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Review

Molecular aspects of isolated and reconstituted carrier proteins from animal mitochondria

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Contents

Abbreviations: *for the carrier proteins:* AGC, aspartate/glutamate carrier; CAC, carnitin carrier; CIC, citrate (tricarboxylate) carrier; DIC, dicarboxylate carrier; OGC, oxoglutarate carrier; ORC, ornithin carrier; PYC, pyruvate (monocarboxylate) carrier; UNC, uncoupling protein, H^+-carrier; *other abbreviations:* CHAPS, 3-[(3-cholamidopropyl)dimethylamino]propanesulfonate; $C_{12}E_8$, octo-ethyleneglycolmono-*n*-dodecyl ether; HA, hydroxyapatite; NEM, *N*-ethylmaleimide; HLB-number, hydrophilic/lipophilic balance; LAPAO, 3-lauramido-*N*,*N*-dimethylpropylamine oxide; SDS, sodium dodecylsulfate; CMC, critical micellar concentration.

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I. Introduction

The inner mitochondrial membrane carries a great number of important transport systems for different substrates such as adenine nucleotides, inorganic anions and cations, mono-, di- and tricarboxylic acids, amino acids and fatty acids. Some of these transport systems, especially those for anionic substrates, have been identified and characterized to a varying extent in the past 20 years.

In this review, we have chosen to concentrate on certain aspects of these carrier systems from animal mitochondria, laying special stress on their solubilization, isolation and/or reconstitution. We will not try to compile all the functional data of carriers, as elucidated in intact mitochondria, since excellent reviews have been published both on mitochondrial carriers in general [122,123,129,143,145,164], and also on some carriers in particular, namely ADP/ATP transport [54,76,77,81,177,178,180] and phosphate transport [50,54,152,184]. Instead, we will discuss on the one hand the 'state of the art' achieved in methods concerning solubilization, identification and purification of the mitochondrial substrate carriers and their characterization in the solubilized state, including data concerning the physical and chemical structure as obtained by analysis of the isolated proteins. On the other hand, we will emphasize studies of these proteins when incorporated into artificial membranes, i.e., after functional reconstitution.

In order to give a closer insight into the state of elucidation in the heterogeneous and sometimes controversial area of mitochondrial carriers, we will review the field by comparing the methods used for solubilization, purification, reconstitution and characterization, as well as comparing the results obtained by these methods. For separate treatment of the function of single carrier systems, the reader is referred to the reviews mentioned above.

To date at least ten transport systems for substrates have been identified in the inner membrane of animal mitochondria. These are the ADP/ATP carrier, the phosphate carrier, the aspartate/ glutamate carrier (AGC), the dicarboxylate carrier (DIC), the citrate carrier (CIC), the oxoglutarate carrier (OGC), the ornithin carrier (ORC), the pyruvate carrier (PYC), the glutamate carrier, the carnitin carrier (CAC) and the uncoupling protein (proton carrier, UNC). Other transport systems, e.g., the glutamine carrier and the neutral amino acid carrier are not yet well characterized. According to the electric nature of the transport reaction, the carriers can be divided into (i) electroneutral systems, i.e., the OGC, DIC, glutamine carrier and CAC; (ii) proton-compensated electroneutral carriers, i.e., the phosphate carrier, PYC, glutamate carrier, ORC and CIC; and (iii) electrophoretic transport systems, i.e., the ADP/ATP carrier, AGC and the UNC. The different types of carriers are not found to be equally distributed in mitochondria of different tissues. The ADP/ATP carrier, phosphate carrier and PYC on the one hand are ubiquitous, the UNC on the other hand is found exclusively in brown fat mitochondria. The other carriers are present only in mitochondria of certain tissues, in agreement with their metabolic function. For example, the activities of the CIC, the DIC and the glutamate carrier are high in liver but low in heart [128,159,166].

The first evidence for the existence of transport systems emerged from investigations on the interaction of added substrates with internal components and from swelling experiments with mitochondria in solutions of various salts [40]. A more detailed characterization of transport was achieved by measuring the distribution of the substrates between the intra- and extramitochondrial space and by investigating the kinetics of transport using the inhibitor stop method [85,86,144]. Obviously, the final proof for the existence of these carriers consists in general in their isolation, purification and functional reconstitution. Around 1970, several laboratories started to focus on the purification of the individual carriers. However, the purification was hindered by the lack of a functional assay for the solubilized transport proteins. In 1975 the ADP/ATP carrier was isolated as the first purified carrier protein by the use of adsorption chromatography on hydroxyapatite [34,87,155,156] in a form stabilized by the tightly bound inhibitor carboxyatractylate. The results of other attempts to identify isolated carriers on the basis of their affinity toward ligands are still questionable, e.g., in the case of the CIC [142] or the glutamate carrier [60]. On the other hand, the UNC has recently been unequivocally identified using its high affinity toward nucleotides [126].

All the other carrier proteins had to be identified solely on the basis of their activity in reconstituted systems. The first carrier protein to be reconstituted from a purified state was the ADP/ATP carrier [109,110]. Later the highly enriched phosphate carrier [98,183] and recently also purified preparations of the UNC [93], the OGC [18], the AGC [118] and the DIC [19,172] were also functionally reconstituted. In the course of these investigations, two experimental findings were of particular importance for the successful purification of mitochondrial carrier proteins. (i) The extraordinary usefulness of adsorption chromatography on hydroxyapatite, first applied for isolating the ADP/ATP carrier [87] and later also for most of the other carrier proteins. (ii) The purification of mitochondrial carriers in functionally active state is greatly facilitated by the addition of cardiolipin (DPG) during solubilization and/or isolation. This important effect was first

discovered in the purification procedure of the phosphate carrier [17,70], and is now generally used for the isolation of mitochondrial carrier proteins.

In summary, besides the ADP/ATP carrier, the phosphate carrier, the OGC, the UNC, the AGC, and the DIC, i.e., the mitochondrial carrier proteins which have been reconstituted from purified preparations, the CIC [169] and the PYC [136] have been reconstituted in partially purified state and the ORC [66] and the CAC [139] have been inserted into liposomes using mitochondrial extracts.

II. Solubilization and isolation of carrier proteins

II-A. Solubilization and the choice of detergent

The classification of detergents with respect to their effectivity in solubilizing membrane proteins on the one hand and their influences on the activity of the respective proteins on the other hand is a field controversely discussed in many papers and reviews [64,79,124,132, 190,192]. In this respect parameters like charge, critical micellar concentration (cmc), hydrophilic/lipophilic balance (HLB-number), and influence of pH and ionic strength on solubilizing effectivity, are used for classification of the detergent.

In the case of mitochondrial carrier proteins, mainly nonionic detergents with long polyoxyethylene tails characterized by low critical micellar concentration have been used for solubilization and purification (see Table I at the end of this section). Few exceptions from this general rule are the application of CHAPS for purification of the ADP/ATP carrier [24] and octylglucoside which has been used for enrichment of the UNC [171] as well as for solubilization of the CAC [139]. It has to be pointed out, however, that the 'standard preparations' of both the ADP/ATP carrier and the UNC are carried out with Triton X-100.

When discussing the choice of detergent for solubilization and purification, examples are of particular interest where the effects of various detergents were directly compared. Similar to other cases where detergent action on membrane proteins was studied, no general concept could be drawn, except perhaps for the necessity to use nonionic detergents with low cmc. The ADP/ATP carrier seems to have the broadest spectrum of detergents which can be used for solubilization and isolation [89]. The first successful isolation of the active protein was carried out with an aminoxide detergent (Aminoxide WS 35) [109], later on Triton X-100 [111] and pure LAPAO [35] was used, recently also $C_{12}E_8$ has been reported to solubilize an active ADP/ATP carrier [119]. In this case also the mild ionic detergent CHAPS can be used leading to a carrier protein with native conformation [24]. The UNC apparently prefers Triton X-100 but also tolerates the related detergent Emulphogen BC720 [127]. More polar detergents like LAPAO and especially cholate lead to inactivation [127,157]. Similar studies have been carried out for the OGC [18], the AGC [118] and the CIC [169]. It is very surprising and emphasizes the completely empirical type of these investigations that detergents which are really very closely related in their physical properties like, for instance, $C_{12}E_8$ and Triton, exhibit completely different effects on typical membrane carrier proteins such as the AGC where $C_{12}E_8$ proved to be much better than Triton, and the OGC, where Triton was successfully used and $C_{12}E_8$ could not be applied at all. Another point to be mentioned in this respect is the difference in use and effect of Triton X-100 and Triton X-114, which are very similar in their physical properties (e.g., HLB number). However, one can speculate on the basis of results presented in subsection II-B that there is a difference between the interaction of DPG with Triton X-100 and that with Triton X-114. It seems that binding of DPG to proteins is more favoured in Triton X-114 than in Triton X-100, thus leading to different results when these two detergents are used in solubilization and purification of membrane proteins, the activity of which depends on this special phospholipid (Palmieri, F., unpublished results).

The only reliable conclusion that can obviously be drawn from these data is that the suitability of detergents for purification of active carrier proteins is more or less completely empirical. Although the detergents can be arranged in a sequence of increasing solubilizing power with respect to mitochondrial membranes [78,89], this sequence differs for other membranes for unknown reasons and does not at all correlate with the effects of these detergents on the native state of the respective membrane proteins. Additionally, there are further factors such as pH and especially ionic strength, which are of importance for the application of detergents. Thus, for example, the observation that linear alkylpolyoxyethylene detergents are unable to disrupt mitochondrial membranes [79] was later found to hold true only in the presence of low or moderate ionic strength, whereas at high salt concentration also $C_{12}E_8$, for instance, is able to solubilize effectively the inner mitochondrial membrane [105,118].

The practical procedures used for solubilization of mitochondrial carrier proteins are generally simple. Usually isolated mitochondria or inner mitochondrial membranes are directly mixed with detergent solutions under defined ionic strength conditions. In some cases the membranes were preextracted with different detergents, e.g., for solubilizing the ADP/ATP carrier [90], the UNC [126] and the AGC [118]. Frequently, lipids have to be added during solubilization, mainly because they are able to stabilize carrier proteins in the solubilized state. These results will be discussed in the following subsection (II-B).

It should be pointed out that discussing the appropriate detergent for solubilizing a carrier protein is not only important with respect to the effectivity of solubilization and the ability of the detergent to retain the native structure and functional activity of the respective protein. There is at least one example, where the choice of detergent also determines the intrinsic conformational state of the carrier protein during solubilization and isolation [24], as will be discussed in more detail below (subsection IV-A).

II-B. Stability of solubilized proteins: role of lipids and ions

One of the main difficulties in handling mitochondrial membrane proteins is their instability in the solubilized state. Thus, the procedures developed for the isolation of functionally active carrier proteins are characterized on the one hand by efforts to minimize the time of exposure to the detergent and on the other hand by the development of various 'tricks' for the stabilization of the isolated carrier proteins.

There are a number of parameters influencing the stability of mitochondrial carrier proteins in detergent solution.

(i) One of the most important factors, the type of detergent used for solubilization, has already been discussed in the preceding section.

(ii) In many cases, addition of lipids is essential for isolating a functionally active carrier protein. This effect in some cases seems to be unspecific, i.e., addition of lipids generally improves the activity of the solubilized protein. This has, for example, been shown for mitochondrial extracts, including the partially purified CIC [169]. A very controversial and equally fascinating point is the apparently specific influence of cardiolipin on several mitochondrial carrier proteins during purification. This was first observed for the phosphate carrier [16,70,130], and later confirmed for the CIC [169], the OGC [18], the CAC [140], the ORC [66] and the DIC [72,162]. The reason for the stabilization by cardiolipin is not yet clear. Controversial results have been published especially in the case of the extensively studied effects on the isolated phosphate carrier. It has been stated that the increase in the activity of the purified phosphate carrier caused by cardiolipin is only due to increased yield of the carrier protein during column chromatography [184]. There is in fact a well-established effect of cardiolipin on the elution of several carrier proteins from hydroxyapatite (see discussion in subsection II-C). However, it seems clear that the presence of cardiolipin in the mixed micelles during isolation of the phosphate carrier enhances the activity of this carrier protein when reconstituted into liposomes [41,70,131,133]. Based on the published data, this observation can be interpreted both as a stabilizing and a stimulating effect [131] (see also subsection IV-C3).

(iii) The influence of ionic strength and of the type of salt added during solubilization and isolation also has to be taken into consideration. Preference for special ionic conditions or definitely negative effects of particular ions have been reported for the ADP/ATP carrier [79,88], the phosphate carrier [130], the UNC [127] and the AGC [118]. In general, addition of high salt concentrations seems to be advantageous, since it leads to reduction of the concentration of detergent necessary for solubilizing the membrane [88].

(iv) In some cases the presence of specific ligands improves the stability of the isolated carrier proteins. This observation is in agreement with the finding that tightly binding ligands in general stabilize the conformation of a protein in solution. This has been noted for the ADP/ATP carrier with respect to the inhibitor ligands carboxyatractylate and bongkrekate [81,88], for the UNC with respect to the tightly binding nucleotides [127] and for the CIC using the inhibitor ligand 1,2,3-benzenetricarbonic acid [168,169]. Stabilization of solubilized carriers by addition of binding ligands can, however, not be generalized, since it is known, for instance, that nucleotides, though substrate ligands of the ADP/ATP carrier, drastically labilize the isolated carrier protein [81].

(v) Due to its reactive SH groups, the phosphate carrier is sensitive to autoxidation and needs the presence of reducing agents for optimal transport activity (for discussion, see Ref. 184). Also the CAC has been solubilized in the presence of reducing agents [139]. In some cases, stabilizing agents such as ethyleneglycol, used for enrichment of the DIC [72], and glycerol, used during the purification of the phosphate carrier [11], improved the stability of isolated carriers from mitochondrial membranes.

II-C. The particular importance of hydroxyapatite for purification

The collection of methods used for purification of the various carrier proteins from mitochondria, as discussed in the next subsection (II-D), seems to be quite a boring list, because nearly always hydroxyapatite column chromatography is used. Ever since the first successful application of hydroxyapatite for isolation of membrane proteins solubilized in nonionic detergents [156], this procedure has proven to be the method of choice for these carrier proteins in general. What is the particular importance of this adsorbent for the purification of mitochondrial carrier proteins?

Although there are extensive theoretical and practical publications on the use and function of hydroxyapatite in adsorption chromatography (e.g., Refs. 58, 59 and

154) the question raised above can in fact not be answered adequately. It is assumed that ionic forces are responsible for the binding of proteins to hydroxyapatite, i.e., hydroxyapatite functions as a mixed-bed ion exchanger. Although intrinsic membrane proteins also exhibit charged amino acids at their surfaces which are normally exposed to the hydrophilic outer or inner side of the membrane, some membrane proteins of this kind, especially carrier proteins, do not bind at all to hydroxyapatite. It must be assumed that the charged amino acids are shielded from binding to hydroxyapatite by the large detergent shell associated with the hydrophobic part of the protein. This argument is further supported by the finding that partial or complete denaturation of the protein leads to increased absorption of the respective carrier to hydroxyapatite. This finding has been elegantly used, for instance, for the separation of the UNC from the ADP/ATP carrier under conditions where the latter protein is partially denatured and thus binds to hydroxyapatite (Refs. 95 and 126; see also Ref. 88). In the same line of arguments is the observation that there seems to be a critical minimum concentration of detergent, below which there is complete absorption of carrier proteins to hydroxyapatite. This general observation has recently been successfully applied for separating the solubilized OGC from the DIC, taking advantage of the fact that different amounts of Triton are necessary for these two carriers to cause binding or nonbinding to hydroxyapatite [19].

In general, mitochondrial carrier proteins are thus not bound to hydroxyapatite during purification procedures. This strategy, however, leads to complete purification of the respective carrier protein only under special conditions. Purification by this procedure was achieved in the case of the phosphate carrier using cardiolipin for specific elution [17], and in the case of the DIC [19] by pretreatment with amberlite in order to reduce the amount of bound detergent. The UNC was found to be nearly pure after an appropriate hydroxyapatite chromatography [126], taking advantage of the stability of this carrier under room temperature.

The other mitochondrial carriers, which have been purified so far, either need additional purification steps, or are bound to hydroxyapatite during purification. The latter type of procedure, i.e., binding to hydroxyapatite, was applied for isolating the phosphate carrier, which was eluted from hydroxyapatite by SDS and urea [100]. Also the AGC [118] and the OGC [19] have been purified after binding to hydroxyapatite. For purification of the AGC, a relatively complicated procedure had to be developed, applying hydroxyapatite-HPLC including salt and cardiolipin gradients. Only in the case of the phosphate carrier, some substantial and interesting variations in the isolation procedure have been introduced. The presence of a reactive sulfhydryl group was the basis for application of affinity chromatography during the purification of the phosphate carrier [43,73,175].

In this respect, an additional method has to be mentioned, namely the purification step using celite as adsorbant. This procedure was first introduced to separate the ADP/ATP carrier from the phosphate carrier in eluates from hydroxyapatite columns [98]. Especially when used in combination with hydroxyapatite, celite leads to complete binding and therefore extraction of the ADP/ATP carrier, the phosphate carrier and the DIC, whereas the OGC (and also the mitochondrial porin) pass through hydroxyapatite and celite [18,45].

Since purification of several mitochondrial carriers is improved by the addition of cardiolipin in various concentrations, a summary of the effects of this phospholipid should be given here. As already mentioned in the preceding section, cardiolipin sometimes enhances the stability of the proteins, i.e., the solubilized carriers retain high activity after reconstitution into liposomes. This more general use of cardiolipin applies to the phosphate carrier [70,130,131], the CIC [169,170], the DIC [19,72,162,172], the OGC [18], and the PYC [136]. Furthermore, the second effect of cardiolipin, i.e., the improvement of the elution of mitochondrial carriers from hydroxyapatite, can be summarized as follows: cardiolipin causes elution of the phosphate carrier [16,17], it causes retention of the contaminating porin from the outer membrane [17,44,45]; cardiolipin leads to retarded elution of the ADP/ATP carrier [17], and it has different effects on the elution of the AGC, depending on the type of salts used in the elution buffer [118].

II-D. Purification and identification: an overview

It is of course beyond the scope of this review to describe the isolation and purification of every single mitochondrial carrier protein in detail. By comparing the general strategies and elaborating the common results in these procedures, the present 'state of the art' in this field will be outlined (see also subsection IV-C2).

When purifying a protein, it is essential to establish a method for its detection during the isolation. This is in fact a basic problem in the case of carrier purification because the activity to be monitored actually is transport, and this requires the presence of two separate compartments. Thus, the purification of a carrier protein has, in general, to be followed by the method of functional reconstitution, which is not only laborious but also leads to considerable inaccuracy. The transport activity measured in the reconstituted system is by no means dependent only on the amount of carrier protein to be identified, but possibly also on the presence and concentration of other proteins and specific lipids and on the ion surroundings. Thus the values of specific activity, used as an indicator for purification during the

TABLE I

SELECTED DATA OF SOLUBILIZATION, ISOLATION, IDENTIFICATION AND STATE OF PURITY OF MITOCHONDRIAL CARRIER PROTEINS

The sources for isolation of the carriers are BHM, bovine heart mitochondrial; BFM, brown fat mitochondria; BLM, bovine liver mitochondria; PHM, pig heart mitochondria; RLM, rat liver mitochondria; AAC, ADP/ATP carrier; PIC, phosphate carrier; DPG, cardiolipin.

Carrier		Source	Solubilization		Purification			State of purity (SDS-PAGE)	Identification	Remarks	References
			detergent	lipid	procedure	addition	factor (see Remark 1)				
AAC	(1)	BHM	TX-100	–	HA-chrom./ gel-filtr.	–	about 10	pure (30 kDa)	inhib. bind. not functionally active		155, 156
	(2)	BHM	TX-100	–	HA-chrom.	–	about 7	70–80% pure	reconstitution		108–110
	(3)	BHM	various det	–	HA-chrom. and others	–	7–10		inhib. bind. and reconst.	2	see remark 2
AGC	(1)	BHM	$C_{12}E_8$	–	HA-chrom./ HA-HPLC	DPG	600	major band at 64 kDa	reconstitution		118
CAC	(1)	RLM	OG	Asolectin	–	–	–	extract	reconstitution		139
CIC	(1)	RLM	TX-100	DPG	HA-chrom.	DPG	25	$\geq$ 5 bands	reconstitution	3	150, 169
DIC	(1)	RLM	TX-114	DPG	HA-chrom.	DPG	226	6 bands (30–37 kDa)	reconstitution		72
	(2)	RLM and others	TX-114 TX-100	DPG	HA-chrom.	DPG	10.5	6 bands (30–36 kDa)	reconstitution		162
	(3)	BHM	TX-100	DPG	HA-chrom./ affin. chrom.	DPG	1350	2 bands (34 + 36 kDa)	reconstitution		172
	(4)	RLM	TX-114	–	HA-chrom./ Celite chrom.	DPG	226	pure (28 kDa)	reconstitution	4, 5, 6	19
OGC	(1)	PHM	TX-114	–	HA-chrom./ Celite chrom.	DPG	247	pure (31.5 kDa)	reconstitution		18
	(2)	BHM	TX-114	DPG	HA-chrom.	DPG	155	> 10 bands	reconstitution		137
	(3)	RLM	TX-114	–	HA-chrom.	DPG	165	pure (32.5 kDa)	reconstitution	4	19
ORC	(1)	BLM	Lubrol WX	–	AS-precip.	–	11	extract	reconstitution		66
PIC	(1)	BHM	OG	–	As-prec.		56	> 10 bands	reconstitution	7	10, 11
	(2)	BHM	TX-100	–	HA-chrom.	–	–	2 bands (30 + 34 kDa)	reconstitution		183
	(3)	PHM	TX-100	–	HA-chrom./ Celite chrom.	–	–	4 bands	reconst./NEM-bind.		98

	(4)	PHM	TX-100	–	Mersalyl/ HA-chrom.	–	–	4 bands	SDS-PAGE	8	175
	(5)	RLM	TX-100	–	HA-chrom.	–	–	2 bands (30 + 35 kDa)	reconstitution		182
	(6)	PHM	TX-100	DPG	HA-chrom./ affin. chrom.	DPG	–	pure (33 kDa)	reconst./NEM-bind.		43
	(7)	BHM	TX-100	–	HA-chrom.	–	–	pure (34 kDa)	SDS-PAGE	9	100
	(8)	PHM	TX-114	–	HA-chrom.	DPG	290	pure (33 kDa)	reconstitution	10	17
	(9)	RLM	TX-100	DPG	HA-chrom./ DEAE/affin. chrom.	DPG	161	pure (33 kDa)	reconst./NEM-bind.		73
	(10)	RLM	TX-114	–	HA-chrom./ Celite (Procion red)	–	–	pure (34 kDa)	NEM-bind./ (antibody)	11	57
PYC	(1)	BHM	TX-114	Asolectin	HA-chrom.	Asolectin	125	$\geqslant$ 6 bands	reconstitution	12	136
UNC	(1)	BFM	TX-100	–	HA-chrom./ ultracentr.	–	16	pure (32 kDa)	reconst./GTP bind.		95, 126, 158

Remarks:

(1) Purification factors are in most cases (except for the AAC and the UNC) based on determination of reconstituted transport activity. They can therefore only be taken as qualitative measure (see discussion in subsection IV-B2).

(2) Many different isolation procedures besides the first one leading to a purified protein [155,156] have been reported, including solubilization by LAPAO [24,35] and CHAPS [24], including preparation of inhibitor ligand complexes with carboxyatractylate [34,87,88,90], bongkrekate [2], and acyl-CoA [188].

(3) Stabilization by 1,2,3-benzenetricarbonic acid.

(4) Additional treatment with amberlite necessary.

(5) Although the DIC has been purified to homogeneity in this preparation, functional reconstitution has to be carried out with another fraction containing small amounts of high-molecular-weight contaminants [19].

(6) A purification procedure starting from liver mitochondria and applying dye-affinity chromatography which leads to a single protein band of 28 kDa in SDS-gels was reported by Nalecz, M.J., Szewczyk, A. and Broger, C., at a recent meeting (1988) at Zakopane, Poland.

(7) The missing data for purification factors of various phosphate carrier preparations are mainly due to difficulties with reconstitution as monitor for purification.

(8) The originally reported pure protein [175] was later shown to consist of several protein bands on SDS-gels [99].

(9) Not functionally identified in this paper [100], however, in a later publication a similar preparation was shown to be reconstitutively active [187].

(10) The purification factor was calculated on the basis of unpublished experiments (Bisaccia, F. and Palmieri, F.) using a phosphate carrier preparation identical to that reported in Ref. 17.

(11) Not functionally identified, antibody inhibits only to about 30%.

(12) By the same authors an improved procedure applying affinity chromatography on cyanocinnamate columns which leads to a single protein band of 34 kDa in SDS-gels was reported at a recent meeting (1988) at Zakopane, Poland.

isolation of carriers, have to be regarded with great caution and should only be used as a qualitative measure. In some cases, the purification could be quantitated by monitoring binding of specific ligands, e.g., carboxyatractyloside to the ADP/ATP carrier [155,156], or GTP to the UNC [126]. More or less specific binding of SH-reagents was used during purification of the phosphate carrier [43,49,100].

The general purification scheme involves (i) solubilization of the mitochondrial membrane with nonionic detergent, in many cases in the presence of phospholipids (see subsection II-B); (ii) chromatography on hydroxyapatite, recovering the carrier protein in the eluate; and (iii) final purification involving further chromatographic steps on celite, hydroxyapatite, affinity columns or ultracentrifugation. The particular importance of hydroxyapatite in the first and sometimes single purification step has already been discussed in detail in the preceding section. It is, however, useful for the comparison of data on carrier purification, as summarized below, to recall a representative polypeptide pattern of hydroxyapatite elution using solubilized heart mitochondria, which is observed when no additional 'tricks' are applied, like addition of cardiolipin or reduction of the amount of detergent. This general pattern is shown in Fig. 1. Four of the five protein bands routinely seen in high-resolution SDS-gels of hydroxyapatite eluates have been identified so far [17,18,45]. Band '1', which is not present in the first fractions is not yet identified. Band '2' represents the mitochondrial porin, band '3' the phosphate carrier, band '4' the OGC, band '5' the ADP/ATP carrier, which usually exceeds the other proteins by at least one order of magnitude. The DIC is eluted only under special conditions [19], the AGC appears to have a very different molecular weight [118]. The UNC is only present in brown fat mitochondria where it represents the major protein band. The other carriers have not yet been correlated to single polypeptides in SDS-gels.

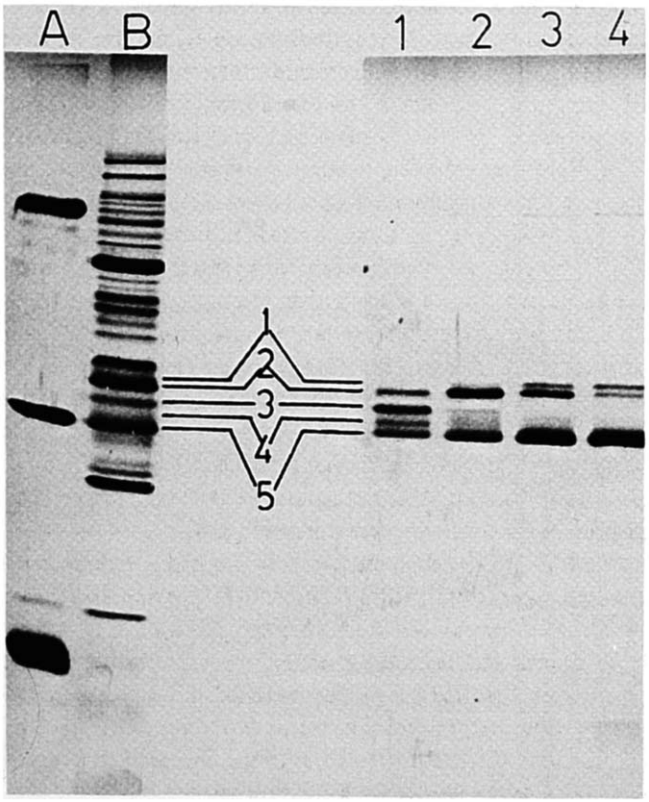

Fig. 1. SDS gel electrophoresis of the eluate fractions obtained by hydroxyapatite chromatography of heart mitochondria solubilized with Triton X-114. Chromatography was carried out without added cardiolipin. (A) Marker proteins (bovine serum albumin, carbonic anhydrase, cytochrome *c*). (B) Mitochondrial extract; 1–4, fractions 1–4 of the eluate from the hydroxyapatite column. For further experimental details, see Ref. 17.

At the end of this section, representative procedures for solubilization, purification and identification of the mitochondrial carriers are summarized in Table I. Some explanatory details are given as 'remarks'. For the detailed purification procedures the reader is referred to the cited literature. The list is incomplete in the case of the ADP/ATP carrier. As already pointed out in the introduction, a separate review would be necessary to summarize the complete data on the work done with respect to purification and characterization of this particular carrier protein. The procedures mentioned in the Table for identification by reconstitution and the characterization of these carriers are discussed in detail in subsection IV-C2.

III. Physical and chemical characterization of the isolated carrier proteins

III-A. Physical characteristics of solubilized proteins

Mainly two carrier proteins have been investigated in the solubilized state with respect to their shape within the mixed micelle, their state of aggregation and the amount of bound detergent and lipid, mainly on the basis of their hydrodynamic properties.

The ADP/ATP carrier has been studied in the stabilized form of the carboxyatractylate-protein complex. Gel permeation chromatography and hydrodynamic methods led to an M_r of 180 000 [61]. This value had to be corrected for the high amount of bound detergent, which was evaluated to be 150 mol Triton X-100/mol carrier protein, i.e., 1.47 g detergent per g protein. Phospholipid binding was determined to be 0.25 g per g protein. In these hydrodynamic studies the state of aggregation of the ADP/ATP carrier was found to be the dimeric form. When the total molecular weight evaluated for the detergent/protein micelle was corrected by appropriate methods for bound detergent, a molecular weight of the residual dimeric protein (M_r 64 000–67 000) was found which exactly correlates with the molecular weight obtained later from the primary structure. The data were interpreted in a model showing

an oblate ellipsoid with a central axis identical to the axis of the dimeric protein [61].

Very similar results were obtained with the isolated UNC [125]. The authors determined a detergent binding of 180 mol Triton X-100 per mol carrier dimer. The shape derived for the protein/detergent micelle was nearly identical to that of the AAC. It is interesting to note that very similar results were also obtained for the phosphate carrier from the chloroplast envelope membrane [52], indicating a common structure of these proteins in dimeric form embedded in a shell of nonionic detergent.

III-B. Lipid interaction in the solubilized state

Although lipid interaction is obviously an important point in the characterization of these hydrophobic carrier proteins, only few data are available. A promising step in the development of methods for elucidating lipid–protein interaction of solubilized and membrane-integrated carrier proteins has recently been reported. By using NMR [13–15] and ESR techniques [15,134] the influence of the hydrophobic surrounding on the conformation and mobility of the carrier protein could be quantitatively examined. These studies are in general directed towards a more fundamental elucidation of lipid—protein interaction and are thus beyond the scope of this review. However, at least in one case they have led to a very important finding of specific lipid binding to a mitochondrial carrier protein (see below).

It has already been mentioned that cardiolipin is important in generally stabilizing mitochondrial carrier proteins, e.g., the phosphate carrier, the CIC, the DIC and the OGC, during isolation and solubilization (see subsection II-B). However, one of the carrier proteins, and interestingly enough it is the ADP/ATP carrier, i.e., just one which is not stabilized by cardiolipin during isolation (Krämer, R., unpublished results), was found to bind cardiolipin very tightly in the solubilized state. By high resolution ^{31}P-NMR studies, as mentioned above, it was shown that, besides loosely bond phosphatidylcholine and phosphatidylethanolamine, about six molecules of cardiolipin remained tightly bound to the dimeric protein during isolation [14]. In general, when carrier proteins are isolated in nonionic detergent, 'unspecific binding' of phospholipids is observed, i.e., the presence of about 10–40 mol phospholipid per mol carrier protein within the mixed micelle. In the case of the isolated ADP/ATP carrier, however, the distinct number of cardiolipin molecules showed unusually tight binding, since they could not be removed by treatment with SDS unless additional denaturation by heat was applied.

There are several reports on specific binding of cardiolipin to membrane proteins of the inner mitochondrial membrane [8,56,160]. Compared to these results, the particular importance of cardiolipin binding to the ADP/ATP carrier becomes obvious when taking into consideration the unusually high amount of phospholipid bound per mol protein, exceeding by far, for example, the amount which is bound to cytochrome oxidase. In this respect it has to be emphasized that the observation of tight cardiolipin binding to the ADP/ATP carrier presumably does not correspond directly to results obtained in reconstituted proteoliposomes which demonstrate stimulation of the ADP/ATP carrier activity by this phospholipid also (see subsection IV-C3).

III-C. Ligand binding and chemical modification in the solubilized state

In general, covalently binding ligands, e.g., SH-group specific inhibitors, can be used to identify or at least to label solubilized carrier proteins. Although this type of binding is in general not specific, considerable effort has been made in the case of the phosphate carrier and the ADP/ATP carrier to analyze the binding of these inhibitors. It should be pointed out here that this review is restricted to investigations using the solubilized carrier proteins and does not cover the multitude of publications on this topic in which labeling of intact mitochondria is reported.

As far as we know, the phosphate carrier has six cysteins per monomer, and only one of them (Cys^{42}) reacts readily with *N*-ethylmaleimide when the protein is in the native state [101]. Further attempts to correlate SH-group modification by oxidation or by covalent modification of the solubilized and/or reconstituted phosphate carrier with structural (state of aggregation) and functional properties (activity) of this carrier have so far not led to conclusive results beyond those obtained by studies with intact mitochondria. The interpretation was put forward that the differing reactivity of the phosphate carrier to SH-modification in the solubilized versus the membrane-integrated form is due to a change between the dimeric and monomeric state of the transporter [67]. This hypothesis, however, cannot easily be distinguished from possible direct effects of the membrane on the reactivity of the respective SH-groups, as has already been stated [184].

Binding of both noncovalent ligand analogs and covalent ligands to the ADP/ATP carrier has been investigated in numerous studies. The aim of these experiments was directed mainly towards elucidation of the carrier function, i.e., the mechanism of conformational transition within the catalytic cycle. Therefore we will discuss these studies in section IV, as far as they were done with the isolated carrier protein.

The same holds true for investigations concerning nucleotide binding to the UNC, which proved to be an interesting tool for the elucidation of the intrinsic mechanism of this carrier (see subsection IV). Besides this, the purified UNC was studied with respect to the kind of amino acids which interact with nucleotides at the

active site of this carrier protein. Experiments using covalent interaction with Rose Bengal (photooxidation), trinitrobenzenesulfonic acid and phenylglyoxal suggested the participation of lysine in the binding of nucleotides to the nucleotide binding center [127].

A promising step towards localisation and characterization of the active site of the phosphate carrier was achieved by the use of 4-azido-2-nitrophenyl phosphate, a photoreactive analogue of phosphate [174]. This compound not only binds to the active site of the phosphate carrier but also inhibits the transport activity in reconstituted proteoliposomes when covalently bound to this carrier protein. It should be mentioned that also the binding site of the relatively unspecific transport inhibitor NEM at the phosphate carrier has recently been localized [101]. By sequencing the N-terminal formic acid fragment of the ^{3}H-NEM-labeled phosphate carrier, binding of NEM to Cys^{42} could be determined.

III-D. Primary structure and conformation

Although studies based on the primary structure of mitochondrial carrier proteins require the use of isolated and purified proteins, at least in the case of amino acid sequence determination, they are nevertheless from a methodological point of view quite distant from the aim of this publication. Sequence determination, establishment of hydropathy profiles and detection of secondary structure elements are beyond the scope of this review as discussed in the introduction. Additionally, it has to be pointed out that especially this topic, i.e., the comparison of mitochondrial carrier proteins on the basis of their primary and secondary structure, has already been excellently reviewed very recently [5,6,97,161,184]. Thus, these results will only be outlined in brief.

It has already become obvious in the former chapters of this section that the availability of substantial structural data is mainly restricted to the ADP/ATP carrier, the UNC and the phosphate carrier. The primary structure of the ADP/ATP carrier from bovine heart mitochondria was determined already in 1982 [3]; this was in fact the first amino acid sequence of a metabolite carrier. Since then the primary structure of the ADP/ATP carrier carrier from other sources, namely from *Neurospora crassa* [7], from *Saccharomyces cerevisiae* [1] and from *Zea mays* [9] have been established by cDNA sequencing. The amino acid sequence of the UNC and the phosphate carrier were determined both by amino acid analysis (Refs. 5 and 6; 92% of the sequence in the case of the phosphate carrier) and DNA sequencing [30,161]. The comparison of the primary structure of these three carrier proteins, including comparison of the ADP/ATP carrier from different sources, is currently one of the most fascinating topics in the field of carriers and membrane proteins. The three carriers show striking similarity not only in direct comparison of the primary sequence but also when showing the unique tripartite structure [6,161,163]. Preliminary data on the primary sequence of the isolated OGC not surprisingly again show similarity to the carrier proteins mentioned above (Runswick, M.J., Walker, J.E., Bisaccia, F. and Palmieri, F., unpublished results). Even more striking are the similarities when the secondary structure is compared. The analysis of secondary structure depends mainly on hydropathy profiles and, in the case of ADP/ATP carrier, also on investigations of the transmembrane folding by covalent labeling of lysine residues [27,28]. The nearly identical pattern of the arrangement derived for the membrane-integrated polypeptide chains of these three carrier proteins (ADP/ATP carrier, phosphate carrier, UNC) led in fact to the suggestion of a common model for mitochondrial substrate carriers [5,6,97,161]. According to this model, the mitochondrial metabolite carriers, including the UNC, should form a 'family' of proteins with similar secondary structure, possibly originating from a common ancestor.

In connection with these ideas about the folding pattern of carrier proteins integrated into the biological membrane, the results obtained on the state of aggregation of these proteins should also be summarized. As already pointed out in subsection III-A, the isolated carrier proteins from mitochondria, as studied by hydrodynamic methods, have so far been found to be dimers in the solubilized state, surrounded by a detergent shell [61,125]. It has to be mentioned that besides these results obtained with isolated proteins, there are other data supporting this general view, mainly concerning an observed 'half-the-site reactivity' towards specific substrates and/or inhibitor ligands. This has been shown for the ADP/ATP carrier [76] and the UNC [127] by binding of specific ligands and also in the case of the phosphate carrier as derived from the reactivity of SH-groups [53]. However, it should be noticed that on the basis of binding experiments using different adenine nucleotide analogues it was concluded that the ADP/ATP carrier in the mitochondrial membrane is organized as a functional tetramer [23]. Thus, although not unequivocally proven, there is good evidence that the mitochondrial carrier proteins in fact exist as functional dimers in the membrane. These results have been summarized in an interesting general concept making plausible the occurrence of a common dimer structure for carrier proteins [78].

IV. Functional analysis of the solubilized and reconstituted carrier proteins

IV-A. Functional analysis in the solubilized state

Although it is obvious that the genuine function of a carrier protein cannot be analyzed in the solubilized

state, there are some examples, especially in the case of the ADP/ATP carrier, where the solubilized protein proved to be well suited for investigations of the conformational state and of special properties of the substrate binding site. It should be pointed out again that the numerous and important studies done on the interaction of covalent and noncovalent binding ligands with intact mitochondria cannot be considered in this review.

In this respect two points already mentioned in other sections shall be discussed here. As described in subsection II-B, the conformation of the ADP/ATP carrier in the solubilized state seemed to be definitely influenced by the type of detergent used for its isolation [24]. A shift from CHAPS to LAPAO as solubilizing agent concomitantly shifted the distribution of carrier proteins that are either in the *c*- or in the *m*-conformation drastically towards the latter. Another result elucidated with the solubilized protein has already been discussed in detail in subsection III-B, namely that a definite amount of cardiolipin tightly bound to the solubilized ADP/ATP carrier has been detected by NMR-techniques [14].

Starting from early observations that the ADP/ATP carrier can be isolated in alternative forms with different inhibitors bound (for a review, see Ref. 81), the isolated protein was used as a tool to study the conformational change between the two states in solution. This was carried out on the one hand by replacing the labelled inhibitor bonkrekic acid (protein in the *m*-state) in the solubilized protein by labelled carboxyatractyloside (protein in the *c*-state) [2]. The same was shown to hold true for the other direction, i.e., replacing labelled atractyloside (*c*-state) by labelled bongkrekic acid (*m*-state) [91]. These results were expanded by experiments concerning the stability of the isolated ADP/ATP carrier against proteases, as well as by experiments using SH-reagents [4] and amino acid-specific reagents (for reviews, see Refs. 81 and 181). Although it is not within the scope of this review, it should at least be mentioned that active site labeling of the ADP/ATP carrier in intact mitochondria has been extensively studied (for reviews, see Refs. 81 and 179). This experimental approach led to interesting results especially for localizing the atractyloside binding site [25,31,179] and the NEM binding site [32]. Very recently, ESR experiments with intact mitochondria and also with the solubilized ADP/ATP carrier have been reported [135] using spin-labeled maleimides which in principle should bind to the same Cys^{56} as determined in Ref. 32. These studies, which led to conclusions concerning the geometry of the surroundings in the vicinity of the maleimide binding site, are a first step in the application of these physical methods for elucidating intrinsic properties of carrier proteins.

The most interesting experiments concerning the conformational change of the isolated ADP/ATP carrier and the characterization of its binding sites were based on fluorescence measurements [22,26,82]. The two conformational states of the carrier could be discriminated in the solubilized state by monitoring the intrinsic fluorescence of the tryptophan residues of the isolated ADP/ATP carrier [36,38]. Although substrate-induced fluorescence changes, i.e., influences on the carrier conformation, could clearly be determined, these studies were complicated by the small difference of fluorescence between the two conformations, which usually does not exceed 5%–10%.

More recently, nucleotide analogues, i.e., naphthoyl-3′-*O*-esters of ADP and ATP were introduced, which led to much better signal discrimination [21,22]. As in the case of another fluorescent derivative of ATP, namely formycin triphosphate, mainly binding studies with these probes were carried out, leading to two and four classes of binding sites at the solubilized protein, respectively [21,37,49]. Since these results do not agree with functional studies of binding sites during the normal action of the carrier protein, the higher number of binding sites has been interpreted in terms of different interactions of the ADP/ATP carrier with transportable and nontransportable nucleotides.

Furthermore, valuable fluorescent probes for the isolated ADP/ATP carrier proved to be the dimethylaminonaphthoyl (dansyl) derivatives of nucleotides. Due to favorable fluorescent properties, i.e., low fluorescence in buffer alone, relatively high change in fluorescence on protein binding and strong discrimination between the two functional states of the carrier, these probes are well suited tools for measuring the true distribution between *c*- and *m*-state of the solubilized ADP/ATP carrier [92], as well as the kinetics of the transition between the two states. It has to be mentioned, however, that there remained difficulties in interpreting the binding specificity of the dansylated nucleotides, since the affinity of the dansyl derivative of AMP turned out to be higher than that of ADP and ATP, whereas AMP is no physiological substrate of the ADP/ATP carrier. By using these fluorescent probes, the influence of environmental factors, such as pH, anions, phospholipids, and temperature could be determined on both the extent of the fluorescence and the kinetics of the fluorescence changes [82,92].

The dimethylaminonaphthoyl derivatives have further been applied for studying the nucleotide binding characteristics of the isolated and solubilized UNC [80,82] and have been successfully used to elucidate the properties of this carrier protein. For this purpose, equilibrium dialysis and a rapid anion-exchange procedure have been applied [96]. Interesting results could be obtained by analyzing the pH dependence of nucleotide binding to the solubilized UNC [80,83,84]. The dissociation constant for binding of the nucleotides ATP, ADP and GTP varied by about two orders of magnitude

when the pH was changed from 4.6 to 7.5. By considering the pK values of the nucleotides and by careful analysis of slopes and 'break points' in the change of measured binding constants of nucleotides to the UNC over this pH range, the binding affinity for protons at the active site of the UNC and the intrinsic affinities for nucleotides could be derived. On the basis of these results a binding site model involving both an anionic (Glu or Asp) and a cationic amino acid (Lys or Arg) could be developed, including additionally the regulating influence of a histidine residue.

IV-B. Reconstitution: practical aspects

IV-B1. Methods for the functional reconstitution of mitochondrial carrier proteins

Most carrier proteins isolated from mitochondria are relatively labile. Thus, methods used for reconstituting these proteins into phospholipid membranes must be fast and effective in creating favorable surroundings stabilizing the conformation and/or function of the isolated carrier proteins. The method of choice should therefore be (i) fast enough so that an immediate transfer of the proteins from the detergent micelle into the phospholipid bilayer is possible; and (ii) mild enough so that denaturation during the reconstitution procedure can be avoided; the method should (iii) lead to more or less complete removal of the detergent from the surroundings of the membrane protein.

A long list of different techniques exists for the reconstitution of detergent-solubilized proteins (for reviews, see Refs. 39, 65, 138 and 153). Out of this list for quite a long time only one single method was used for the reconstitution of mitochondrial carrier proteins, namely the freeze/thaw/sonication method, first developed for the glucose carrier from erythrocytes [74] and the ADP/ATP carrier from inner mitochondrial membranes [107,109]. Since then most of the other mitochondrial carriers have also been reconstituted by the use of this method: the phosphate carrier [98,130,182,183], the CIC [150,168,169], the OGC [18,68,137], the DIC [72,162,172], the AGC [105,118], the ORC [66], and the PYC [136]. In some cases the last sonication step could be omitted, for example in the reconstitution of the ADP/ATP carrier [189] and the phosphate carrier [187].

In general, dialysis methods for reconstitution of carrier proteins would be very favourable due to controlled conditions and reproducible size and homogeneity of the proteoliposomes. However, it has always been a disadvantage that these dialysis methods could in general not be applied to the labile carrier proteins, due to the long exposure times to detergents with high critical micellar concentration which were necessary when using these reconstitution procedures. Only the phosphate carrier has been successfully reconstituted by the classical dialysis method using CHAPS as dialyzable detergent [187]. The activity of the phosphate carrier reconstituted by this method was relatively low when compared to proteoliposomes prepared by the freeze/thaw/sonication technique. However, different protein preparations were used in these studies.

In recent years methods for detergent removal have also been applied to some of the mitochondrial carrier proteins. It has long been known that nonionic detergents can be removed from solubilized proteins by the use of absorption to polystyrene beads. However, this method is not readily applicable in functional reconstitution of membrane proteins, since direct removal normally leads to aggregation and inactivation of the carrier proteins. Thus, reconstitution of mitochondrial carriers in a functionally active state was only possible by modifying these methods, leading to controlled and relatively fast removal of the detergents from the solubilized proteins with a simultaneous integration of the carriers into the phospholipid bilayer. Methods of this type have been developed for the UNC [29,93,94], and in an elaborate procedure for the AGC and the ADP/ATP carrier, consisting of a controlled recycling chromatography on polystyrene beads [119]. This method has then also been applied to the OGC [69] and the DIC [19].

It has to be mentioned that also the CAC has been reconstituted from solubilized membranes by a procedure of this kind [139]. Furthermore, this carrier is the only example where a detergent dilution method using octylglucoside as solubilizing agent has been successfully applied [139].

Especially the data published for the AGC and the ADP/ATP carrier demonstrate that the reconstitution method of fast and controlled detergent removal by recycling chromatography on polystyrene beads [69,119,176] fulfills the criteria listed above as essential for effective reconstitution of labile mitochondrial carrier proteins.

In all these experiments mainly two types of lipids have been used, i.e., soy bean lipids (asolectin) and egg yolk phospholipids. The specific influence of certain kinds of phospholipid on the activity of reconstituted carrier proteins is discussed in detail below (see subsection IV-C3).

IV-B2. Efficiency of reconstitution

The efficiency of reconstitution of mitochondrial carrier proteins has considerably improved since the first reports. Nevertheless, one has to take into account that there is always a severe problem when discussing reconstitution efficiencies: the calculations are based mainly on measurements of the transport activities. These values are, however, by no means reliable or accurate. On the one hand, they may be underestimated due to several facts, since the surroundings of the protein may

not be ideal when compared to the original mitochondrial membrane or some unknown stimulating factors may be missing (see discussion in subsection IC-C4). On the other hand the obtained values may also be overestimated due to particular activation by special conditions of the proteoliposomal surroundings (see subsection IV-C4). In general, it is more likely that the values derived for transport activity in the reconstituted systems are underestimated due to constraint caused by the small size and internal volume of the phospholipid vesicles and also due to inappropriate hydrophilic and hydrophobic surroundings of the protein inserted into the membrane. Therefore, the calculated values for the efficiency of reconstitution in general represent the lower limits of the true situation, when compared to the original activity observed in intact mitochondria. However, when the activity of reconstituted crude mitochondrial extracts is used as a basis for comparison, the values for the efficiency of reconstitution are in general overestimated, since the transport activity of carriers reconstituted from crude extracts is often disproportionately low. This may explain the frequently made observation of an increase in the total carrier activity during the purification procedure (see, e.g., Refs. 72, 118 and 137).

It has further to be mentioned that useful estimations of the efficiency of reconstitution can only be made with purified or at least highly enriched proteins, since any unknown and presumably undesired interactions may occur in crude and undefined extracts. The values given for reconstitution of the CAC and the ORC from solubilized mitochondrial membranes which amount to 3% [139] and 18% [66], respectively, of the original mitochondrial activity have thus to be considered in this light.

Taking into account the restrictions discussed above concerning the significance of reconstituted transport activities (in comparison to the original activity in mitochondrial membranes), the efficiency of reconstitution for the purified carriers can be summarized as follows. The turnover numbers of the reconstituted OGC and the reconstituted DIC have been determined to amount to about 700 min^{-1} [68] and 300 min^{-1} (Indiveri, C. and Palmieri, F., unpublished results), respectively, at room temperature. These values are of the same order of magnitude as those reported for the ADP/ATP carrier and the AGC. However, they cannot be directly compared to the original mitochondrial activity, since the turnover numbers in mitochondria cannot be determined. A calculation of the true molecular activity would only be possible if the original amount of the carrier within the mitochondrial membrane could be estimated. Determinations of this type, when possible, have to be carried out by using specific ligands, which in general have to be applied radioactively labelled. This was possible in the case of the ADP/ATP carrier on the basis of binding data with the labelled inhibitors (carboxy) atractylate [35,81,111], and in the case of the UNC by binding of labelled GTP [63,93]. Another successful titration of a carrier protein was carried out by specifically labelling SH-groups in the case of the phosphate carrier [42,55,62].

It has been estimated that the majority of the reconstituted UNC is in fact functionally active [93]. This was based on a comparison of the rate of H^+-flux in mitochondria and reconstituted liposomes in relation to the amount of bound GTP, which is a tightly binding inhibitor ligand of the UNC.

A similar estimation has been carried out for the phosphate carrier, although in this case the determination of molecular activity in intact mitochondria is somewhat uncertain because it is based on titration of specific SH-groups. The molecular activity, calculated in reconstituted proteoliposomes to be about 14000 min^{-1} [186], nearly matches the corresponding molecular activity as estimated in intact mitochondria [184]. This would mean that in the case of the phosphate carrier also the majority of reconstituted protein molecules are active in the reconstituted system.

Obviously, the situation is not so favourable in the case of the ADP/ATP carrier. This carrier has been most extensively investigated with respect to the effectiveness of reconstitution. Elaborate titrations of transport activity were correlated to inhibitor binding, which is in fact a reliable method for determining the molecular activity of the reconstituted protein. These determinations led to the result that normally only 5–10% of the reconstituted carrier proteins are active in the original function [35,111]. In the course of these experiments, an important observation was made, which again confirms the precautions necessary when interpreting data of reconstitution efficiency, as mentioned above. It was found that, based on the measured values of reconstituted activity of the ADP/ATP carrier, the specific activity in the reconstituted system under optimal conditions with respect to phospholipids and ions nearly matches the original specific activity as determined in mitochondria. However, when titrating this activity with the tightly binding ligand carboxyatractylate, it was surprisingly found that the observed transport activity could be correlated with only about 10% of active carriers. These carrier molecules, however, showed a value for the turnover number about 10 times higher than that calculated in mitochondria. Similar titration experiments have also been carried out using labeled atractyloside [35].

Thus, in conclusion, it can at least be said that the mitochondrial carrier proteins reconstituted so far from purified preparations show significant activity which in all cases is definitely above the borderline of experimental significance. In some cases the reconstitution seems to be very effective in reaching the same molecular

activity as that of intact mitochondria. However, due to difficulties in the interpretation of data from reconstituted systems in comparison to intact mitochondrial membranes, exact quantitative values for the efficiency of reconstitution cannot be given.

IV-B3. State of analysis in reconstituted systems

The expression reconstitution actually covers a very broad spectrum of methods and may describe quite different states of analysis of protein function. Reconstitution in the correct sense of the word includes a purification step before incorporation of the isolated protein back into an artificial membrane. This, however, applies only to some of the mitochondrial carrier proteins.

Thus, the CAC and the ORC, which have only been solubilized in crude preparations, will not be treated further in the following section on results of reconstitution studies. Few data are available on the PYC, the reconstitution of which was done from an enriched preparation. The remaining carrier proteins have all been purified (except the CIC) and were identified, during the isolation procedure, by reconstitution. Preliminary functional data have been published for the DIC and the CIC, somewhat more complete findings in the case of the phosphate carrier, the OGC and the AGC. Many experimental results from reconstitution studies are available for the UNC and particularly elaborate data have been published in the case of the ADP/ATP carrier. The latter examples will therefore form the basis for discussing the analysis of carrier mechanisms by functional reconstitution in the following section.

IV-C. Analysis of carrier function in reconstituted systems

IV-C1. Are reconstituted carriers suitable model systems?

Arguments have been put forward to the effect that proteoliposomes are not suitable model systems for analyzing protein functions in vitro [75]. These arguments were, however, based on a very special experimental case. Not only has an overwhelming amount of data already been accumulated by the use of this method – data, which can be well compared with the results obtained from native mitochondria – but there are also additional arguments in favour of proteoliposomes as model systems for transport reactions.

(i) Since the molecular activity of carriers is in general relatively low, i.e., turnover numbers from a hundred to a few thousand per minute, it is necessary to incorporate a large amount of protein into the phospholipid bilayer membrane in order to achieve a signal large enough to be measured. This cannot be accomplished, for example, in planar films, which are, however, ideally suited for electrical measurements of channels, which have considerably higher molecular transport activity than the carrier proteins.

(ii) During isolation and purification of carriers from solubilized preparations of mitochondrial membranes, one has always to consider interfering activities of other proteins, which in some cases may have deleterious influences on the transport activity of the carrier to be measured. In this respect we only want to mention the presence of mitochondrial porin in unpurified preparations, which would cause immediate loss of all accumulated low-molecular-weight substances when coincorporated into the membrane. However, the high number of small proteoliposomes in standard preparations of reconstituted systems in general leads to extensive dilution of these interfering proteins. Thus the activity of at least a considerable fraction of the reconstituted carrier proteins can be measured; in fact of that part incorporated into liposomes without coinserted porin.

These arguments emphasize why reconstitution into proteoliposomes has been the method of choice at least for identification of carrier proteins during isolation and to a remarkable extent also for the elucidation of the molecular transport mechanism of these proteins.

IV-C2. Identification of mitochondrial carriers by reconstitution and characterization of their orientation within proteoliposomes

As mentioned above, functional reconstitution of a protein leads to its identification only if the protein under study is purified beforehand. This is in fact not the case for several mitochondrial carriers on the list. In the following, an overview of the state of identification of the carrier proteins will be given, without any details on the purification procedures which have already been described in subsection II-D. For the following discussion the reader is again referred to the summarizing Table I.

Beginning with the ORC, we find that only the presence of reconstitutable transport activity has been shown in a crude extract [66]. Kinetic and functional properties of the reconstituted carrier resemble the function known from intact mitochondria.

The CAC, too, has been reconstituted only from crude extracts [139]. Especially in this case a preceding purification would be of great interest, since the question whether the acyltransferase activity is part of the carrier (mechanism) or not has been the subject of a long-standing discussion. This would be a classical example of the use of reconstitution studies to solve questions about the functional entity of membrane proteins in general and carrier systems in particular.

Although experiments showing the binding of a specific inhibitor of the PYC in mitochondria have been published [151,165,173], the protein itself has not yet been clearly identified. The monocarboxylate transport activity was reconstituted from a significantly enriched fraction showing, however, still several different polypeptide bands in SDS-gels [136]. The presence of the

PYC in this enriched fraction was shown by functional reconstitution which demonstrated substrate and inhibitor specificity of the original monocarboxylate transport system known from mitochondria. Also preliminary data on the orientation of the protein were given [137]. Based on inhibition studies with *N*-ethylmaleimide and other SH-reagents added before and after reconstitution, a right-side out orientation of the carrier in the membrane was suggested. However, it is difficult to draw these conclusions on the basis of differentiation between the solubilized and the membrane-integrated state. The reason is that a different accessibility of these reagents towards hydrophobic groups, rather than side-specific effects, cannot easily be ruled out. It should be mentioned that an enriched preparation of the PYC from plant mitochondria has also been reported recently [33].

Doubtless, the CIC in functionally active form is present in the fractions enriched by classical hydroxyapatite chromatography. Substrate specificity and inhibitor sensitivity clearly resemble the properties known from intact mitochondria [150,168,169]. However, in this case, too, when starting from liver mitochondria, the enriched fraction consists more or less of the standard pattern of polypeptides after HA-chromatography, i.e. about 6–7 bands. Since several of these bands can clearly be correlated with known carrier proteins, the authors speculate that a peptide with M_r 27000 may be a likely candidate for the CIC [169].

For the next carrier protein, the DIC, conflicting results have very recently been published. In one report [172], the authors achieved a highly enriched preparation of the DIC from bovine heart, showing substrate and inhibitor specificity similar to the DIC measured in mitochondria. The preparation still contains two bands with M_r 34000 and 36000, respectively. The authors speculate that the two bands may in fact both be correlated to the DIC, based on assumptions concerning proteolytic degradation or precursor forms. Another group, however, has purified the same carrier from rat liver, also showing properties absolutely specific for this transport system [19]. This purified preparation nearly exclusively consists of a protein band of M_r 28000. It is very unlikely that small differences in the gel system would cause such a difference in the molecular-weight determination. It has to be taken into account, however, that the starting material of the former preparation [172], i.e., bovine heart, contains a much lower activity of the DIC as compared to rat liver, which was used in the latter preparation [19]. Furthermore, the activity of the DIC purified from rat liver [19] was found to be much higher than the transport activity using the carrier protein from bovine heart for reconstitution [172].

Fortunately, the situation is much clearer in the case of the OGC. This carrier protein has been identified by functional reconstitution in liposomes to be correlated to a protein band of M_r 31 500 when isolated from pig heart [18] and M_r 32 500 when purified from rat liver [19]. As usual, the carrier was functionally characterized by its substrate specificity and inhibitor sensitivity. Using eosin-5-maleimide as a nonpermeable labeling reagent, it could be shown that the reconstituted OGC is oriented in right-side-out direction in proteoliposomes [191].

The AGC has been purified by a relatively laborious purification procedure and was identified in a way similar to that used with other carrier proteins, based on its catalytic function in the reconstituted system [118]. When taking into account the difficulties with respect to unequivocal correlation of the Asp/Glu exchange function with a definite protein band in the SDS-gels, the correlation of the carrier to a band with M_r 64000 in the gels cannot be accepted with the same reliability as in the case of the phosphate carrier, the OGC, the UNC and the ADP/ATP carrier. There are apparently some contaminating protein bands present in the purified preparation of the AGC, however, only to a small extent [118]. Although specific inhibitors are lacking for this carrier protein, it was nevertheless possible by elaborate kinetic studies to determine that the orientation of the membrane-integrated AGC was nearly exclusively in an inside-out direction [47].

Identification of the following carrier proteins, the phosphate carrier, the UNC and the ADP/ATP carrier, is no longer based simply on proteins bands in gels and on reconstituted carrier activities, since the primary sequences of these proteins are known, either from protein sequence determination or from analysis of the gene structure (see subsection III-D). However, the primary identification of the carriers was nevertheless only possible by clear correlation of purified polypeptides, shown as single protein bands in SDS-gels, and functional activity, when reconstituted in proteoliposomes.

For a long time, there was a controversy about the occurrence of two protein bands in purified preparations of the phosphate carrier. The presence or absence of the two bands seems to be dependent of the addition of reducing agents [73,101,149]. This question has been dealt with in great detail in a recent review [184], however, in contrast to the somewhat confusing situation as described in the paper mentioned above [184], it is clear now that the reconstitutively active phosphate carrier correlates with a single protein band of M_r 33 000 in the presence of reducing agents [17,43,73,148,149]. This has been convincingly proven by isolating several mitochondrial carriers in parallel and demonstrating reconstituted phosphate exchange to be exclusively dependent on the presence of a homogeneous polypeptide with M_r 33000 in SDS gels [149]. Although the phosphate carrier is unequivocally identi-

fied by functional reconstitution, no clear experiments have been published about the orientation of the reconstituted protein within the liposomal membrane.

Functional reconstitution of the UNC seems to be comparatively easy due to the relative stability of this protein in the solubilized state. However, the substrate flux to be measured, which in this case is proton translocation across liposomal membranes, made it very difficult to prove by functional reconstitution that the correct and complete UNC has been isolated. Elaborate procedures for the transport measurements of protons had to be developed until a clear functional correlation of the 32 kDa protein with the proton translocator function was possible [93,94].

The identity of the prominent 29 kDa protein band from bovine heart mitochondria with the isolated ADP/ATP carrier has in fact been known for more than 10 years, documented by the pioneering isolation of the carboxyatractylate-binding protein from mitochondria [155,156]. The functional reconstitution later clarified the exclusive dependence of the complete carrier activity on the single polypeptide [109,111]. This, however, was the first purified substrate carrier protein to be functionally reconstituted.

The orientation of both the UNC and the ADP/ATP carrier in the reconstituted proteoliposomes was relatively easy to determine, since specific, tightly binding inhibitors are available for the two proteins, GTP in the case of the UNC and (carboxy)atractylate and bonkrekate in the case of the ADP/ATP carrier. Whereas the UNC is oriented in a clear right-side-out manner [93], the ADP/ATP carrier unfortunately shows both orientations [35,111], however not in random fashion (see also subsection IV-C4).

IV-C3. Influence of lipids on reconstituted carrier proteins

In this section we will discuss the specific influences of particular hydrophobic surroundings of reconstituted carrier proteins on their transport functions. It should be mentioned that there are also observations of a definite influence of lipids on the solubilization and isolation of mitochondrial carriers and especially on the stability of the solubilized proteins. These results have been discussed in section II.

Since cardiolipin has been reported to have particular effects on the stability of several carrier proteins during isolation and solubilization, the influence of especially this phospholipid on mitochondrial carrier proteins was intensively investigated. The first carrier which was found to be stimulated by the presence of cardiolipin or mitochondrial phospholipids in the liposomal membranes used for reconstitution was the CIC [168]. Just the opposite could be observed with the isolated OGC. This carrier functioned well in liposomes made from egg yolk lipids and was inhibited by nearly all lipid additions including cardiolipin and especially cholesterol [68]. In the case of the phosphate carrier the situation is quite complicated. Apart from the drastic effects of cardiolipin during isolation and solubilization (see subsection II-B) the reconstituted phosphate transport activity was stimulated to an extent of about 50% by the addition of cardiolipin and mitochondrial phospholipids to reconstituted proteoliposomes [16,70,130]. Activation by cardiolipin was shown not to be due to increased stability during the isolation procedure, since the respective phospholipid exerts its influence on the transport function when added to the purified protein just before incorporation into the liposomes [131]. Furthermore, the influence of cardiolipin was found to be specific for this particular phospholipid. These interpretations of phosphate carrier activation by cardiolipin were questioned by another group, reporting drastic activation of this carrier by addition of the calcium salt of phosphatidic acid and only minor stimulation by cardiolipin [186]. There is, however, further support for the former observation of transport stimulation by cardiolipin, based on interesting studies using the inhibitors doxorubicin and daunomycin, known to bind cardiolipin tightly, thereby counteracting its influence [41,133]. These investigations also demonstrated both stimulation of phosphate carrier function by addition of cardiolipin to the reconstituted liposomes and reversal of this stimulation after addition of the cardiolipin-specific inhibitors.

The influence of the hydrophobic surroundings on the activity of the reconstituted phosphate carrier has been studied and reported in a series of publications. It should be remembered that the ADP/ATP carrier has furthermore been shown to carry about six tightly bound molecules of cardiolipin when isolated in detergent [14]. This extraordinary specific binding of phospholipids has been discussed above (see subsection III-B), and should not be confused with the observations of carrier activation in the reconstituted state. On the one hand, the solubilized ADP/ATP carrier, when reconstituted into phospholipid membranes, still carries the tightly attached cardiolipin molecules, and on the other hand, it was shown in the reconstituted system that activation by cardiolipin was not saturable, which should be expected when specific binding sites have already been occupied [112].

The influence of negatively charged lipids in general was also thoroughly investigated. The ADP/ATP carrier is substantially activated by negatively charged phospholipids; the activation is, however, not specific for cardiolipin. Phosphatidylserine, phosphatidylinositol and even phosphatidic acid also stimulate the adenine nucleotide exchange in reconstituted proteoliposomes [112]. The unspecific character of this stimulation is in agreement with the observation that addition of doxorubicin to proteoliposomes, which included several types of negatively charged phospholipids, does not

result in inhibition of transport (Krämer, R., unpublished results). However, activation by negatively charged lipids proved to be very specific in another respect. For the first time, a side-specific activation by lipids was shown in this case [104]. Stimulation by anionic phospholipids was only observed when these lipids were present at the matrix side of the ADP/ATP carrier, i.e., the side of the protein which is originally oriented to the inside of the mitochondria. This is a further argument for the clear functional asymmetry of this carrier and presumably also other transport proteins [78,117].

The activity of the ADP/ATP carrier is not only modulated by negatively charged lipids. This mitochondrial exchange carrier was shown to require the presence of phosphatidylethanolamine for proper function [35,112]. Activation by this phospholipid is a quite unusual observation and must presumably be explained by effects on the lipid phase structure, since the stimulation by phosphatidylethanolamine was shown to be not saturable within experimental limits [35,112].

It should be mentioned in this respect that another quite unexpected observation was made in these investigations concerning the lipid interaction of the reconstituted ADP/ATP carrier. The transport function of this protein was substantially stimulated by the presence of cholesterol within the bilayer membrane of proteoliposomes leading to molecular activities definitely higher than in intact mitochondria [103]. Although this observation presumably has no physiological relevance, since the inner mitochondrial membrane normally contains no or only negligible amounts of cholesterol, it sheds further light on the importance of the physical state of the surrounding membrane for proper carrier function. This more general parameter is, however, by no means less important than the often postulated and sometimes overestimated specific influences and direct interactions of lipids with membrane proteins.

IV-C4. Elucidation of carrier mechanism in the reconstituted systems

There are at least three important reasons for using functional reconstitution of carrier proteins: (i) unequivocal identification of the functional entity of the respective transporter; (ii) elucidation of aspects of its molecular mechanism, i.e., aspects which are not easily accessible on the basis of experiments with intact mitochondria; (iii) investigation of the functional asymmetric properties of the carrier proteins within the artificial membrane of proteoliposomes. The last argument requires the oriented reconstitution of the respective carrier protein in the liposomal membrane or at least the possibility to differentiate experimentally between the two possible orientations. Experiments of this kind, i.e., experiments leading to results which are not easily available in intact mitochondria, have been reported mainly for the ADP/ATP carrier and the UNC and to some extent also for the AGC and the phosphate carrier. Nevertheless, functional studies in reconstituted systems are in general used to define the basic kinetic properties, such as K_m values, substrate specificity and inhibitor sensitivity. These basic experiments have been carried out for most of the proteins discussed here, and are mentioned in subsection IV-C2. Investigations of this kind are necessary for carrier identification, but of course do not add new information with respect to carrier mechanism.

When studying the reconstituted phosphate carrier it was important to demonstrate the correct mechanism of the purified and reconstituted protein, since there are several possibilities of phosphate flux through the inner mitochondrial membrane [54,71,145]. The expected influence of the pH-gradient was demonstrated both for phosphate uptake into [41,133,146,147,185] and phosphate efflux from reconstituted proteoliposomes [141,147,186]. The results of these studies are consistent with a phosphate/proton symport by the isolated and reconstituted phosphate carrier.

The functional data for the purified OGC have also recently been determined by kinetic analysis in the reconstituted system [68]. Interestingly, an influence of the pH on the apparent substrate affinity but not on the transport rate could be observed in the case of this electroneutral carrier system. The PYC, too, has been functionally characterized in the reconstituted state [136]. Especially for this carrier a reliable functional analysis was necessary for a clear identification, since phospholipid membranes are permeable for pyruvate to some extent, which may cause severe experimental problems in these studies.

The basis for functional studies of the reconstituted AGC was the finding that this protein is oriented unidirectionally (inside-out) when reconstituted by a detergent adsorption method [47]. This was essential for functional studies with the reconstituted protein, since no specific, tightly binding ligands for the AGC are known, which could be used to differentiate the orientations of the protein. The finding that the membrane potential exerts its influence specifically on the aspartate-loaded carrier, whereas the pH-gradient mainly influences the glutamate binding and transport steps, was more or less expected from the results obtained in intact mitochondria. However, careful kinetic analysis in the reconstituted system led to a clear decision between two possibilities for the molecular mechanism of the AGC favoured by two different groups [123]. Functional studies with the reconstituted AGC unequivocally showed that the intrinsic mechanism of this transport protein is a sequential one, i.e., a ternary complex of carrier, aspartate and glutamate occurs during the catalytic cycle [46]. It could further be shown that this ternary

complex is organized most probably in a form where the two substrates are simultaneously bound at opposite sides of the membrane [48].

An extended approach for the elucidation of carrier mechanisms by functional co-reconstitution of the AGC and the OGC within the same liposomes was recently reported, thereby creating the main part of the mitochondrial aspartate/malate shuttle within proteoliposomes [69]. This system thus provides a basis for characterizing not only mechanisms of single carriers but also the influence of regulating and modulating parameters of complete shuttle systems.

The UNC is another example of oriented insertion of a carrier protein into reconstituted proteoliposomes. In this case, the orientation could be proven by specific interaction of the tightly binding ligand GTP with the reconstituted protein from the outside, i.e., the UNC is oriented right-side out [93,94]. The interesting results of the pH-dependence of ligand binding to the reconstituted UNC have already been discussed (see subsection III-C). Furthermore, at least two important findings concerning the catalytic mechanism of the UNC were made using the reconstituted protein.

(i) The proton flux through the membrane-inserted UNC was measured in both possible directions. When analyzing the influence of the membrane potential on the proton-transport activity, it was found that the electric gradient modulates the carrier activity in exactly the opposite way when applied in the opposite direction [93]. The observed influence was mainly on the V_{max} of the transport activity. These observations can be explained by assuming that the UNC has no 'valve effect', and the regulation by membrane potential can be described by an 'electrophoretic control mechanism'. This description quite closely resembles the mode which has been developed for the ADP/ATP carrier (see below).

(ii) There is a long-standing controversy about the function of the UNC as Cl^- carrier. In careful investigations with the purified and reconstituted protein no significant Cl^- cotransport could be detected [93]. It should be pointed out, however, that in experiments with partially purified and reconstituted UNC, Cl^- transport in fact was found [171]. A speculative explanation for this discrepancy was suggested in assuming contamination by another protein in the latter preparation which would be responsible for the Cl^- transport activity [93].

The most extensive studies concerning elucidation of transport mechanisms have been carried out with the ADP/ATP carrier. The results of these studies can only be discussed in brief here. Basically, the situation is not very favourable for these investigations, since it can be shown that the reconstituted ADP/ATP carrier is not unidirectionally oriented in the liposomal membranes. The two orientations can, however, not only be quantitated by using the side-specific inhibitors (carboxy)atractylate and bongkrekate, but these inhibitors also proved to be suitable tools for analyzing the function of the membrane-inserted ADP/ATP carrier separately in its two orientations [104,111]. Although it is clear that both possible directions of insertion are present in proteoliposomes, different groups have published different ratios of orientation. The total activity of the carrier oriented right-side-out was generally found to be higher [35,111,189]. However, by careful examination it could be shown that the ADP/ATP carrier molecules oriented inside-out have a significantly lower specific activity and that therefore the true ratio of orientation is more in favour of the inside-out orientation [104,111].

In the course of years, a long list of regulating and modulating factors of the reconstituted ADP/ATP carrier has been elucidated. Table II gives an overview of the main results obtained by these studies. Some important points will be discussed in more detail here.

The regulation mechanism of membrane potential on the ADP/ATP carrier activity was quantitatively elucidated in the reconstituted system. A strictly linear

TABLE II

SUMMARY OF REGULATING AND MODULATING PARAMETERS OF THE RECONSTITUTED ADP/ATP CARRIER

Parameter	Effect	Reference
Membrane potential	electrophoretic regulation of rate constants (symmetric)	113–115
Surface potential	modulation of apparent substrate affinity	104
Surface charge	side-specific activation/inhibition (matrix side)	104
Anions	side-specific modulation of rate constants (cytosolic side)	116, 121
Polyvalent cations	activation of nucleotide exchange	120, 121
Inhibitors (CAT,BKA)	side-specific transport inhibitors	35, 111
Acyl-CoA	competitive inhibitor	189
Pyrophosphate	competitive inhibitor as well as transport substrate	106
Divalent cations	modulation of true substrate concentration	35, 102
Phosphatidylethanolamine	activation (rate constant)	35, 112
Anionic phospholipids	activation (rate constants)	35, 104, 112
Cholesterol	activation (rate constants)	103

relation between the logarithm of the uptake velocities for ADP and ATP ($\log(V_{ATP}/V_{ADP})$) and the membrane potential was obtained [113–115]. This finding was not obvious, since the functionally active carrier molecules are oriented asymmetrically right-side-out. Thus, it must be concluded that the membrane potential controls the exchange reaction symmetrically, which can be interpreted as a simple electrophoretic mechanism of ADP/ATP exchange. The influence of the membrane potential on the kinetic constants could be quantitatively determined [115]. Out of the list of possible affinity constants for the two substrates and for the two carrier sides on the one hand and of possible rate constants for ADP and ATP, respectively, for both inward and outward transport on the other, almost exclusively the rate constants for the carrier-ATP complexes were modulated by the membrane potential. Since the substrate affinities were found to be more or less unaffected, a model for regulation of ADP/ATP transport by membrane potential could be derived, which does not include a major conformational change of the carrier due to potential effects, but a velocity control by the electric gradient.

These experiments, although elucidating aspects of the regulation by membrane potential, are not suited to decide the important question about the kinetic mechanism of ADP/ATP carrier. There are still conflicting interpretations [81,180]. Since these problems have not yet been addressed using isolated and reconstituted ADP/ATP carrier, we will give only a brief overview about the state of discussion. On the one hand the data mainly obtained by characterizing substrate and inhibitor binding sites led to the fundamental concept of the reorientating site mechanism (for a review, see Ref. 81). This interpretation, whether based on binding to a single reorientating site or to multiple sites assuming exclusive availability of only one site at one time [180,20], leads to ping-pong kinetics of carrier action. However, results of nucleotide exchange kinetics in heart [51] and liver [12] have been published, which are not in agreement with the ping-pong mechanism. Recently in a thorough study of initial transport kinetics using isolated rat-heart mitochondria [167], data were reported which are clearly in favor of a sequential mechanism of transport kinetics including a ternary complex during the catalytic cycle of the ADP/ATP carrier (see also the discussion on the transport mechanism of the AGC).

Another quite unexpected result was the functionally asymmetric influence of modulating parameters at the two sides of the ADP/ATP carrier. This has already been mentioned with respect to stimulation by phospholipids (see preceding subsection), but it could also be detected in the case of stimulation/inhibition by negative/positive surface charges, which exert their influence only at the *m*-side of the protein [104]. The activation by anions, on the other hand, was shown to influence the carrier protein only from the c-side [116,121]. These examples clearly demonstrate that functional studies with a reconstituted protein may in fact lead to definite statements concerning the asymmetric structure and the intrinsic mechanism of a carrier protein.

Even investigations of functional properties that have long been known from intact mitochondria can lead to new and interesting results when carried out in the relatively simple and defined surroundings of a reconstituted system. It was clear from results obtained in mitochondria that the ADP/ATP carrier does not transport the Mg-complexes of nucleotides. Apart from simple confirmation and quantitation of these results in the reconstituted system, it was detected that the complexes of nucleotides with divalent cations are not only not transported but are not even competitors at the active site, i.e., the surroundings of the active site have structural properties which completely exclude the cation complexes from reaching the true nucleotide binding site of the ADP/ATP carrier [102].

Basic data known from intact mitochondria concerning aspects of substrate and inhibitor specificity could also be extended and completed using reconstituted systems. The inhibiting properties of various long chain acyl-CoA derivatives could be quantitated in liposomes carrying the reconstituted ADP/ATP carrier [189]. These experiments are difficult and ambiguous in intact mitochondria due to the presence of internal acyl-CoA compounds and also due to the presence of other proteins and carriers which may bind these substances. Another interesting example is the quantitative determination of an alternative activity of the ADP/ATP carrier. In view of the very high substrate specificity of this transport system, it is very surprising that the ADP/ATP carrier is also able to accept and transport pyrophosphate instead of nucleotides [106]. It could be shown that this substrate, although bound with low affinity, can be transported at relatively high rates reaching 10% of the carrier activity when ADP or ATP is used as substrate.

V. Conclusions and Perspectives

The first and obvious aim of this review has been to document the 'state of the art' in isolation, reconstitution and characterization of mitochondrial carrier proteins. A reasonable hierarchy of achievement can be given on the basis of the data presented here: the ADP/ATP carrier is by far the most extensively investigated and best-known carrier protein, followed by the UNC. This is in fact surprising, in view of the relatively limited period of time since the UNC has been isolated. The next level is occupied by the phosphate carrier, characterized by a considerable amount of structural data but lacking functional results. In de-

creasing order, with respect to the state of elucidation, the OGC, the AGC, the DIC, the CIC and the PYC should be mentioned, followed by the poorly characterized CAC and ORC. As far as the glutamate and the glutamine carrier are concerned, data are still completely lacking.

The second and perhaps more important aim of this review was a comparison of strategies and methods used in the solubilization, purification and reconstitution of these carrier proteins. In view of the summarized data, it must be said that the general procedures and strategies of carrier purification are surprisingly similar. In our opinion, this may be caused by the structural homology and the similarity of chemical and physical properties of these membrane proteins. Although the basic data for purification and reconstitution are – with some exceptions – in fact relatively similar, the reader may at least take advantage of the collection of experimental 'tricks' used in the studies, as reported here.

The third and presumably most important aim of this review is, however, to focus on the possible lines of future investigations which – on the basis of the collection and comparison of data here – seem to be both necessary and promising. In this respect, it has to be emphasized that even in the case of the ADP/ATP carrier, where an impressive amount of data has been accumulated in the course of the years by numerous careful and detailed investigations, we have in fact no conclusive idea about the true intrinsic transport mechanism, i.e., how the passage of the substrates ADP and ATP is mediated by/through the carrier and which processes do in fact occur at/in the carrier molecule. In view of the large amount of functional data, which apparently do not suffice for describing the intrinsic carrier mechanism, we have to conclude that we essentially need more structural data. Promising steps in this direction have been made, predominantly in the case of the ADP/ATP carrier, but also for the UNC and the phosphate carrier. A good example is the effort which has been made to explore the nature of the respective substrate binding sites. It is an obvious, but nowadays by no means novel statement, to point out that for definitive improvement in understanding carrier mechanisms we probably need the complete structure of the respective proteins. That would mean crystallization and structural analysis. We do not dare to decide here whether in the case of these relatively small and hydrophobic integral membrane proteins this experimental approach could be successful.

Coming back finally to the functional data presented in this review, we would like to emphasize a further argument. Mainly on the basis of the results obtained for the ADP/ATP carrier and the UNC, it should have become clear that a careful and complete functional analysis not only leads to a reliable basis for description and understanding of the carrier mechanism in kinetic and energetic terms, but also provides a set of functional data which have to be matched and explained by all further results to be obtained concerning protein structure. In this respect, the unifying concept of mitochondrial carrier catalysis should be considered (see subsection III-D); however, the broad diversity of different carrier functions should not only be taken into account, but also investigated by the appropriate methods. The variety of substrates, for example, ranges from the smallest substrate, i.e., the proton (UNC), to one of the largest among all the substrates transported by carrier proteins, i.e., adenine nucleotides ADP/ATP carrier. The variety of transport mechanisms includes uniport, symport and antiport, electroneutral, proton-compensated or electrophoretic mechanisms, kinetics of sequential type or ping-pong mechanisms. Thus it seems absolutely necessary, in order to provide a solid basis for the interpretation of structural data, to accumulate also a set of functional data, as reliable and complete as possible, at least for some of the different carrier molecules. One way to obtain these results, and perhaps one of the most obvious and promising methods, is the investigation of properties and function of these carrier proteins in the solubilized and reconstituted state. We hope that the justification and the importance of this experimental strategy has been conclusively demonstrated in this review.

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