Reconstitution of Adenine Nucleotide Transport from Beef Heart Mitochondria†

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ABSTRACT: The adenine nucleotide translocator isolated from beef heart mitochondria with Triton X-100 is incorporated into the membranes of lecithin liposomes. Reconstitution of ADP–ATP transport is achieved in this artificial system similarly as described previously [Krämer, R., & Klingenberg, M. (1977) *FEBS Lett. 82*, 363]. The reconstitution process and the system are characterized for their functional and molecular properties. (1) The activation by sonication can now be explained. On addition, first the carrier protein is preferentially incorporated into small liposomes. Thereby larger and presumably multilamellar structures are formed which have reconstituted inhibitor binding but no exchange activity. By short sonication these vesicles are converted into unilamellar liposomes of medium size with reconstituted adenine nucleotide exchange activity. (2) By correlating carboxyatractylate (CAT) and bongkrekate (BKA) binding with adenine nucleotide transport, three portions of the inserted protein can be identified. (a) A portion (up to 5%) with high exchange activity, comparable to the original transport rates, was differentiated. This part of the protein is inhibited by CAT and BKA. The binding data provide evidence that this portion is oriented in the same direction as it is in mitochondria. (b) A population of very weakly exchanging, though still active, carrier molecules (about 25%), which are inhibited only by BKA and not by CAT, was differentiated. This portion of carrier molecules appears to be oriented in the opposite direction as it is in the mitochondria and reaches only about 1/500 of the activity of the carrier in population a. (c) The rest of the carrier protein (about 70%) is irreversibly denatured during isolation and reconstitution. (3) Reconstitution of adenine nucleotide transport with a highly purified CAT–protein complex instead of the unliganded carrier protein is reported. Thus, it is confirmed that only the protein with a molecular weight of 30 000 is responsible for the ATP–ADP transport in the artificial system. Possible contributions of the minor peptide impurities to the exchange activity are thereby excluded. (4) Whereas reconstitution with the CAT protein has a low yield due to harsher methods for removing CAT, good results are obtained with the ATR– and BKA–carrier complexes. Not only protection against denaturation is achieved but also the same activity as with the free carrier is obtained after removal of the inhibitors.

Attempts to reconstitute transport processes across phospholipid membranes have gained wide interest since they can be powerful tools, when pure carrier proteins are used, in elucidating transport functions without the complications of the natural membrane. In most cases, only partial reconstitution of the labile systems as compared to the original activity was possible. Precautions must be taken to be sure that in the artificial system the measured functions are comparable to those in the original membrane. The following are some of the problems. (1) Is there an unequivocal correlation of the reconstituted function with the isolated and purified protein? (2) Is the protein to be studied pure enough to exclude possible contributions of impurities or copurified peptides? (3) What is the turnover number of the actively reconstituted molecules as compared to that of the intact system?

In the case of the ADP–ATP carrier system of the inner mitochondrial membrane, reconstitution studies have been reported by us and by another group (Krämer et al., 1977; Krämer & Klingenberg, 1977a,b; Shertzer & Racker, 1976; Shertzer et al., 1977). As it was already discussed (Krämer & Klingenberg, 1977b), the state of purity of the carrier preparation is very different after both isolation procedures. On the basis of our nearly homogeneous carrier preparation (Krämer et al., 1977), it is now possible to characterize the reconstituted system of adenine nucleotide transport on a molecular level and to obtain a fairly accurate insight into de- and reactivation during solubilization, purification, and reconstitution.

Materials and Methods

Materials. The sources of chemicals were the following: Triton X-100 (Sigma); egg yolk lecithin (Merck); carboxyatractylate, atractylate, and nucleotides (Boehringer); radioactive nucleotides (NEM), Dowex 1-X8, and Bio-Beads SM2 (Bio-Rad); Sephadex, Sepharose, and Ficoll (Pharmacia). Bongkrekate was a gift from Professor W. Berends (Delft) and [^{3}H]Triton was a gift from Rohm and Haas. All other chemicals were of analytical grade.

Preparations. Lecithin was purified from egg yolk phospholipids according to Beyer & Klingenberg (1978), omitting the silica gel chromatography; hydroxylapatite was prepared as described by Bernardi (1971) with one modification: the material was stored in 10 mM Na_2HPO_4/NaH_2PO_4, pH 6.8. [^{3}H]Bongkrekate (Babel et al., 1976) and [^{35}S]carboxyatractylate (Riccio et al., 1973) were prepared as described earlier. The synthesis of [^{3}H]carboxyatractylate from unlabeled carboxyatractylate by an oxidation/reduction procedure will be published separately.

Determinations. Protein concentration was determined by the method of Lowry in the presence of 1% sodium lauryl sulfate (Helenius & Simons, 1972), and phosphorus was estimated by the method of Chen et al. (1956). Triton X-100 was either determined by its UV absorption or by the use of tritiated detergent.

Isolation of the Adenine Nucleotide Carrier. The carrier protein was isolated basically as described by Krämer et al. (1977). However, it is now possible to isolate also the unliganded carrier protein with the same procedure, only using Triton X-100 (2.5%) instead of LAPAO[1] (1.0%) by application

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of hydroxylapatite prepared in our own laboratory (see above). This consists of rather coarse crystals compared to the material from Bio-Rad Laboratories, which was first used for the isolation of the CAT–protein complex. The isolated protein, though showing the same properties as that previously prepared with laurylamine *N*-oxide, offers some advantages for the incorporation and makes possible an easy determination of detergent present in the reconstituted system (see below). The isolation of the CAT–, ATR–, and BKA–protein complexes followed exactly the same procedure as described for the CAT–protein complex (Riccio et al., 1975a,b). Excess BKA is not extracted before solubilization because it is removed during the Dowex chromatography before the exchange assay.

Reconstitution of Adenine Nucleotide Exchange and Inhibitor Binding. Preparation of liposomes and incorporation of the solubilized protein were performed as described (Krämer et al., 1977; Krämer & Klingenberg, 1977a), using a different buffer solution (100 mM NaCl, 10 mM ATP, 0.5 mM $MgCl_2$, and 10 mM Tricine–KOH, pH 7.5). Furthermore, the isolation of the unliganded carrier protein with Triton X-100 made it possible to incorporate nearly twice as much protein as with LAPAO (Krämer et al., 1977), since Triton X-100 proves to be less harmful to the structure of the liposomal membranes. The reconstitution procedure was modified first by introducing a freeze–thaw step after incorporation of the protein (Kasahara & Hinkle, 1977) to create larger liposomes and second by drastically reducing the sonication time to 15–20 s by a Branson sonifier, Model B-15 (cf. Figure 4).

Binding of the inhibitor ligands CAT and BKA was measured according to the previously used gel filtration method (Krämer & Klingenberg, 1977a). The assay for reconstituted adenine nucleotide transport was essentially the same as that described by Krämer & Klingenberg (1977b), omitting the added $MgCl_2$ (already present in the sonication buffer). "CAT-insensitive transport" (see Results) was determined with BKA as the stopping agent, added in varying time intervals after CAT, which is the usual stopping agent for the "CAT-sensitive transport". The assay for the CAT-insensitive transport is described in detail in Figure 6.

Characterization of Liposomes. Liposomes were separated by chromatography on Sepharose 4B columns (1 m × 1 cm) in 0.13 M NaCl and 5 mM Tricine–KOH, pH 7.5. For gradient centrifugation, 0.5 mL of the liposome fractions was layered on a linear gradient of 0–4% Ficoll in 60 mM sucrose. The tubes were spun for 4 h at 50 000 rpm in a Beckman 50 Ti rotor (0 °C).

Removal of Triton X-100 by Bio-Beads SM2. Procedure 1. The simplest method was incubating the solubilized carrier protein (50 mg of Triton per g of Bio-Beads) with washed Bio-Beads (Holloway, 1973) and gentle shaking for 30 min, at 0 °C. *Procedure 2.* An improved method (see Results) consists of passing 500 μL of carrier preparation (1% Triton) through a 5 × 0.5 cm column filled with Bio-Beads. These were previously saturated with phospholipids by incubating them with liposomes for 30 min. The flux rate has to be very slow, about 2 mL/(cm² h). *Procedure 3.* For removal of Triton from liposomal membranes, the first procedure was modified, lowering the ratio of Triton to Bio-Beads to about 10 mg/g.

Results

Characterization of the Reconstituted Carrier Protein. A crucial point in reconstitution studies is to assure that the observed effects are exclusively based on the particular protein to be reconstituted and not caused or influenced by contaminating proteins. Although the protein preparation used in this system is about 70% pure and therefore represents a progress as compared to former reconstitution studies, there remains some uncertainty about the 30% contaminating material. It has been shown (Krämer et al., 1977) that the isolated carrier protein, without bound inhibitor, is highly unstable and cannot be purified further without loss of activity. On the other hand, the isolated ADP–ATP carrier protein is rather stable when complexed with its specific inhibitor CAT. Also, the inhibitors BKA and ATR stabilize, yet they are not as effective as CAT (Aquila et al., 1978).

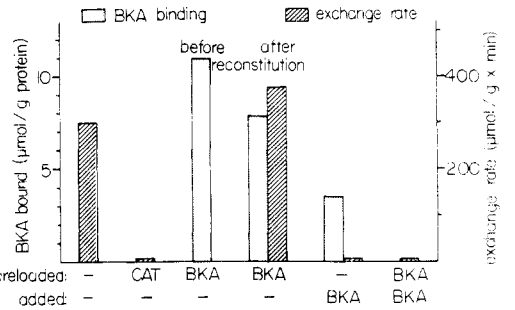

FIGURE 1: Reconstitution of the ATP exchange with the BKA–protein complex. Binding of BKA and reconstitution of adenine nucleotide transport are measured with different carrier protein preparations. The reconstituted exchange activity of the routinely used unliganded carrier protein (column 1) is completely inhibited by preloading with CAT (column 2). Binding of BKA to the carrier, when isolated from BKA-preloaded mitochondria (column 3), is slightly reduced after the reconstitution (column 4); simultaneously, adenine nucleotide exchange activity appears. In control experiments, BKA added after the reconstitution still blocks the exchange activity with both the unloaded (column 5) and BKA-loaded (column 6) carrier protein.

Because of its stability, the CAT–protein complex can be highly purified (Riccio et al., 1975a,b; Klingenberg et al., 1978). When substituting the unliganded carrier protein by this almost pure inhibitor complex in the reconstitution, we did not observe any transport activity. Only after detachment of about 10–30% of the tightly bound ligand by prolonged incubation with ADP (45 min at 21 °C) and sonication (15 s at 0 °C) does reconstituted transport activity appear. It reaches only a relatively low activity of about 30 μmol/(g min) since removal of the inhibitor CAT is accompanied by extensive denaturation of the carrier protein. Because of its varying results, this method cannot be routinely used. Nevertheless, these experiments clearly demonstrate that the adenine nucleotide transport, measured in the artificial system, depends solely on the 30 000 molecular weight protein, first known as the CAT binding protein (Riccio et al., 1975a).

When performing these experiments with the isolated BKA– or ATR–protein complexes, we reconstituted adenine nucleotide exchange simply by incorporation into the liposomes (10 mM ATP present) without further treatment for inhibitor detachment (Figure 1). The easy removal of these inhibitors as compared to CAT is in accordance with the less tight and therefore more ATP sensitive binding of ATR and BKA to the carrier (Aquila et al., 1978). On the basis of these experiments, the stability of the isolated carrier protein in its different ligand complexes can be determined. For this purpose the time dependence of the reconstitution capability is followed over several days (Figure 2).

The reconstitutional activity of the protein is markedly dependent on the interaction of BKA or liposomes with the protein. Significant stabilization is already observed by incorporation of the unliganded protein into liposomal mem-

[1] Abbreviations used: LAPAO, 3-lauramido-*N*,*N*-dimethylpropylamine *N*-oxide; CAT, carboxyatractylate; BKA, bongkrekate; ATR, atractylate; Tricine, *N*-tris(hydroxymethyl)methylglycine.

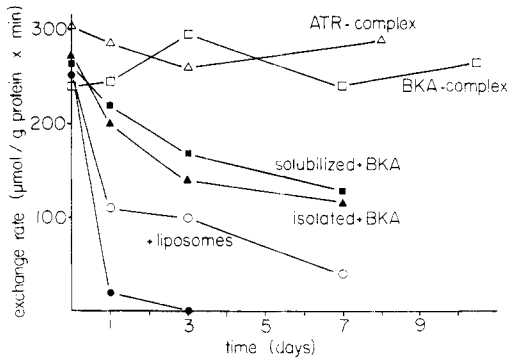

FIGURE 2: Stability of the adenine nucleotide carrier in different complexes. The adenine nucleotide carrier protein is isolated in different forms and incorporated into liposomes after incubation at 0 °C for various times. Whereas the reconstitutive activity of the unliganded protein is rapidly lost (●), the isolated BKA (□) and ATR complexes (△) show undiminished activity in reconstitution after storage for more than 1 week. BKA stabilizes, although somewhat less, when added after solubilization (■) or after isolation (▲) of the carrier protein. In another experiment, the unliganded adenine nucleotide carrier is incorporated at time zero, and the exchange activity of the system is tested after different time intervals (○).

branes. On the other hand, BKA, when added before solubilization, protects the carrier against inactivation. In order to exclude the possibility that BKA leads to more complete solubilization of an activating or stabilizing cofactor of the carrier, in additional experiments we added the inhibitor after solubilization or after isolation of the ADP–ATP carrier. In this case, BKA still causes pronounced stabilization, thus confirming that this effect actually depends on the interaction of BKA with the carrier protein. The diminished stability in these experiments, as compared to the situation with BKA-preloaded mitochondria, is in accordance with the reduced ability of the isolated protein to bind BKA (Krämer & Klingenberg, 1977a). The same stabilization of the isolated adenine nucleotide carrier is obtained when ATR instead of BKA is added before solubilization of the mitochondria (Figure 2).

Characterization of the Reconstituted Exchange Activity. The procedure used for reconstitution of adenine nucleotide transport has several common features with other reconstitution methods: incorporation by mixing liposomes and protein (Eytan et al., 1975), a freeze and thaw step (Kasahara & Hinkle, 1977), and a second sonication (Kasahara & Hinkle, 1977; Crane et al., 1976). Whereas the activating effect of this additional sonication is mostly explained to improve incorporation of the protein, in the case of the adenine nucleotide carrier, this procedure seems to be necessary for other reasons. Incorporation has proven to be complete after mixing the protein with the liposomes (Krämer et al., 1977); however, no exchange activity could be measured in this system. The sonication itself shows a rather distinct optimum in the range of 10–20 s (Figure 3). The highest transport rates are obtained by sonication times (about 20 s) longer than those required to obtain the largest extent of exchange (optimum at about 10 s). This can be explained by the fact that the reconstituted liposomes created by shorter sonication periods are larger than those formed after longer sonication. Nevertheless, the biphasic dependence of the reconstituted transport on sonication time seems to be a superposition of the reconstitution process on the one hand and protein inactivation by prolonged ultrasonic treatment on the other.

For a further analysis, liposomes were separated into small and large particles on Ficoll gradients (Figures 4 and 5). By mixture of the solubilized protein with homogeneous large

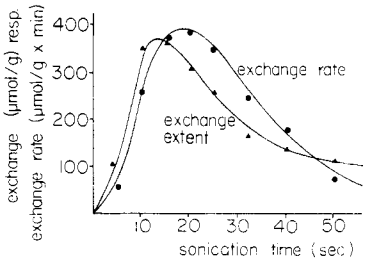

FIGURE 3: Effect of sonication on the reconstituted exchange activity. The maximum extent of exchange (▲) and the exchange rate (●) are measured after increasing sonication times (see Methods). The extent of exchange is determined by measuring the amount of transported adenine nucleotides after 15 min at 21 °C, whereas the exchange rate is calculated from a kinetic determination with CAT stops at 0, 10, 20, and 30 s.

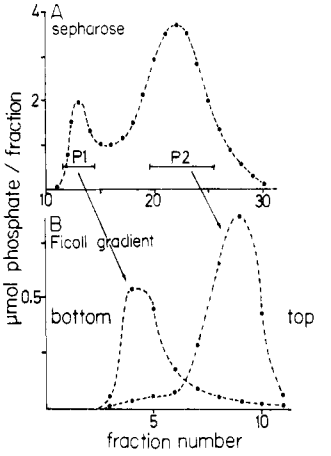

FIGURE 4: Separation of liposomes into homogeneous small and large vesicles. Liposomes are separated on Sepharose 4B (A). The two pools (P1, large; P2, small) are used for the incorporation studies in Figure 5. To make sure that the separation of the liposomes on ultracentrifuge gradients leads to similar results as compared to gel filtration on Sepharose, the indicated pools 1 and 2 from Figure 5A are subjected to Ficoll gradient centrifugation (Figure 4B).

(Figure 5A) or small (Figure 5B) vesicles, separated before on Sepharose 4B (Figure 4A), it is seen that the ADP–ATP carrier is incorporated to a much larger extent into the membranes of small liposomes, thereby generating a new kind of larger structures. A preferential incorporation of cytochrome oxidase into small liposomes is also reported by Eytan & Broza (1978).

The structures formed by insertion of the protein are also closed vesicles as demonstrated by entrapped [^{14}C]sucrose (experiments not shown). Obviously, the protein–detergent micelle induces a fusion of small liposomes. When sonicated again, the large vesicles are now reconstitutively active and have an intermediate size (Figure 5C). With diminishing size of the vesicles after sonication, there is an unexpected increase of the specific internal volume. At first the incorporation of the solubilized ATP–ADP carrier generates larger and closed vesicles with low internal volume, which are presumably multilamellar liposomes. It can be visualized that membrane-inserted carrier molecules are unable to transport hydrophilic anions across more than one layer. By sonication these structures are probably transformed into unilamellar vesicles with reconstituted transport activity.

The preferential insertion into small liposomes is furthermore confirmed by use of very large phospholipid vesicles formed in an ether evaporation method described by Deamer & Bangham (1976). Nearly no incorporation of the protein into these large unilamellar liposomes with a defined structure could be achieved.

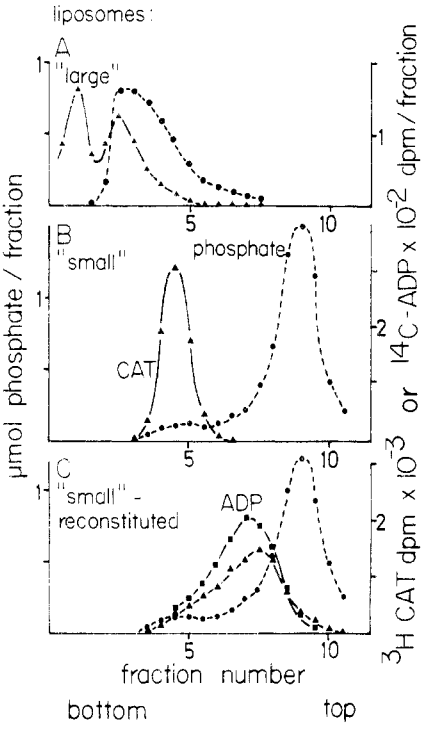

FIGURE 5: Incorporation of the adenine nucleotide carrier into different liposomal fractions. (A) Incorporation of the isolated CAT–protein complex [CAT (▲)] into the population of large liposomes [phosphate (●)] derived from Figure 4A. More than half of the protein is not incorporated into these vesicles and appears as free CAT–protein in front of the liposomes. The firmly bound [^{3}H]CAT is used for detection of the carrier protein. (B) Incorporation of the isolated CAT–protein complex into the population of small liposomes derived from Figure 4A. (C) A mixture of CAT–protein and unliganded carrier protein is incorporated into small vesicles according to Figure 4C. After additional sonication (10 s) adenine nucleotide exchange is performed with these reconstituted vesicles. [^{14}C]ADP, transported into the vesicles (■), [^{3}H]CAT (▲), and phosphate (●) are measured after Ficoll gradient centrifugation.

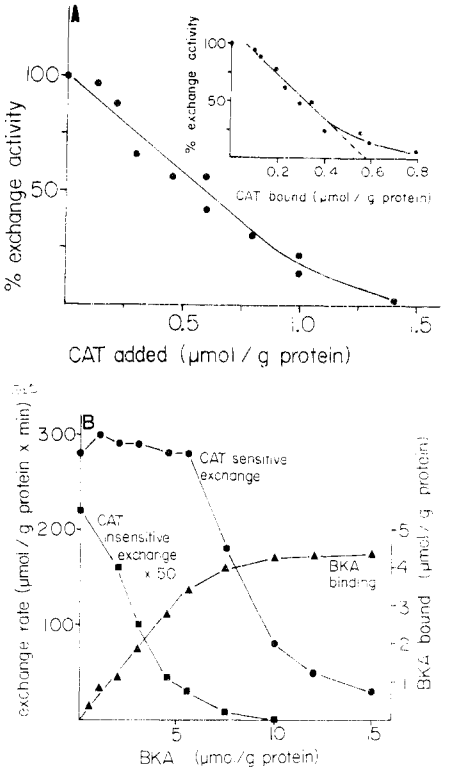

FIGURE 6: Titration of reconstituted exchange with CAT and BKA. (A) The reconstituted adenine nucleotide exchange is titrated with CAT. The CAT-insensitive exchange (cf. Figure 6B) is subtracted. CAT binding to the carrier protein is correlated with the exchange inhibition in the insert. (B) The reconstituted exchange is titrated with BKA. The CAT-sensitive exchange (●) is determined as follows. Increasing amounts of [^{3}H]BKA are added to the reconstituted system as indicated on the abscissa. The exchange is subsequently measured by the use of the CAT stop (see Methods). The corresponding blank is obtained by adding CAT (10 μM) before the nucleotide; its value never exceeds 10% of the exchange values. The binding of BKA to the carrier protein is analyzed by gel filtration. The CAT-insensitive exchange (■) is determined as follows. The same amounts of BKA as in the measurement of CAT-sensitive exchange are added to the reconstituted system together with CAT (50 μM). Adenine nucleotide transport is started by addition of [^{14}C]ATP and stopped by excess BKA (50 μM). The corresponding blank is obtained by adding CAT (10 μM) and BKA (10 μM) before the nucleotide. Although very low rates of the CAT-insensitive exchange were obtained, the blanks never exceed 25% of the exchange values.

Though adenine nucleotide transport is functionally reconstituted and unequivocally related to the 30000 molecular weight protein, there are still questions left concerning this artificial system. What is the explanation of the difference between the rather large extent of reconstituted binding for CAT and BKA (up to 30%) and the relatively small extent of reconstituted transport (up to 5–6%) (Krämer & Klingenberg, 1977a,b)? Does the low transport rate in liposomes depend on a small number of carrier proteins with high activity or on a large number of less active carrier molecules?

Elucidation of these questions is possible by the fact that one of the specific inhibitors, CAT, is highly hydrophilic and therefore not membrane permeable and another, BKA, is lipophilic when protonated and thus permeates phospholipid membranes, particularly at lower pH (Erdelt et al., 1972). Taking into account that there is a mutual exclusion of CAT and ADP binding (Klingenberg et al., 1978), one can expect that only those active sites that are available for CAT binding are also able to interact with adenine nucleotides. This statement does not hold for the inhibitor BKA, since this ligand can also interact with active sites hidden by or buried in the liposomal membrane.

On the basis of these arguments, the observed transport rate can now be correlated with the true amount of active carriers. The extent of reconstituted adenine nucleotide exchange was titrated with CAT, and the binding of CAT was simultaneously measured (Figure 6A). A corresponding titration of the reconstituted activity with BKA is shown in Figure 6B.

All transport measurements are routinely performed with the inhibitor stop method employing CAT. In what has been regarded as a blank value, obtained as CAT-insensitive exchange, an unexpected inhibition by BKA is found. This shows that the small CAT-insensitive activity, which might have been considered as a carrier-independent transport, is also carrier-mediated. Usually in transport assays the CAT-insensitive exchange, being part of the blank value, can be neglected because it amounts to only 1–2% of total exchange activity. Furthermore, the CAT-insensitive transport shows absolute dependence on internal ADP or ATP as it has been demonstrated for the CAT-sensitive transport (Krämer & Klingenberg, 1977b) and, therefore, also represents a counter exchange.

BKA in low concentration (Figure 6B) first acts exclusively on the CAT-insensitive transport. Only after complete inhibition of this kind of exchange is the much more active CAT-sensitive transport affected. The discrimination between two types of the reconstituted transport activities leads to the result that both kinds of exchange have opposite sensitivity to CAT and BKA at low inhibitor concentration. The implication of this finding will be discussed below.

Table I: Correlation of Reconstituted Exchange and Inhibitor Binding

	CAT or BKA binding (μmol/g of protein)	exchange (21 °C) [μmol/(g min)]	correlated[a] turnover no. (1/min)
reconstituted carrier			
portion 1[b]	0.6 (3.2%)	265	500
portion 2	4.9 (28%)	4	0.9
portion 3	rest of protein (70%)		
isolated CAT-protein	17.5[c] (100%)	beef heart mitochondria	600[d]

[a] The turnover number of the reconstituted system is related to the corresponding amount of CAT or BKA binding (column 2), assuming a molecular weight of 60 000 for the active dimer (Klingenberg et al., 1975). [b] For the definition of the different carrier portions see Figure 6 and the text. [c] According to Klingenberg et al. (1978). [d] According to Klingenberg (1976).

Table II: Removal of Triton X-100

procedure[a]	residual CAT binding[b] (μmol/g of protein)	exchange rate[c] [μmol/(g min)]	residual Triton bound (%)
	14.5	320	
1	2.3	5	6
2	12.8	25	8
3	12.5	285	5.5

[a] For procedures 1–3 see Methods. [b] The CAT binding is used for monitoring the intactness of the carrier protein. Mitochondria are preloaded with [^{3}H]CAT. The amount of inhibitor bound to the isolated protein is measured after the various procedures for Triton removal. [c] The adenine nucleotide exchange is reconstituted with the unloaded carrier protein (unlike the experiment in column 2, where CAT-protein is used) after Triton is removed by the different methods.

In Table I the reconstituted parameters are correlated with those of the mitochondria. The carrier molecules in the reconstituted system can be divided into three parts. (1) One portion is functionally reconstituted carrier molecules, inhibited by CAT and also by BKA, when added in high concentrations. They represent up to 5% of carrier molecules and nearly reach the same turnover number as those in intact mitochondria. (2) A second part is carrier molecules with reconstituted binding capacity for BKA. They show very low, though specific, counter exchange and are inhibited only by BKA. This population comprises 25–30% of carrier molecules. (3) The rest of the carrier proteins (about 70%) seem to be inactivated.

Since both inhibitor binding and transport activity (Krämer & Klingenberg, 1977a,b) are severely affected by detergents used to solubilize the carrier, it is tested whether reducing the amount of detergent increases the reconstituted activity of the ADP–ATP carrier (Table II). If the detergent Triton X-100 is removed by incubation with Bio-Beads SM2, the carrier is largely inactivated as monitored by the loss of CAT-binding capacity. With a column technique using phospholipid-saturated Bio-Beads, the intactness of the CAT–protein complex is preserved, whereas the amount of reconstituted adenine nucleotide exchange with the unliganded protein is very small. As tested by gradient centrifugation (experiments not shown), the unsuccessful reconstitution is due to a low extent of incorporation of the carrier molecules into the phospholipid membranes caused by the removal of the detergent.

We succeeded, however, in reducing the amount of Triton X-100 after incorporation into the liposomes by incubating the reconstituted liposomes with Bio-Beads under certain conditions (see Methods). After removal of about 95% of Triton X-100, the reconstituted transport activity remains nearly unaffected. These experiments have been repeated with the carrier protein isolated by the use of LAPAO (Krämer et al., 1977). It can be shown that Bio-Beads SM2 are also able to remove this detergent from the solubilized protein and from the vesicles. Nevertheless, also in this case no stimulation of the reconstituted exchange activity after removal of the bulk amount of detergent could be seen.

Discussion

It has been unequivocally shown by the reconstitution experiments with the highly purified CAT–protein complex that adenine nucleotide transport in the reconstituted system depends exclusively on the protein characterized by 30 000 molecular weight polypeptides (Riccio et al., 1975a,b), which is known as the CAT-binding protein. Although the extent of reconstituted transport with the CAT–protein complex is rather low, caused by the drastic procedures necessary for removal of the tightly bound CAT, the reconstituted exchange activity clearly appears only after partial detachment of the specific inhibitor.

In the previously reported reconstitution of the adenine nucleotide transport, the unliganded carrier protein was used (Krämer & Klingenberg, 1977b) which is very unstable and rapidly loses its reconstitutive activity. The introduction of BKA- and ATR-loaded protein complexes for reconstitution of adenine nucleotide exchange, however, represents an improvement in handling this very labile protein. The carrier is much more stable when isolated in these forms and therefore to be preferred in larger experimental series. Nevertheless, the extent of reconstituted transport could not be increased over 5–6% of the original mitochondrial activity, the same extent as achieved with the unliganded carrier. The isolated adenine nucleotide carrier cannot be stored by freezing in liquid nitrogen, since its reconstitutive activity is lost after thawing.

Solubilization, isolation, and reconstitution of the unliganded carrier seem to be rather harsh procedures leading to inactivation of the largest part of the protein. By testing the reconstituted binding and transport activity throughout the course of isolation and reconstitution of the ADP–ATP carrier protein, it is possible to detect the most critical steps with regard to inactivation. The marked influence of temperature during the solubilization on the binding capacity of the adenine nucleotide carrier (Klingenberg et al., 1978) suggests that this step involves considerable inactivation. Also, purification of the protein by adsorption chromatography on hydroxylapatite seems to be critical. When solubilization is performed at room temperature, reconstitution hardly reaches 10% of the value when carried out at 0 °C (experiments not shown). Nevertheless, this procedure is indispensible due to its rapidity and good purification in a single step. Another important point is sonication. Although it is essential for reconstitution, partial inactivation cannot be avoided, as shown in Figure 3.

Several other attempts have been made to obtain more complete reconstitution by varying possible factors influencing the carrier activity in the artificial system. (1) Reduction of the large amount of detergent incorporated together with the protein into the membrane of phospholipid vesicles does not lead to an augmentation of reconstituted transport. (2) Mitochondria in different metabolic states (energized/

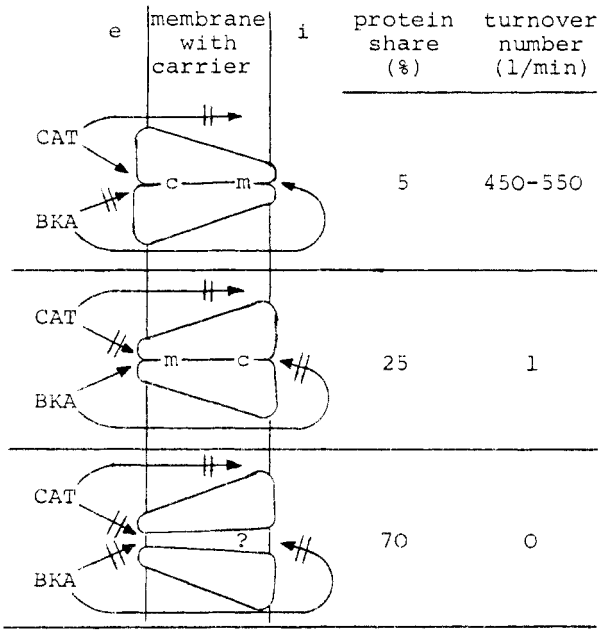

FIGURE 7: Illustration of the binding and exchange properties of the three carrier populations. The carrier protein in its functional state is shown as a dimer with the c (cytosolic) or m (matrix) binding site exposed to the external (e) or internal (i) side of the liposomal membrane. For further details see the text.

deenergized) were used for carrier isolation. This variation, however, does not influence the reconstituted binding or transport activity (experiments not shown). (3) The effect of various types of phospholipids on the extent of reconstitution has already been tested and discussed (Krämer & Klingenberg, 1977b). Egg yolk phospholipids proved to be much better than soy bean phospholipids. An optimum mixture consisting of phosphatidylcholine and extracted mitochondrial phospholipids enabled reconstitution up to 7% of the original mitochondrial carrier activity, but higher rates were not observed. (4) Possible influence of cofactors that may be lost during the isolation procedure has also been tested. Addition of crude extracts of beef heart mitochondria, solubilized with Triton, laurylamine *N*-oxide, or cholate, however, does not show any stimulation, when added in various steps during the reconstitution procedure.

In the reconstituted system the adenine nucleotide exchange can be analyzed for the amount of participating carrier molecules by relating CAT and BKA binding to the inhibition of transport. When the carrier is incorporated into liposomes before sonication, binding of CAT and BKA to 30% of the inserted protein molecules is found (Krämer & Klingenberg, 1977a); however, no transport activity is reconstituted. The reconstitution of about 5% transport activity after sonication can now be correlated with a correspondingly decreased binding of CAT. Also, the question can be answered whether the reconstitution reflects a small portion of fully active or a large population of only partially active carrier molecules. The total amount of incorporated carrier protein can be divided into three parts: (1) a small portion (up to 5%) of carrier protein with nearly the same specific activity as observed in mitochondria, (2) a larger population (25–30%) of very weakly though specifically exchanging carriers, and (3) the largest part (about 70%) of carrier molecules, inactive both in transport and binding.

The two active populations of incorporated proteins could be distinguished by their ability to bind CAT and BKA. About 5% of the carrier protein binds CAT, and this binding causes 98% inhibition of the exchange activity. BKA binds to about 30% of the protein, resulting in complete inhibition of transport. BKA is also able to inhibit that portion of carrier activity which is not blocked by prior addition of CAT. The inhibition of CAT-insensitive activity corresponds to the binding of BKA to about 25% of the protein. These results can be interpreted in terms of the orientation of carrier molecules in the reconstituted system, as illustrated in Figure 7, by taking into account the results of binding studies with mitochondria: CAT was found not to permeate the mitochondria and to bind exclusively to the cytosolic ("c") side of the membrane, looking to the outside (Klingenberg et al., 1973). BKA as a permeant ligand is able to bind to the matrix ("m") side. The binding site can reorient between both sides and change its specificity accordingly (Klingenberg & Buchholz, 1973; Scherer & Klingenberg, 1974).

Hence, it can be assumed that the 5% of fully active molecules expose the c side to the outer surface of the liposomes and therefore can bind CAT. BKA, when added in higher concentration, is bound to the m side of these protein molecules after penetrating the liposomes. The 25% weakly active carriers are oriented in the opposite direction with the m side exposed to the outer surface such that CAT is rejected and BKA is readily accepted. Only the relatively small portion of 5% of the incorporated carriers is oriented in the same manner as in mitochondria, and it appears reasonable that only this portion is reconstituted with full transport activity.

The most important conclusion, however, that can be drawn from the correlation of binding with transport, made possible by the use of the two inhibitor ligands, is the following. On the molecular level the activity of the functionally reconstituted carrier protein is very similar to that known from intact mitochondria. Therefore, forthcoming results concerning regulation, kinetic properties, etc. obtained with the reconstituted system can directly be transferred to the conditions in mitochondria.

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Steroidogenic Activity of High Molecular Weight Forms of Corticotropin†

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ABSTRACT: The high molecular weight forms of adrenocorticotropic hormone (ACTH) produced by mouse pituitary tumor cells (AtT-20/D-16v) were separated from each other by gel filtration; their ability to stimulate steroidogenesis by isolated rat adrenal cortical cells was studied. Pools of pro-ACTH/endorphin, ACTH biosynthetic intermediate, and glycosylated ACTH(1–39) were obtained; on the basis of $NaDodSO_4$–polyacrylamide gel electrophoresis, over 97% of the immunoactive ACTH was found to have the expected molecular weight. Suspensions of isolated rat adrenal cortical cells were incubated overnight in tissue culture medium and used in a 2-h steroid production assay. Synthetic human ACTH(1–39) [hACTH(1–39)] was used as a bioassay and immunoassay standard; 60 pM hACTH(1–39) stimulated half-maximal production of fluorogenic steroid. The amount of pro-ACTH/endorphin, ACTH biosynthetic intermediate, or glycosylated ACTH(1–39) added was estimated with an ACTH(17–24) immunoassay. All three high molecular weight forms of ACTH are capable of stimulating the same maximal level of steroidogenesis as hACTH(1–39). Glycosylated ACTH(1–39) is equipotent with hACTH(1–39); ACTH biosynthetic intermediate and pro-ACTH/endorphin are, respectively, 100- and 300-fold less potent than hACTH(1–39). Steroid production in response to all four forms of ACTH is linear in time. All of the different forms of ACTH stimulate the synthesis of corticosterone and related steroids; no significant production of cortisol or aldosterone was observed. β-Lipotropin (βLPH) and 16K fragment, which comprise the non-ACTH regions of pro-ACTH/endorphin and are secreted by the pituitary tumor cells, did not stimulate or interfere with steroidogenesis. Brief incubations of pro-ACTH/endorphin and ACTH biosynthetic intermediate with trypsin generated lower molecular weight forms of ACTH and increased biological activity 50-fold; thus, the decreased steroidogenic potency of these forms of ACTH is thought to be due to structural constraints on the ACTH(1–39)-like sequence in these larger precursor molecules.

Pulse-labeling studies using a mouse pituitary tumor cell line (AtT-20/D-16v), as well as isolated rat anterior and intermediate pituitary cells, have shown that glycosylated and nonglycosylated forms of adrenocorticotropic hormone [ACTH(1-39)] are synthesized as part of a larger glycoprotein molecule (Mains & Eipper, 1976; Mains et al., 1977; Eipper & Mains, 1978a). Since this glycoprotein is the biosynthetic precursor for both ACTH- and endorphin-containing molecules, it is referred to as pro-ACTH/endorphin. Quantitative pulse–chase experiments have shown that the major pathway for ACTH biosynthesis in mouse pituitary tumor cells is as shown in Scheme I. The ACTH peptide backbone makes up roughly the middle third of the pro-ACTH/endorphin molecule. The peptide extension to the carboxyl terminal side of the ACTH(1–39)-like region is similar to known βLPH molecules; a β-endorphin-like sequence [βLPH(61–91)] lies at the carboxyl-terminal end of the precursor. The amino-terminal third of the precursor molecule is referred to as 16K fragment, and at present very little is known about its structure or possible biological activity (Eipper & Mains, 1978b).

Pro-ACTH/endorphin, ACTH biosynthetic intermediate, and glycosylated ACTH(1–39) are secreted by pituitary tumor cells (Eipper & Mains, 1975; Eipper et al., 1976; Allen et al., 1978) and by isolated rat anterior and intermediate pituitary cells (Eipper & Mains, 1978a; Vale et al., 1978); in addition, high molecular weight forms of ACTH have been demonstrated in normal human plasma and the plasma of patients with pituitary or ectopic ACTH-secreting tumors (Yalow & Berson, 1973; Gewirtz & Yalow, 1974; Orth & Nicholson,

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