SOLUBILIZATION OF THE CARBOXY-ATRACTYLATE BINDING PROTEIN FROM MITOCHONDRIA *

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1. Introduction

The discovery of a specific ADP, ATP transport through the inner mitochondrial membrane [1] suggested early the existence of a specific carrier catalyzing this transport. At a subsequent stage the carrier was defined by the binding of the substrates, ADP and ATP, or the highly specific inhibitors, atractylate (ATR), carboxy-atractylate (CAT) and bongkrekate (BKA), which are assumed also to bind specifically to the ADP, ATP carrier [2–5]. For the isolation of the ADP, ATP carrier it appeared most promising to use for its identification the tagging of the protein with the high affinity ligand carboxy-atractylate which is available in the ^{35}S-labelled form. Moreover, ADP, ATP binding had been found to be very sensitive to relatively minor alterations of the mitochondrial membrane in contrast to binding of ^{35}S-CAT.

At first the identification of the CAT-binding protein by ^{35}S-CAT binding was unsuccessful because no specific binding of CAT was found after solubilization of the membranes with a variety of detergents. As reported a year ago [6] a breakthrough was achieved by loading the carrier in the parent mitochondria with CAT before extraction. By this means it was possible in particular with Triton X-100 to solubilize the CAT–protein complex and to apply standard fractionation procedures as reported in a following letter [7]. These communications should be read in context with the earlier report [6] on our first results on the solubilization and partial purification of the CAT-binding protein.

* This publication is report II, in a series of reports on the isolated ADP,ATP carrier. Report I is reference 6.

2. Materials and methods

Triton X-100 was obtained from Sigma Chemical Co. ^{35}S-CAT was prepared as described earlier [8].

Procedure for the solubilization of the CAT-binding protein: Beef heart mitochondria (10 mg protein/ml) were loaded with CAT by incubation with 20 to 50 μM ^{32}S- or ^{35}S-CAT for 10 min at 0°C in a medium (pH 6.8) containing 0.25 M sucrose, 0.5 mM EDTA, 2.5 mM $MgCl_2$ and 20 mM morpholino-propane sulfonic acid (MOPS).

After short time centrifugation, the sediment was suspended in 4% Triton X-100, 0.5 M NaCl, 20 mM MOPS at pH 7.2 and extracted 30 min at 0°C. The mixture was then centrifuged at 143 000 g for 1 hr and the precipitate was discarded. The supernatant fraction contained the solubilized CAT–protein.

In some experiments the CAT-binding protein was first enriched in the mitochondrial membrane by extraction of the more soluble proteins with 4% cholate in 0.1 M ammonium sulfate at pH 8 or with 0.5% Triton X-100 in 0.4 M NaCl at 7.2 for 30 min at 0°. After centrifugation (143 000 g, 1 hr), the CAT-binding protein was extracted from the residue with the 4% Triton medium as described above.

Equilibrium dialysis: Determination of the protein-bound ^{35}S-CAT in solution was carried out by dialysing the extracts in 200 μl cells of a 'DIANORM'-apparatus (Dr Weder, ETH Zürich) against the same medium used for extraction. For binding studies

with the solubilized protein, ^{35}S-CAT was added to the protein-free half cell. Dialysis was performed at room temperature for 3 to 5 hr.

Results

3.1. *Comparison of various detergents*

The procedure commonly applied for the isolation of a specific membrane protein consists of solubilization with a detergent and subsequent identification by its capacity to bind specific ligands added to the mixture of solubilized proteins or to protein fractions precipitated after removal of detergents. As has been briefly reported previously [6] and as will be discussed in more detail elsewhere, these experiments revealed nonspecific binding of CAT to the solubilized and precipitated proteins but failed to identify a specific CAT-binding protein. In contrast to such unspecific CAT binding, the binding of CAT to mitochondria has been shown to be highly specifically linked to the ADP,ATP carrier and strictly limited to these carrier sites [4]. Therefore, the membranes were solubilized with detergents after preloading the carrier in the mitochondria with CAT.

Fig.1 shows a comparison of various detergents on the solubilization of ^{35}S-CAT protein under conditions where detergents are applied in amounts giving near maximum solubilization of total protein. The ionic detergents are quite effective in solubilizing CAT whereas among the non-ionic detergents of the polyglycol-group, only Triton X-100 is effective. The solubilization with Triton X-100 stands out among all of these detergents because it is the only case where the solubilized CAT remains bound largely to a protein. In all other cases as determined by equilibrium dialysis the solubilized CAT appears to be free. It is obvious that in contrast to Triton X-100 the ionic detergents denature the CAT loaded protein.

The extraction of CAT, in its dependence on the concentration of Triton X-100 (fig.2), demonstrates that usually high concentrations of Triton X-100 are required for appreciable solubilization. In the presence of 0.1 M KCl up to 70% of the ^{35}S-CAT is solubilized with 6% Triton X-100. Only above 2% Triton X-100 does a significant amount of the ^{35}S-CAT become

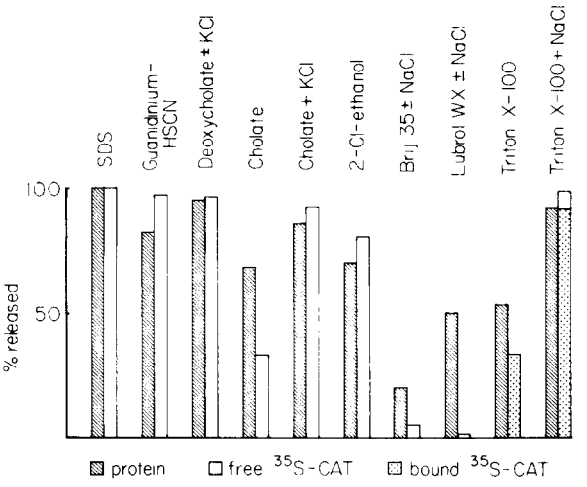

Fig.1. Effectiveness of different detergents and solvents in release of protein and free and protein-bound ^{35}S-CAT from beef heart mitochondria (BHM) preloaded with ^{35}S-CAT. Mitochondria were incubated under conditions optimal for each treatment: buffered detergent, 30 to 60 min at 0 or 37°C, protein concentration ranging between 1 and 20 mg/ml. Milligrams of detergent per milligram of protein: SDS, 2; DOC, 5.3; DOC (+1 M KCl), 1.1; cholate, 4.1; cholate (+ 0.5 M KCl) 5.2; 2-chloroethanol, 95%; Brij 35, 45; Lubrol, 8; Triton X-100, 3; Triton X-100 (+ 0.5 M NaCl), 3.5.

solubilized. Below that range, first a relative enrichment of CAT binding in the membrane is obtained as a result of the removal of other proteins. The dependence on the concentration of KCl in the presence of 6% Triton X-100 illustrates that full solubilization is obtained only above 0.2 M KCl. In all these cases solubilized CAT is found by equilibrium dialysis to be bound to protein. These results show that the CAT binding protein belongs to the most hydrophobic proteins of the mitochondria.

It was already documented in the previous publication [6] that the binding of CAT to mitochondria is a prerequisite for retaining the CAT binding capability after the solubilization with Triton X-100. This is further demonstrated in fig.3 where the capability to bind ^{35}S-CAT is compared in solubilized mitochondria which have either been loaded with CAT or remained unloaded. At the same time the experiments demonstrate that in solution the bound CAT can be exchanged against

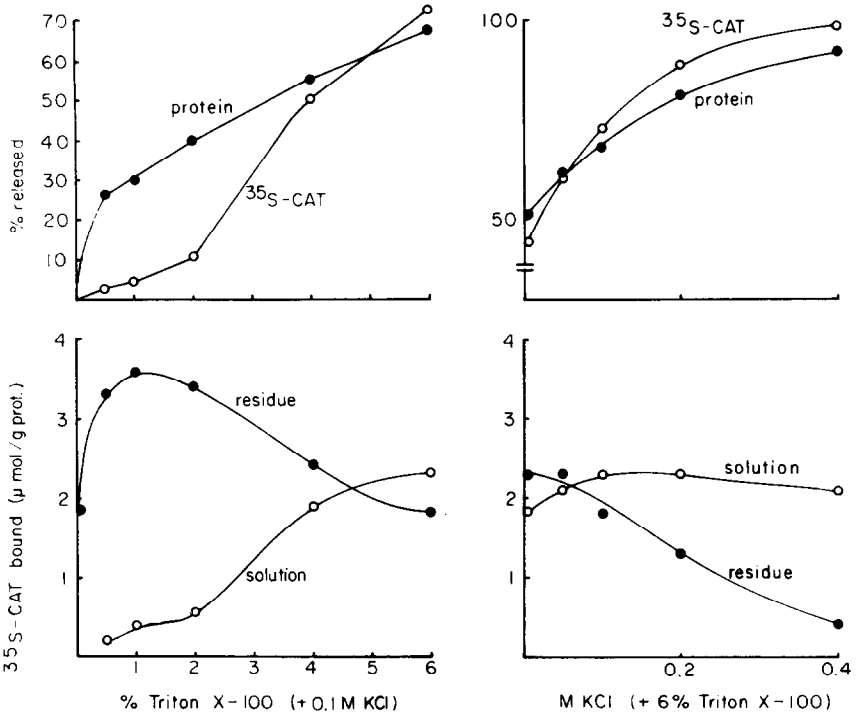

Fig.2. Effect of detergent and salt concentration on the solubilization of protein and ^{35}S-CAT by Triton X-100 from mitochondria preloaded with ^{35}S-CAT. 16 mg protein/sample incubated for 30 min at 0°C.

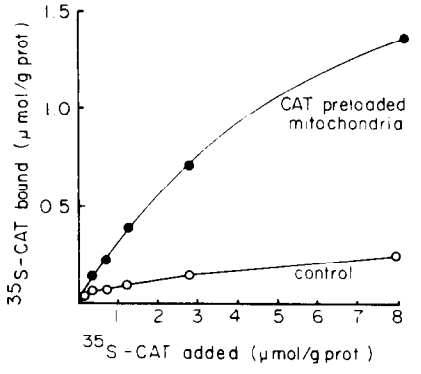

Fig.3. Demonstration of ^{35}S-CAT binding to Triton X-100 extracts obtained from control and ^{32}S-CAT-preloaded mitochondria. Binding was measured by equilibrium dialysis in 6% Triton X-100, 10 mM MOPS, 0.5 M NaCl at pH 7.2 by adding ^{35}S-CAT to the protein-free half cells. No correction of specific activity was made for the exchange with the unlabelled CAT already bound to the mitochondria.

added CAT. There is practically no binding of ^{35}S-CAT in the unloaded extracts.

The experiment shows again that on solubilization with Triton X-100 the binding of CAT to protein is retained and that CAT is not trapped in a Triton X-100 micelle together with a protein. Otherwise ^{35}S-CAT should also bind to the extract of unloaded mitochondria. Furthermore the binding of CAT to the solubilized protein appears to be less strong than in mitochondria where CAT cannot exchange against ^{35}S-CAT [9].

3. Discusssion

The most difficult problem for the planned isolation was the solubilization of a functional and identifiable CAT binding protein. This became possible by loading the carrier with CAT in the intact mitochondrial membrane and then obtaining a solubilized CAT–protein–complex. Besides having

an identification marker tagged to the protein, two unexpected benefits resulted from the binding of CAT to the protein in the intact membrane. The bound CAT protects against denaturation by Triton X-100 and facilitates greatly the separation from the other mitochondrial proteins as will be shown in a following publication [7].

The protection of the protein by CAT in preserving a certain conformational state after solubilization, has been stressed by us in our first communication [6]. In particular it was stated that CAT cannot bind to the solubilized protein. Only when the protein is loaded with CAT before solubilization by Triton X-100, can CAT bind and then only by exchange with the originally bound CAT, as shown in fig.3. Some time after these results were communicated by us [6], the group of Vignais [10], adopting the same principle of preloading the parent mitochondria before solubilization with ATR, published a partial purification of the ATR binding protein from liver mitochondria by affinity chromatography with succinyl ATR attached to Sepharose.

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