)
	RLM	TX-114	HA-chrom.	32.5	reconstitution	Bisaccia *et al.* (1988)
5. Dicarboxylate	RLM	TX-114	HA/Celite chrom.	28	reconstitution	Bisaccia *et al.* (1988)
6. Citrate	RLM	TX-100 + DPG	HA/Celite chrom.	30	reconstitution	Bisaccia *et al.* (1989)
	BLM	TX-114 + DPG	HA/silica chrom.	37	reconstitution	Claeys and Azzi (1989)
7. Pyruvate	BHM	TX-100	HA/affin. chrom.	34	reconstitution	Bolli *et al.* (1989)
8. Carnitine	RLM	TX-100	HA/Celite chrom.	32.5	reconstitution	Indiveri *et al.* (1990)
9. Aspartate/glutamate	BHM	$C_{12}E_8$	HA/Celite chrom.	31.5	reconstitution	Bisaccia *et al.* (1992)
10. Ornithine	RLM	TX-100	HA-chrom. and others	33.5	reconstitution	Indiveri *et al.* (1992a)

[a] Only the first publications reporting complete purification and/or functional reconstitution are mentioned. Abbreviations for different sources of mitochondria are: BHM, beef heart; BLM, beef liver; RLM, rat liver; PHM, pig heart; BFM, brown fat. Other abbreviations: $C_{12}E_8$, octaethyleneglycol mono-n-dodecyl ether; DPG, cardiolipin, HA, hydroxyapatite; SPAGE, SDS-polyacrylamide gel electrophoresis.

for every single carrier protein in order to obtain maximum transport rates (Krämer and Heberger, 1986; Indiveri *et al.*, 1987b, 1989a, 1991a, b; Bisaccia *et al.*, 1990).

BASIC KINETIC DATA

For characterization of the basic kinetic properties of all those mitochondrial carrier proteins which were purified in our laboratories, we analyzed their transport function in the reconstituted system (Table II). Transport activities in general were measured both in the "forward mode" (uptake of label) and in the "backward mode" (efflux of label), both methods leading to closely similar results. The dependence of the transport rates on external and internal substrate concentrations in each case followed simple Michaelis–Menten kinetics which was the basis for the determination of transport affinities (apparent K_m values) and maximum transport rates (V_{max}). Several important restrictions have to be considered when comparing the data of Table II. The V_{max} values of course primarily depend on the molecular activity of the respective reconstituted carrier protein. In principle, they also depend on the purity of the reconstituted protein, which, however, in all cases reported in Table II, is high. Nevertheless, molecular activities cannot be calculated from the V_{max} values listed in Table II, since they critically depend on the efficiency of reconstitution which clearly will not be 100% and will presumably not be identical for the different carrier proteins. The efficiency of reconstitution has been determined in the case of the AAC to be about 5–10% (Krämer and Klingenberg, 1979). Furthermore, the maximum transport rates essentially depend on the type of lipid used for the reconstitution (see below). As an example, in the case of the AAC, where the determination of molecular activity was possible on the basis of inhibitor titration (Krämer and Klingenberg, 1979), a wide variation of the reconstituted transport activity was observed, from zero to values significantly higher than in the natural surrounding of intact mitochondria (Krämer and Klingenberg, 1980b; Krämer, 1982). In spite of these restrictions, it is obvious that the reconstituted PiC shows the highest specific activity, as also known from intact mitochondria. Since the measured transport affinities do not depend on the carrier purity and the efficiency of reconstitution, these values are more reliable. This is corroborated by the fact that they are in general very close to the values measured in intact mitochondria, as far as they are known for the intact organelle under these conditions. In particular for the internal (matrix) side of these carrier proteins in the case of several carrier proteins, the data obtained from these studies in reconstituted systems provided the first determination of the respective K_m values. It should again be taken into account, however, that the lipid composition may significantly influence the K_m values measured. This is due to the fact that binding of highly negatively charged substrates, e.g. nucleotides, may be critically influenced by the surface properties (surface charge and surface potential) of the surrounding phospholipid bilayer (Krämer, 1983). Furthermore, in some cases the pH significantly influences the measured K_m values, especially when protons are involved in the transport function, e.g., for the AGC or the PiC (Table II).

As a further important functional property, the activation energy of the reconstituted mitochondrial carrier proteins is listed. This value is especially high for CAC and for the AAC (only below 20°C), probably due to the more complex conformational change within the catalytic cycle of the carrier protein necessary for catalyzing transport of these bulky substrates. In the cases where the activation energy for carrier proteins was determined in intact mitochondria, the measured values are in general very close to that listed in Table II.

As stated above, it is an advantage of the reconstituted system that K_m values can be accurately determined on both sides of the membrane. This is in general difficult in intact mitochondria for the internal surface, since internal substrate concentrations cannot be varied freely and interference by metabolism as well as by other carriers sharing the same substrate usually occurs. By using purified proteins in the reconstituted system we obtained a complete set of K_m values on both sides of the liposomal membrane for the carriers listed in Table II. Importantly, for at least one substrate of each carrier, the apparent K_m on the inside is significantly different from that on the outside, as determined both by "forward" and "backward" exchange. In the case of the AGC, the CAC, and the PiC, the difference between the internal and external K_m is almost one order of magnitude. Except for the AAC, where the different sides were discriminated by using side-specific inhibitors, in all other cases no significant amount of the carrier population with "internal K_m" was found on the outside and vice versa. This conclusion

Table II. Kinetic Data of Purified and Reconstituted Mitochondrial Carrier Proteins

Carrier	Substrate	K_m (mM) external	K_m (mM) internal	V^a_{max} (mmol/min × g)	Activation energy	Kinetic mechanism	References[b]
AAC	ATP	0.01	0.025	up to 7.1	160/62	n.d.	Krämer and Klingenberg (1980a); Krämer (1982);
	ADP	0.01					Krämer and Klingenberg (1982)
AGC	Aspartate	0.12 ± 0.02	2.8 ± 0.6	n.d.[c]	77	Simultaneous	Krämer *et al.* (1986); Dierks and Krämer (1988);
	Glutamate	0.25[d]	3[d]			Simultaneous	Dierks *et al.* (1988)
PiC	Pi/Pi	1.8 ± 0.1	9.4 ± 0.1	92	64	Simultaneous	Palmieri *et al.* (1990);
	Pi/OH^-		11.2 ± 0.9	53		Simultaneous	Stappen and Krämer (1993a, b)
OGC	Oxoglutarate	0.31 ± 0.08	0.17 ± 0.06	9.5 ± 1.5	54	Simultaneous	Indiveri *et al.* (1987a); Indiveri *et al.* (1991a)
	Malate	1.36 ± 0.14	0.71 ± 0.18	10.0 ± 1.1		Simultaneous	
DIC	Malate	0.49 ± 0.05	0.92 ± 0.16	6.0 ± 1.6	96	Simultaneous	Indiveri *et al.* (1989a, b); Indiveri *et al.* (1993a)
	Malonate	0.54 ± 0.10	n.d.	5.9 ± 0.9		n.d.	
	Phosphate	1.41 ± 0.35	0.93 ± 0.18	6.0 ± 1.4		Simultaneous	
CIC	Citrate	0.032 ± 0.004	0.027 ± 0.004	10.5 ± 1.9	70	Simultaneous	Bisaccia *et al.* (1990); Bisaccia *et al.* (1993)
	Malate	0.025 ± 0.03	0.060 ± 0.006	11.5 ± 1.8		Simultaneous	
CAC	Carnitine (exchange)	1.1 ± 0.1	10 ± 0.8	1.7 ± 1.3	133	Ping-pong	Indiveri *et al.* (1991b, c); Indiveri *et al.* (1993b)
	Carnitine (uniport)	0.53 ± 0.12	2.2 ± 0.4	0.2 ± 0.09	115		
ORC	Ornithine	0.16 ± 0.016	n.d.	3.3 ± 0.9	86	n.d.	Indiveri *et al.*, unpublished results

[a] V_{max} values may significantly depend on the type of lipids, pH, reconstitution efficiency, etc.
[b] The most recent data with respect to the particular carrier proteins are referred to. All data are from studies using hydrophobic chromatography for reconstitution except those for the AAC.
[c] True V_{max} value has not been determined with the purified protein (see Bisaccia *et al.*, 1992).
[d] Value given for pH 6.5; the K_m for glutamate significantly depends on pH due to H^+-cotransport (see Dierks *et al.*, 1988).

was corroborated by the fact that by scrambling the carrier proteins by an additional freeze/thaw/sonication step, carrier populations with the two different K_m values on the outside of the proteoliposomes were found (Indiveri *et al.*, 1991a, 1993b). These results allow the conclusion that all carriers investigated, except the AAC which in fact is inserted in both orientations and the ORC were the internal K_m has not yet been determined, are inserted unidirectionally into the liposomal membrane. By comparing the internal and the external transport affinities and comparing them with the values measured in intact mitochondria, we have concluded that the AGC (Dierks and Krämer, 1988), the CIC (Bisaccia *et al.*, 1993), the DIC (Indiveri *et al.*, 1993a), the CAC (Indiveri *et al.*, 1993b), and PiC (Stappen and Krämer, 1993a) are oriented right-side-out, as compared to their orientation in intact mitochondria, whereas the OGC (Indiveri *et al.*, 1991a) seems to be inserted in the opposite direction. In the case of the AGC, this type of determination of sidedness has been directly controlled by a similar approach in intact mitochondria (Sluse *et al.*, 1991).

It is interesting to note that knowledge of the affinity of the carriers for their substrates on the cytosolic side and (derived from the reconstituted proteins) on the matrix side of the inner mitochondrial membrane has important physiological implications. For example, the high affinity of the CAC for substrates on the cytosolic side of the mitochondrial membrane facilitates the entry of acylcarnitines into the matrix space. On the other hand, the low affinity on the internal side prevents their re-efflux, making them available to the mitochondrial acylcarnitine transferase. Furthermore, the matrix K_m for carnitine is higher than carnitine concentration inside the mitochondria, and therefore it is likely that the intramitochondrial level of carnitine regulates fatty acid translocation into mitochondria.

FURTHER KINETIC PROPERTIES

The isolated and reconstituted carrier proteins also offered the possibility to investigate the kinetic interaction of the various substrates and inhibitors with the respective carrier not only on the external side (as generally carried out in mitochondria) but also on the internal side of the proteoliposomes. From the competitive nature of the mutual inhibition exhibited by the substrates of the AAC, AGC, OGC, CIC, and CAC the presence of a single substrate-binding site has been inferred both at the external and the internal membrane side (Krämer and Klingenberg, 1982; Dierks *et al.*, 1988; Indiveri *et al.*, 1987a, 1991a, b, 1993b; Bisaccia *et al.*, 1990, 1993; and unpublished data). As compared to other mitochondrial antiport carriers, the situation with the dicarboxylate carrier is more complex. On each membrane side of the reconstituted protein two separate binding sites exist, one specific for phosphate (thiosulfate, arsenate, and phenylphosphate), the other for dicarboxylates (sulfate and sulfite) (Indiveri *et al.*, 1989b, 1993a). The two different binding sites can be occupied by the respective substrates without mutual interference as demonstrated by the non competitive interaction of these substrates with the carrier (Table III). It should be recalled that these kinetic data obtained in the reconstituted system support the conclusion of early studies in intact mitochondria. Thus, long ago it was suggested that on the cytosolic mitochondrial surface both the CIC and OGC have a single substrate-binding site for all the transported metabolites (Palmieri *et al.*, 1972a, b), whereas the DIC has two different binding sites, one specific for phosphate and the other for dicarboxylates (Palmieri *et al.*, 1971; Quagliariello *et al.*, 1974; Crompton *et al.*, 1975).

Considerable differences between the various carriers were found when considering the influence of pH on the transport activity. The reconstituted OGC, DIC, and CAC (Indiveri *et al.*, 1987a, 1989a, 1991b) are practically independent of the applied pH at high substrate concentrations in a rather wide range of pH, as found previously in intact mitochondria (Palmieri *et al.*, 1972a, b; Meisner *et al.*, 1972). The ORC and the CIC, on the other hand, are more sensitive to variation of pH. The activity of the ORC exhibits an optimum at pH 8.0–8.5 (Indiveri *et al.*, 1992a). The reconstituted CIC shows a marked pH optimum between 7 and 8, being strongly inactivated at higher and lower pH values (Bisaccia *et al.*, 1990). Between pH 7 and 8 the substrates are predominantly present as citrate^{3-} and malate^{2-}. To answer the question related to the substrate species transported by the carrier (citrate^{2-} or citrate^{3-}), the dependence of the reconstituted CIC activity on pH was investigated in the range between 7.0 and 7.8 (Bisaccia *et al.*, 1993). Both homologous citrate/citrate antiport and heterologous (electroneutral) malate/citrate antiport were kinetically analyzed. The maximal rates of the two exchange modes did not vary significantly between pH 7.0 and

Table III. *Ki* (mM) Values of Substrates and Inhibitors for Purified and Reconstituted Carriers

Substrate or inhibitor	OGC (competitive)	CIC (competitive)	CAC (competitive)	DIC			
				Malate uptake		Phosphate uptake	
				Competitive	Non competitive	Competitive	Noncompetitive
Malate	0.27						0.5
Malonate	1	3.1		0.5			
Succinate	1.2	2.5		0.95			
Phthalonate	0.06						
Phenylsuccinate	0.4			1.6			
Butylmalonate	0.7			0.4			0.4
cis-Aconitate		0.04					
threo-D_s-Isocitrate		0.08					
Phosphoenolpyruvate		0.18					
1,2,3-Benzenetricarboxylate		0.08					
Phosphate					0.8		
Sulfate				0.6			0.5
Sulfite				0.2			0.3
Thiosulfate					0.8	0.4	
L-carnitine			0.5				
DL-acylcarnitines (12–18 C)			0.004–0.03				

7.8. When the apparent transport affinity was calculated for the different citrate species at the applied pH values, a fairly constant K_m value was observed only when citrate^{2-} was considered as the transported species. The K_m for both citrate^{3-} and citrate^{1-} varied significantly; furthermore, the K_m for citrate^{1-} was extremely low. In the case of the malate/citrate antiport, the K_m for malate^{2-} did not significantly change on variation of pH; in contrast, the K_m value for malate^{1-} varied considerably. These results indicate that only the single protonated form of citrate (H-citrate^{2-}) and the unprotonated form of malate (malate^{2-}) are the species transported by the citrate carrier.

INFLUENCE OF PHOSPHOLIPIDS ON CARRIER ACTIVITY

As already mentioned above, lipids significantly modulate the activity of reconstituted mitochondrial carriers. Detailed studies in this respect were first carried out in the case of the reconstituted AAC. A variety of factors exert a strong influence on both the transport activity and affinity. Among these are the nature of the phospholipid headgroups (Krämer and Klingenberg, 1980b), the headgroup charge (Krämer and Klingenberg, 1980b; Krämer, 1983), as well as the physical state of the membrane (Krämer, 1982). A clear preference for PE in general as well as negatively charged phospholipids at the internal surface was observed. The surprising additional stimulating effect of added cholesterol, which is not present in significant amounts in the physiological surrounding of this carrier, i.e., in the inner mitochondrial membrane, indicates that the presence of "non bilayer forming" lipids (de Kruiff, 1985) clearly enhances the catalytic activity of the reconstituted AAC. At about the same time, it was found that cardiolipin stimulates the reconstituted PiC, whereas other acidic and unsaturated phospholipids have no effect (Kadenbach *et al.*, 1982; Mende *et al.*, 1982). Subsequently, detailed studies on the influence of the lipid surroundings on carrier activity have been carried out with several other mitochondrial carriers (Indiveri *et al.*, 1987a, 1989a, 1991b; Bisaccia *et al.*, 1990). We investigated these effects in general by adding various phospholipids to the basic egg yolk phosphatidyl phospholipid mixture used in these experiments, which consisted of PC and PE. In all cases, transport was measured both as initial rate and as amount of label taken up after equilibrium. The former parameter is related to the specific carrier activity, the latter to the efficiency of carrier insertion (and to the size of the reconstituted liposomes). Synthetic phosphatidylcholines with different fatty acids (DOPC, DPPC, and DSPC) have no effect on the CAC and a slight inhibitory effect on the CIC. Interestingly, the activity of the OGC (but not its incorporation) is inhibited only by DOPC, whereas incorporation into liposomes (but not the activity) of the DIC is negatively influenced only by DPPC. This suggests that even a slight change in the phospholipid composition plays, in some cases, an important role in the behaviour of the transport proteins. In contrast to the reconstituted AAC, PE has no significant effect on these carriers, except on the OGC, the incorporation of which is strongly reduced. Very interesting too is the influence of cardiolipin and other acidic phospholipids like PI and PS on the mitochondrial carriers. A positive effect is exerted by all three acidic phospholipids on the AAC (see above), the CIC, and only by cardolipin on the CAC. The latter effect is due to activation of the carrier molecules without any influence on their incorporation, whereas the effect of the acidic phospholipids on the CIC is largely due to an increase in protein incorporation into liposomes. Finally, cardiolipin and PI inhibit the DIC and the OGC. This effect is due to a reduction in activity in the case of the DIC and to a decrease in incorporation in the case of the OGC.

ANALYSIS OF THE KINETIC MECHANISM OF TRANSPORT

The asymmetric orientation of the membrane-embedded carrier proteins, on the one hand, and the independence of the exchange reactions from the type of countersubstrate, on the other hand, provided well-defined conditions for studying the transport mechanisms of the reconstituted mitochondrial carriers using the kinetic approach. The counterexchange of substrates catalyzed by an antiport carrier can be described in terms of a bisubstrate reaction involving different steps of binding, transport and release of substrates. The sequence of these steps follows one of the two basically different bisubstrate mechanisms, the ping-pong or the simultaneous (sequential) type. In the ping-pong mechanism, the first transported substrate has to leave the transport site before the second substrate (transported in the opposite direction) is bound. The simultaneous mechanism,

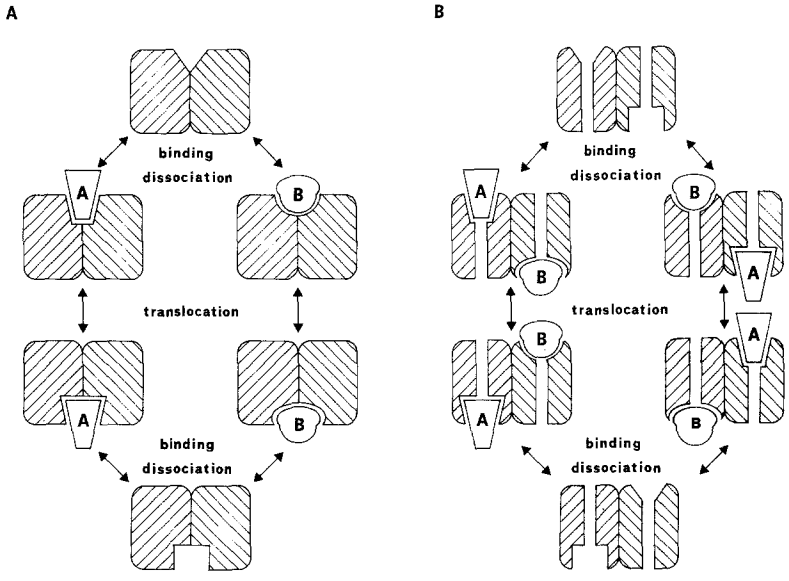

Fig. 1. Functional models for mitochondrial exchange carriers. The different substrates A and B are bound to the active sites at different sides of the membrane. (A) "single site gated pore mechanism" as suggested for the adenine nucleotide carrier (Klingenberg, 1989). The single binding site is exposed either on the inside or on the outside of the carrier protein. The transport pathway is formed between the two monomers. The kinetic mechanism is of the ping-pong type. (B) Transport mechanism as suggested for the aspartate/glutamate carrier (Dierks *et al.*, 1988). The dimer contains two binding sites, one on each side of the membrane. A separate substrate pathway in each monomer is assumed; the kinetic mechanism is simultaneous.

on the other hand, involves the binding of the two substrates at the same time, leading to the formation of a ternary complex with the carrier protein before transport catalysis occurs. These two possibilities can be discriminated by two-reactant initial-velocity studies varying both the internal and external substrate concentrations. For ping-pong reactions the ratio of K_m/V_{max} (i.e., the slope of Lineweaver–Burk plots) is not influenced by the concentration of the countersubstrate; for simultaneous reactions this ratio diminishes as the countersubstrate increases (Cleland, 1970). By using this analysis, we have conclusively shown that the reconstituted AGC (Dierks *et al.*, 1988), OGC (Indiveri *et al.*, 1991a), CIC (Bisaccia *et al.*, 1993), DIC (Indiveri *et al.*, 1993a), and PiC (Stappen and Krämer, 1993b) function according to a simultaneous mechanism, whereas the CAC proceeds via a ping-pong mechanism (Indiveri *et al.*, 1993b). It should be emphasized that the distinction between ping-pong type and sequential type kinetics is not trivial; in fact, it has important structural consequences. If a carrier protein accepting two substrates, e.g., a classical antiport carrier, functions according to a ping-pong mechanism, basically only one binding site is necessary, alternatively exposed to the two different sides of the membrane. On the other hand, if a simultaneous type of kinetics is detected, including binding on opposite sides at a given time within the transport cycle, two binding sites must be exposed and occupied (Fig. 1). It has to be taken into account that the problem could in principle be solved by simply assuming a functional dimer composed of two monomers with one substrate binding site and one transport channel each. This, however, would be in contrast with the postulate assuming a transport channel between the two putative subunits (Klingenberg, 1981), a hypothesis not proved so far. A sequential type of mechanism has also been shown for the OGC and the AGC in studies with intact mitochondria (Sluse *et al.*, 1979, 1991). It was therefore assumed that the mitochondrial metabolite carriers form a homogeneous functional family based on a common type of kinetic mechanism (Krämer and Palmieri, 1992). There are, however, two problems. First, in the case of the AAC, the well-documented observation of site recruitment (Klingenberg, 1989) during the catalytic cycle clearly argues for a ping-pong mechanism, in spite of conflict-

ing kinetic data (Duykaerts *et al.*, 1980). Importantly, recent results obtained for the kinetic mechanism of the reconstituted CAC indicate that this carrier differs functionally from all other mitochondrial carriers studied in the reconstituted system so far. Two-reactant initial velocity studies showed that the reconstituted CAC functions in a ping-pong type of mechanism (Indiveri *et al.*, 1993b). Obviously, knowledge of the primary structure of the CAC is needed in order to ascertain whether the CAC also falls into the family of mitochondrial carrier proteins, which has been suggested on the basis of sequence homologies between the AAC, the PiC, the OGC, and the uncoupling protein from brown adipose tissue (Runswick *et al.*, 1987, 1990; Aquila *et al.*, 1987), as well as on the basis of the common simultaneous mechanism found so far (Krämer and Palmieri, 1992).

AN ALTERNATIVE MODE OF TRANSPORT: MERCURIAL INDUCED UNIPORT

The construction principle of antiport carriers, like the AAC and many other mitochondrial transporters, in general guarantees tight coupling during transport, i.e., the rate of slippage (unidirectional transport) is low. On the other hand, some carriers, e.g., the PiC or the PYC, mainly catalyze proton-coupled unidirectional substrate flux (in addition to homologous exchange), whereas the CAC also mediates carnitine uniport (in addition to homologous and heterologous exchange). Surprisingly, a dramatic functional switch upon modification of specific cysteine residues has been observed for several mitochondrial carrier proteins. This reversible switch to an unphysiological transport mode of uncoupling efflux was first characterized for exclusive antiport carriers (AGC and AAC; see Dierks *et al.*, 1990a, b), but, interestingly, later on this was also found for the multifunctional CAC (Indiveri *et al.*, 1992b) and the PiC (Stappen and Krämer, 1993a). This functional conversion after addition of particular SH-reactive reagents is characterized by a complete inhibition of the "physiological" transport functions. By modifying an additional (second) cysteine, a unidirectional substrate flux can be induced, as clearly shown in particular for the AGC and the CAC. This reversible change in transport function is characterized by a more or less complete loss of substrate specificity which can in principle be interpreted in terms of a channel-type function. On the other hand, the obvious properties of a carrier protein, such as high activation energy as well as kinetic trans-effects of substrates, are retained. These results clearly indicate that the respective carrier proteins can change their transport mode from coupled antiport to unspecific uniport after chemical modification of specific amino acid residues. This new type of activity has been interpreted to indicate the presence of an intrinsic unspecific channel within the antiport carrier proteins, which is normally hidden by appropriate gates. This interpretation may be reconciled with the emerging general concept of the evolution of carrier proteins, starting from simple pores and evolving gradually to more and more complex structures. Thus, the basic function of a channel-type mechanism is still present also in the mitochondrial antiport carriers and may have been artificially brought to light by the modification of these particular cysteine residues.

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