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Isolation of the Unliganded Adenosine 5′-Diphosphate,Adenosine 5′-Triphosphate Carrier-Linked Binding Protein and Incorporation into the Membranes of Liposomes†

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ABSTRACT: The ADP,ATP carrier-linked binding protein of beef heart mitochondria was isolated free of ligands, using the detergent 3-lauramido-*N,N*-dimethylpropylamine oxide. Unlike the preparation of the carboxyatractylate (CAT)–protein complex described earlier, this protein enables direct binding studies to be made with the inhibitor ligands. The protein was characterized with respect to its polypeptide composition, stability against degradation, and immunological properties; the identity of the binding protein with the previously isolated CAT–protein complex was thereby shown. As a step toward reconstitution studies, the isolated binding protein was incorporated into liposomes by a simple rapid mixing process. The complete insertion into the vesicular membrane was demonstrated by chromatography on Sepharose 6B and by immunoprecipitation reactions.

The characterization of a transport system isolated from biomembranes depends to a large extent on the reconstitution of its function, which requires reincorporation into artificial membranes. As a step toward this aim the isolation of the ADP,ATP carrier-linked binding protein free of ligands is shown in this paper, and the incorporation of the protein into liposomes is demonstrated.

Previously, the successful isolation of the ADP,ATP carrier as the CAT[1]–protein complex has been achieved with Triton X-100 as detergent. The protein is protected by the tightly binding CAT against denaturation during solubilization (Riccio et al., 1975a,b). Under the same conditions in the unliganded or ADP-loaded form the protein became denatured. The BKA–protein complex could be isolated with intermediate stability.

In the present paper the isolation of the ATP,ADP carrier protein in the unliganded form and in the undenatured state will be demonstrated using other detergents, in particular LAPAO. This protein enables the binding of the inhibitors CAT and BKA to be studied directly, and provides a starting point for reconstitution studies in artificial membranes.

Materials and Methods

Triton X-100 was obtained from the Sigma Chemical Co. LAPAO (Aminoxide WS-35) was a gift from the Theo Goldschmidt AG; all concentrations were calculated for pure detergent (the substance is provided as a 35% w/v solution). Egg-yolk phospholipids were obtained from E. Merck, Darmstadt, and [^{14}C]sucrose was from the New England Nuclear Company. [^{35}S]CAT and [^{3}H]BKA were prepared as described earlier (Riccio et al., 1973; Babel et al., 1976).

Hydroxylapatite was prepared according to A. Tiselius as described by Bernardi (1971). Polyacrylamide gel electrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) was carried out as described by Neville and Glossmann (1974). Gels (5 × 100 mm) with 11% acrylamide and 0.1% cross-linker were used. The gels were stained with Coomassie brilliant blue G-250.

Protein was determined according to Lowry et al. (1951) or Bode et al. (1967), and phosphorus was estimated by the method of Chen et al. (1956).

LAPAO was assayed by a redox titration method (Gawargious and Ashworth, 1971), which was kindly provided by the Theo Goldschmidt AG. Under nitrogen and continuous stirring, CH_3COONa and NH_4SCN were added to the solution with LAPAO. With $TiCl_3$ solution the LAPAO was now reduced and after addition of H_2SO_4 the excess of Ti^{3+} was titrated with Fe^{3+} until the orange color of $Fe(SCN)_3$ appeared.

Liposomes were prepared by sonication with a Branson sonifier with microtip at a concentration of 50 mg of phospholipid per mL of sonication buffer (usually 0.1 M NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 7.5). The internal volume as determined with trapped [^{14}C]sucrose amounts to about 0.45 μL per μmol of phospholipid. The preparation of antibodies and other immunological methods were described by Buchanan et al. (1976).

Isolation of the binding protein with LAPAO was basically similar to that previously described with Triton X-100 (Riccio

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[1] Abbreviations used are: CAT, carboxyatractylate; BKA, bongkrekate; LAPAO, 3-lauramido-*N,N*-dimethylpropylamine oxide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

TABLE I: Comparison of Chromatography on Hydroxylapatite of Various Forms of the ADP,ATP Carrier Protein.[a]

Mitochondria charged with	Solubilizing detergent	% of total protein in pass-through	Ligand bound (μmol/g of protein)	Calcd purity in %
CAT	Triton X-100	9.4	13-14	75
	LAPAO	10.1	13-14	75
BKA	Triton X-100	10.5	*b*	
	LAPAO	9.6	13-14	75
	Triton X-100	4.9		40
	LAPAO	8.9		70-80

[a] Solubilization was performed by Triton X-100 (4%) or LAPAO (1%) in the solubilization buffer (for details see Materials and Methods). [b] Not directly measurable because of unspecific BKA adsorption.

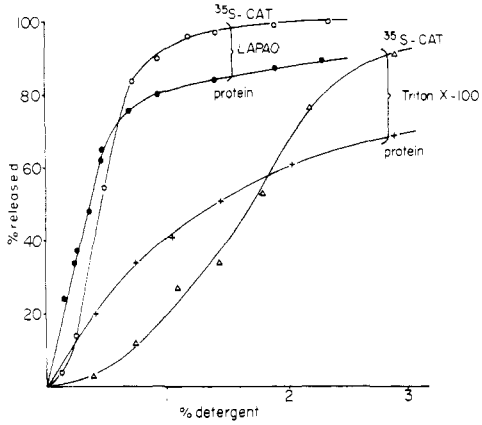

FIGURE 1: Effect of LAPAO and Triton concentration on the solubilization of total protein and protein-bound [^{35}S]CAT from mitochondria preloaded with [^{35}S]CAT. The samples were incubated in the usual buffers (see Materials and Methods) with increasing concentration of detergent, as indicated on the abscissa.

et al., 1975a,b). The column chromatography in this work was essentially the same, only using a solubilization buffer containing 1% LAPAO, 0.1 M Na_2SO_4, 10 mM Tricine-KOH, 1 mM EDTA, pH 7.5. For further speeding up of the isolation a batch procedure was applied. The solubilized mitochondria were added directly to a suspension of hydroxylapatite in the solubilization buffer. After a 10-min incubation at 0 °C, the hydroxylapatite is spun down (15 min at 20 000*g*); further centrifugation (60 min at 140 000*g*) of the supernatant, which contains the solubilized ADP,ATP carrier-linked binding protein, does not give any sediment.

Incorporation of the isolated binding protein into liposomes was achieved by incubation of the solubilized protein with liposomes for 30 min at 0 °C. The ratio of phospholipid to detergent (mol/mol) is kept above 10.

Results

Isolation of the Unliganded ADP,ATP Carrier-Linked Binding Protein

In the present work for the solubilization and purification of the ADP,ATP carrier-linked protein the detergent LAPAO is used instead of Triton X-100, as previously. The effect of varying the detergent concentration on the solubilization of beef heart mitochondria saturated with [^{35}S]CAT is shown in Figure 1, comparing the release of protein and [^{35}S]CAT by LAPAO and Triton X-100. LAPAO is effective at considerably lower concentrations than Triton X-100 in the release of protein and [^{35}S]CAT. For optimum solubilization a high ionic strength is required (0.3 M NaCl), as was shown before with Triton X-100. Between pH 6.5 and 8.0 solubilization is nearly independent of the pH. The standard conditions used for the extraction of the binding protein (1% LAPAO, 100 mM Na_2SO_4, pH 7.5) permit the solubilization of 80% of the mitochondrial protein and more than 90% of [^{35}S]CAT. As with Triton X-100, the [^{35}S]CAT remains tightly bound to the solubilized protein, as checked by equilibrium dialysis. In other words, the amount of CAT bound to the protein after release with LAPAO is as high as the specific binding of CAT in a Triton X-100 extract. The same can be said for the solubilization of the BKA-protein complex from BKA-preloaded mitochondria.

The release of unliganded protein shows approximately the same dependence on detergent and salt concentrations. However, it will be shown below that only with LAPAO is the unliganded form of the carrier released in the undenatured state with a survival time of a few hours, whereas with Triton X-100 the protein is denatured on solubilization within a few minutes. This ability of LAPAO to release undenatured forms of the carrier protein will be a major concern of this paper.

For the purification of the ADP,ATP carrier-linked binding protein in the presence of LAPAO, the same procedures as those reported earlier using Triton X-100 as detergent can successfully be applied. After the first chromatographic step on hydroxylapatite, the undenatured forms of the carrier protein have been found to accumulate in the pass-through fractions, and they are not absorbed by hydroxylapatite in contrast to most other proteins. It was found, using Triton X-100, that the protein yield of the pass-through is decreased when the mitochondria are not preloaded with inhibitors CAT and BKA, as confirmed by the experiments of Table I. However, with LAPAO a high protein yield in the pass-through is obtained also in the extracts from untreated mitochondria reaching about 10% of mitochondrial inner membrane protein, similar to results with CAT-loaded mitochondria (Riccio et al., 1975a,b), and similar to these of other groups (Boxer, 1975; Capaldi et al., 1973). This indicates that the unliganded protein is not denatured by LAPAO, since it had been shown previously (Klingenberg et al., 1976) that the nonabsorptivity to hydroxylapatite of the ADP,ATP carrier is linked largely to its undenatured state.

The amount of ADP,ATP carrier-binding protein in the pass-through can be estimated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the appearance of the polypeptide with mol wt 30 000. Virtually the same polypeptide pattern is seen (Figure 2) in the pass-through of LAPAO extracts from CAT-loaded mitochondria and unloaded mitochondria as previously described for the CAT protein, extracted with Triton X-100. The protein derived from unloaded mitochondria has a small additional shoulder with a mol wt of about 27 000. This compound is likely to be derived by proteolytic degradation from the binding protein.

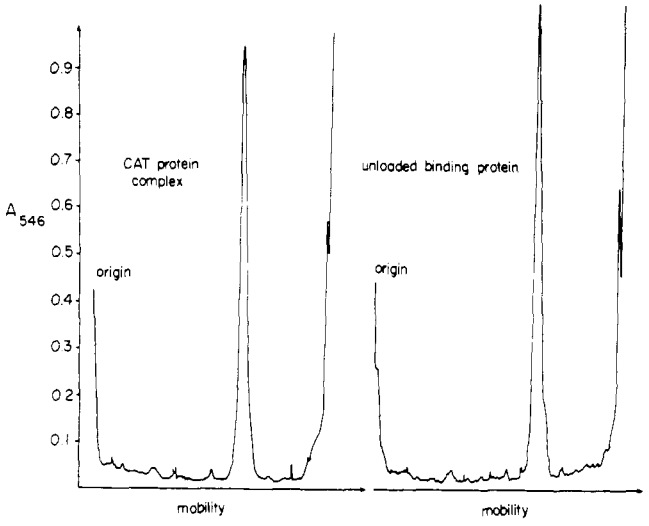

FIGURE 2: Polypeptide composition of binding protein isolated from CAT-loaded or -unloaded beef heart mitochondria. (a, left) CAT binding protein isolated with LAPAO after hydroxylapatite column chromatography. (b, right) ADP,ATP carrier-linked binding protein free of ligands; isolation identical with a. Densitometric traces of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The peak near the front of the gel is caused by staining of the detergent LAPAO. For molecular weight determinations see Riccio et al. (1975b).

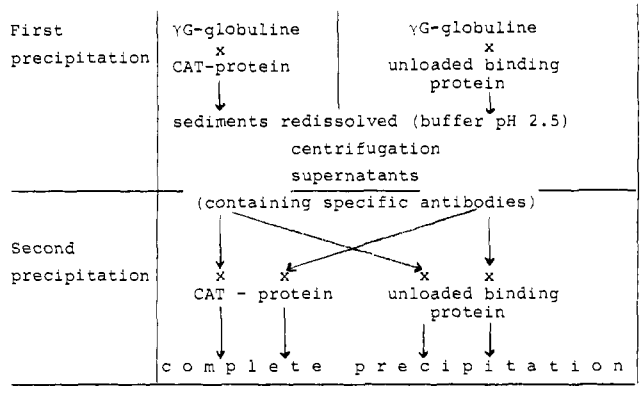

FIGURE 3: Cross-reaction of isolated antibody against CAT-protein complex and with unloaded binding protein. Immunoprecipitates of the first precipitation reaction were redissolved in glycine buffer (pH 2.5) (McCans et al., 1975). The complete precipitation of CAT-protein complex and of unloaded binding protein is checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sediments and of supernatants from the second precipitation reaction (excess of dithioerythritol was added). More than 80% of the 30 000 mol wt protein bound is found in each of the four precipitates.

The amount of ligand bound to the protein after the pass-through of the hydroxylapatite column is determined both with [^{3}H]CAT and [^{3}H]BKA. With Triton X-100 a determination of [^{3}H]BKA binding is not possible because free [^{3}H]BKA is retained largely in overlapping Triton X-100 micelles. Assuming that the purified CAT- and BKA-protein complexes contain about 18 nmol of ligand/mg of protein, one can calculate that the ligand-protein complex amounts to about 75% of the pass-through (Table I). With these values one can standardize the polypeptide bands measured in gel electrophoresis at mol wt 30 000 and, by comparing the extracts from unloaded with CAT-loaded mitochondria, calculate the purity of the ligand-free protein in the hydroxylapatite pass-through at about 70%.

Additional gel filtration on Sepharose 6B can be used to further purify the protein but in the process a new difficulty arises. Whereas the CAT-protein complex could be purified by this procedure, the unloaded protein is considerably degraded leading to an increase of the shoulder with a mol wt of 27 000 in gel electrophoresis.

The unloaded protein solubilized with Triton X-100 is too unstable to endure the purification steps. Addition of diisopropyl fluorophosphate at any time of isolation had no inhibitory effect on the appearance of the band with a mol wt of 27 000. In order to avoid the substantial degradation of the unloaded binding protein during the isolation procedure for the binding experiments, the second step of purification (Sepharose 6B column) is omitted. For further acceleration of isolation in most experiments a batch procedure for adsorption chromatography on hydroxylapatite was used, which shortens the preparation time from 3 h to about 20 min. The protein isolated with this fast method shows substantially better activity in binding experiments (Krämer and Klingenberg, 1977) compared to the protein isolated by column procedure.

To demonstrate the identity of the CAT-binding protein and the unloaded binding protein, further experiments were performed. In co-electrophoresis (sodium dodecyl sulfate-polyacrylamide) of these two protein preparations only one peak with a mol wt of about 30 000 is seen. Amino acid analysis of both proteins shows nearly identical composition.

Further evidence for the identity of these two proteins is obtained by immunological studies (Buchanan et al., 1976). In Ouchterlony double diffusion tests, both CAT-binding protein and unloaded binding protein precipitate with antibody against the isolated CAT-binding protein. Even clearer similarity of antigenic determinants can be demonstrated in a more refined immunological analysis, shown in Figure 3, by cross-reacting the precipitated and redissociated antibodies with the CAT-protein and unloaded protein.

Incorporation of the ADP,ATP Carrier-Linked Binding Protein into the Membrane of Liposomes

Small amounts of detergents (Triton X-100, LAPAO) added to liposomes are incorporated into the membranes of the vesicles without destruction of the membrane. An incorporation of LAPAO was observed also in parallel NMR studies (K. Beyer, manuscript in preparation), in accordance with reports on the interaction of Triton X-100 with liposomes (Inoue and Kitagawa, 1976). Increasing amounts of detergent finally destroy the vesicular structure and make the internal volume accessible. Detailed studies of the effects of detergent and protein on liposomes are important in order to assure the intactness of the vesicles used in the following reconstitution experiments (Krämer and Klingenberg, 1977). The residual internal volume (indicating the amount of intact vesicles) and the absorbance at 300 nm are plotted as a function of added LAPAO (Figure 4). The vesicles evidently collapse at a distinct ratio of phospholipid to LAPAO. The simultaneously increasing absorbance corresponds to the formation of larger vesicular and multilamellar structures. These findings are again supported by NMR experiments (K. Beyer, manuscript in preparation). The additional presence of isolated carrier-linked binding protein lowers the critical detergent concentration for the breakdown of liposomes only slightly.

In the following experiments for the incorporation into liposomes the amount of detergent-solubilized protein was adjusted to a phospholipid: LAPAO ratio between 10 and 15. At this ratio the intactness of the vesicles is not yet affected by the added detergent.

Binding of the isolated protein to liposomes can be demonstrated by chromatography on Sepharose 6B (Figure 5). Figure

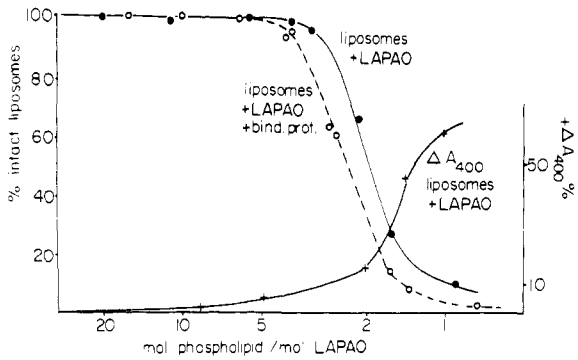

FIGURE 4: Influence of the detergent LAPAO on the intactness of liposomes. Liposomes were sonicated in the presence of [^{14}C]sucrose. External sucrose was removed to more than 99% by dialysis against the sonication buffer for 16 h. Varying amounts of LAPAO either to pure liposomes or to vesicles with incorporated protein were added. After 1 h at 4 °C, aliquots were separated on Sephadex G75 or measured in a spectrophotometer. The amount of [^{14}C]sucrose eluted in the void volume of the column is used as a measure of the amount of intact vesicles.

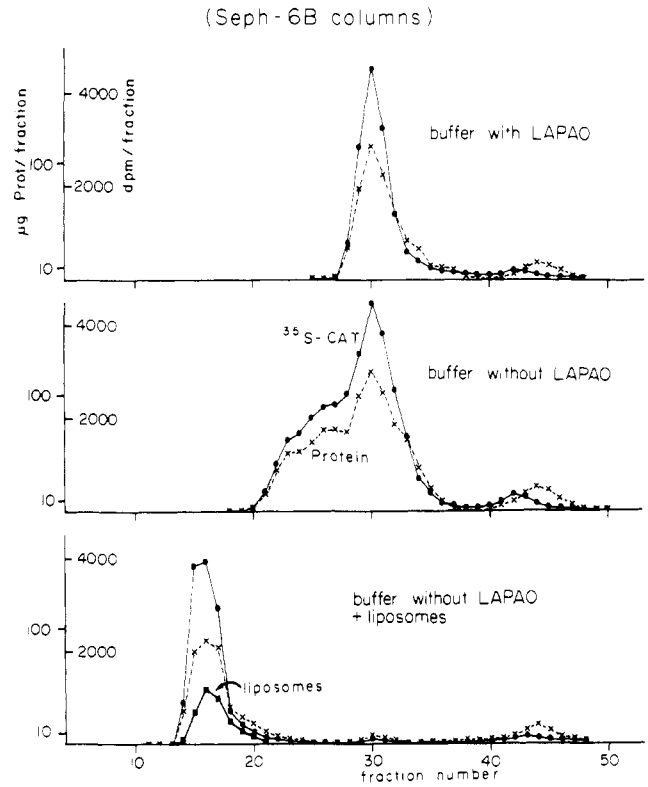

FIGURE 5: Chromatography of the ADP,ATP carrier-linked binding protein and liposomes on Sepharose 6B columns (10 × 500 mm): (a, top) CAT binding protein loaded with [^{35}S]CAT in 50 mM NaCl, 5 mM Tris-Cl, 0.5 mM EDTA, 0.1% LAPAO, pH 7.5; (b, middle) same as a, in buffer without detergent; (c, bottom) liposomes with incorporated CAT binding protein in buffer without detergent.

5b illustrates the problem arising from the role of detergent for solubilization of the protein. Liposomes cannot be chromatographed in buffer containing LAPAO without damage, and isolated binding protein can hardly be chromatographed in detergent-free buffer without precipitation. With detergent-free buffer (Figure 5b) the protein appears with an additional shoulder probably containing aggregated protein which is eluted with an apparently higher molecular weight. Nevertheless, after incubation with liposomes (Figure 5c) all the protein is now eluted together with the phospholipid vesicles in the void volume, arguing for an incorporation or at least a tight attachment of the protein to the liposomes. Similar results are obtained by corresponding experiments with the unloaded binding protein.

The reaction with antibodies gives a more specific test for the incorporation. Reaction of antibody against the binding protein with the isolated ADP,ATP carrier-linked binding protein can be followed by monitoring the absorbance increase on forming the precipitate. Under identical conditions, the isolated binding protein precipitates completely with the antibody, whereas the incorporated protein does not give any precipitation, demonstrating the incorporation into the vesicles.

Discussion

The use of LAPAO as detergent for the isolation of the CAT binding protein to be used in reconstitution studies has several advantages.

(1) LAPAO enables the isolation of the ADP,ATP carrier-linked binding protein free of bound ligands. With this form of the protein the reconstituting activity of the isolated carrier can be shown in respect to both its binding and its conformational properties (Krämer and Klingenberg, 1977).

(2) The amount of detergent necessary for solubilization can be reduced to one-fourth compared with Triton X-100 (Riccio et al., 1975a) which is important for incorporation into liposomes without disintegration of the vesicular structure.

(3) Similar to Triton X-100 (but not to cholate and deoxycholate) the ligands CAT and BKA remain bound to the protein extracted from CAT or BKA preloaded mitochondria during solubilization. Among the nonionic detergents not only nonpolar but also polar substances such as LAPAO are apparently less harmful to the isolated carrier protein.

The application of a short-cut batch procedure for the isolation of the ADP,ATP carrier-linked binding protein seems to be essential because of the instability of the isolated, unliganded protein. The stabilizing effect of ligand binding is an effect which is known also for many other proteins (Goldberg and Dice, 1974). For isolating undenatured binding protein, high-affinity ligand binding has been used for the CAT protein as protection against the destabilizing effect of detergent. With LAPAO, however, the protein appears to be less dependent on protection as compared to Triton X-100. Loss of binding capability and degradation that cannot be prevented by protease inhibitors remains a problem in the work with this protein.

What is the evidence that the isolated protein amounting to 70% of the protein content in the hydroxylapatite supernatant is homogeneous unloaded binding protein? The corresponding purified CAT-protein complex has been shown to respond quantitatively to specific conformation changes linked to the CAT binding. Moreover, when isolating the same protein in another conformation, such as the BKA-protein complex, it has changed completely its resistance to proteases. Whereas the isolated CAT-protein is resistant against trypsin, the BKA protein is fully degraded in a short time (H. Aquila, manuscript in preparation). Such drastic responses to ADP,ATP carrier-linked ligands are not to be expected for other proteins, if possibly present in the isolated fraction.

Which criteria can be given for real insertion of the protein into the membrane instead of simple attachment to the vesicles? Although with the applied methods a clear incorporation cannot be proved, several results argue for an insertion of the binding protein into the phospholipid membrane of the liposomes. (1) In experiments with Sepharose 6B columns all the added protein is eluted with the added liposomes. (2) The

ADP,ATP carrier-linked binding protein which can react instantly with its specific antibody does not show any precipitation when liposomes are present in the incubation medium. (3) In the following paper (Krämer and Klingenberg, 1977) it is demonstrated that the protein dramatically changes its binding and its conformational properties on addition of phospholipid vesicles to the isolated binding protein.

Acknowledgment

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