PURIFICATION OF THE CARBOXY-ATRACTYLATE BINDING PROTEIN FROM MITOCHONDRIA*

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1. Introduction

The solubilization of the CAT** binding protein complex from mitochondria reported in the preceding letter [1] made possible the purification of this protein by standard fractionation procedures. The present report gives a brief account of a new simple purification method which is a modification of that previously published [2]. A preliminary characterization of the isolated protein, which is tentatively identified with the ADP,ATP carrier, will be presented.

2. Materials and methods

Sources of chemicals: Triton X-100, Sigma Chemical Co.; hydroxylapatite (Bio Gel HTP) and Bio Gel A 1.5 m, BIO-RAD Laboratories; Sepharose 6B, Pharmacia; mol. wt calibration proteins, Böhringer.

2.1. *Analytical procedure*

Protein determination: Protein was determined by a modified biuret method or by a modified method of Lowry in the presence of 0.5% SDS to prevent precipitation of Triton X-100 [3]. BSA was used as standard.

Polyacrylamide Gel Electrophoresis in SDS (SDS-PAGE): Electrophoresis was carried out essentially as described by Weber and Osborn [4]. Usually 5 × 60 mm gels with 8% acrylamide and 0.35% crosslinker were used in the presence of 0.1% SDS. The gels were stained with Coomassie brilliant blue G-250.

Preparation of samples: 1.5 to 80 μg of protein was dissolved in a solution (pH 7.2) containing 2% SDS, 10 mM phosphate, 5% sucrose, 2 mM dithioerythreitol and heated to 100°C for 2 min prior to application to the gels.

Removal of Triton X-100 from protein sample: For electrophoresis and amino acids analysis, Triton X-100 was extracted from the samples with diethyl ether.

Procedure for the purification of the CAT-binding protein: The Triton X-100 extract from mitochondria [1] was applied directly to a hydroxylapatite column, 0.8 to 2 mg protein per ml packed HTP, and eluted with a solution (pH 7.2) containing 0.5% Triton X-100, 100 mM NaCl, 10 mM MOPS. The pass-through fractions were collected, concentrated by pressure dialysis and applied to a Bio Gel A 1.5 m or to a Sepharose 6B column. The major ^{35}S-CAT-containing fractions were pooled and tested electrophoretically for purity of the protein.

3. Results

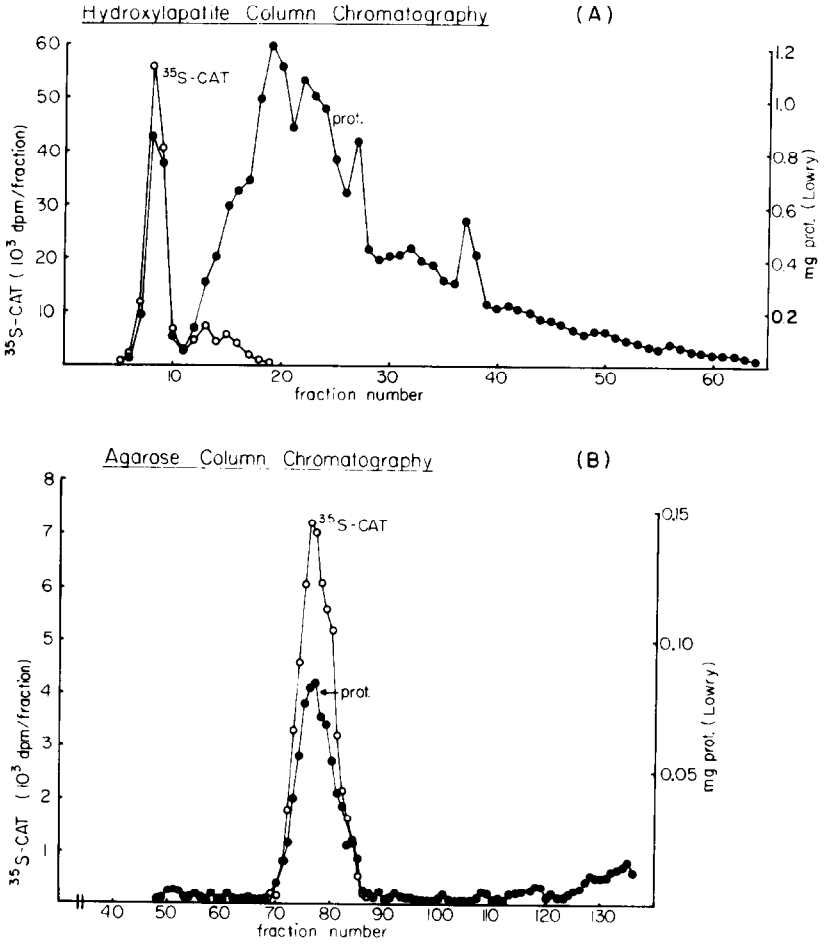
Initially the purification of the CAT binding protein started with the removal by cholate extraction of the more soluble membrane proteins (cf. procedure 1, table 1). In some cases a three-fold enrichment of the CAT-binding protein was achieved with this procedure

*This publication is report III, in a series of reports on the isolated ADP,ATP carrier. Report II is reference 1.

** *Abbreviations.* ATR, atractylate: BKA, bongkrekate: CAT, carboxy-atractylate.

Table 1
Summary of the purification of the CAT-binding protein by two similar procedures

	Protein mg	Yield protein % of total	Yield ^{35}S-CAT %	μmol CAT / g protein	Fold enriched
Procedure I					
^{35}S-CAT loaded BHM	115	100	100	1.88	1
Cholate Residue	40	35	58	3.14	1.7
Triton X-100 Extract	31	28	57	3.90	2.1
Ca-Phosphate Gel	4.5	3.5	36	16.9	9.0
Sepharose 6B	2.9	3	32	18.6	9.9
Procedure II					
^{35}S-CAT loaded BHM	85	100	100	1.76	1
Triton X-100 Extract	61	72	90	2.30	1.3
Hydroxylapatite	7.5	6.8	64	12.8	7.3
Sepharose 6B	2.5	5.9	57	17.4	9.9



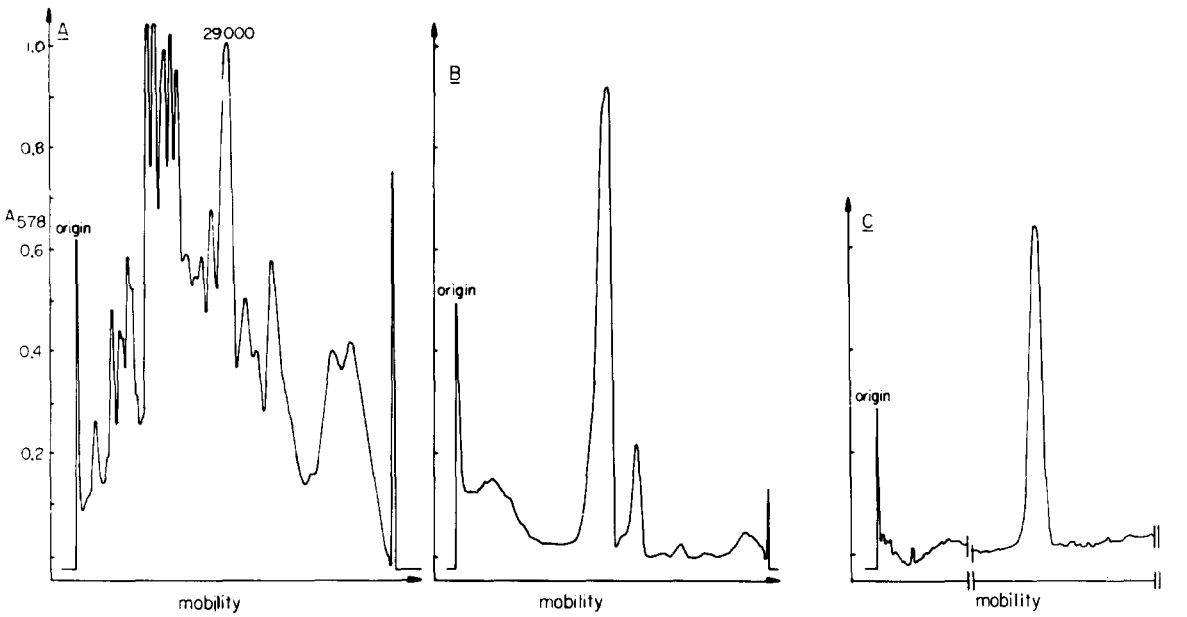


Fig. 2. Densitometric traces of SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). (A) Triton X-100 extract of mitochondria preloaded with CAT; 5 × 60 mm gel. (B) Pass-through of the HTP column; 5 × 60 mm gel. (C) Pooled fractions of the Bio Gel 1.5 m column; 5 × 240 mm gel.

(cf. fig.9, ref. 2) before the residue was subsequently solubilized.

A more convenient procedure starts with a Triton X-100 solubilization of the whole mitochondria according to the results shown in fig.2 of the preceding report [1]. The solubilized extract is then chromatographed on a hydroxylapatite column. The elution profile both of protein and the ^{35}S-CAT, is shown in fig.1A. Nearly all the ^{35}S is not absorbed to the column and appears in the pass-through fractions (peak at fraction 12). The major part of the protein is eluted with a linear gradient of phosphate. The pass-through contains only about 10% of the solubilized protein, resulting in a remarkable enrichment (by a factor of 6.9) of the CAT binding protein. The protein collected from these fractions is concentrated and then applied to a Bio Gel 1.5 m (fig. 1B) or Sepharose 6B column. The protein and the ^{35}S activity are eluted (together) in a single peak with a distinct slower moving shoulder. This results in a further enrichment (by a factor of 1.4) of the CAT-binding protein.

In addition to ^{35}S activity the progress of purification is further controlled by SDS polyacrylamide gel-electrophoresis (SDS-PAGE). As shown in fig.2, one single peptide band is enriched out of the multitude of different proteins observed in the crude extract from CAT loaded beef heart mitochondria. Already in the crude extract the same peptide can be observed and is obtained in the apparent purified form at the last stage. This illustrates the quantitative importance of this protein in the mitochondria.

Fig. 1. Column chromatography of a Triton extract from beef heart mitochondria preloaded with ^{35}S-CAT. (A) Hydroxylapatite (HTP). 1.2 mg protein of the Triton extract was added, per ml packed HTP. Proteins were eluted with 0.5% Triton X-100, 10 mM MOPS, 100 mM NaCl, pH 7.2 in a linear phosphate gradient (0 to 0.4 M phosphate). To isolate only the ^{35}S-CAT protein, the phosphate gradient may be omitted. (B) Agarose (Bio Gel 1.5 m) 1.6 mg protein of the pass-through fractions collected from the HTP column was applied to a 80 ml agarose column and eluted with 0.5% Triton X-100, 5 mM MOPS, 50 mM NaCl, pH 7.2.

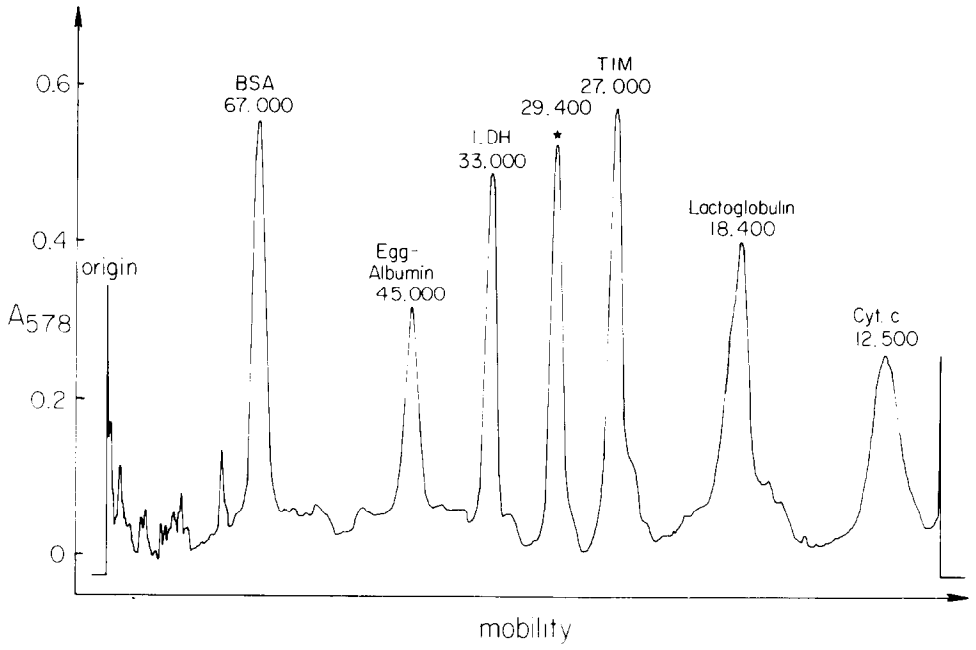


Fig.3. Determination of molecular weight of purified CAT-protein by SDS-PAGE. 5 X 240 mm gel.

The results of two typical purification experiments by slightly varied procedures are given in table 1. The main enrichment is obtained by the chromatography on calcium-phosphate gel or hydroxylapatite. On agarose the protein is further purified 1.2 to 1.5-fold with an overall enrichment of about 10-fold. At this state the protein is electrophoretically virtually pure. The protein then has a content of about 18 μmol CAT/g. It might be added that this preparation does not contain spectroscopically detectable heme. The occurrence of cytochromes is thus excluded.

For the determination of an apparent mol. wt the CAT binding protein was subjected to gel electrophoresis in SDS and appeared between lactate dehydrogenase and triose-phosphate isomerase (fig.3). From this calibration an apparent mol. wt = $29 \cdot 10^3$ is estimated.

The amino acid composition assayed in three hydrolysates of the purified CAT-protein is in % of total (without tryptophan): Asx, 8.2; Thr, 4.4; Ser, 8.3; Glx, 8.2; Pro, 2.6; Gly, 13.4; Ala, 11.1; Val, 6.9; Met, 1.9; Ile, 4.7; Leu, 7.5; Tyr, 3.4; Phe, 5.5; His, 1.3; Lys, 7.1; Arg, 5.7. As far as can be stated with the present accuracy, there is no cysteine present. This is confirmed by a control measurement with dithiobisnitrobenzoic acid (DTNB). One single N-terminal has been found and has been identified with high probability to be histidine.

3.1. *The interaction of CAT binding with other ligands of the ADP, ATP carrier*

The interaction of other ligands with the isolated ^{35}S-CAT binding protein should give information about the specificity of the binding site in the solubilized protein as compared to that known from the carrier in the membrane. For this purpose the removability of ^{35}S-CAT from the protein by various ligands was studied by equilibrium dialysis (fig.4). CAT removes nearly all ^{35}S-CAT, whereas ATR is much less effective corresponding to the lower affinity observed also in mitochondria [5]. BKA displaces the CAT also only to a small extent but is much stronger together with ADP. This is analogous to the situation in mitochondria where removal of ATR by BKA requires the presence of ADP [6,7]. The high specificity for ADP and ATP as compared to other nucleotides, such as ITP, known from mitochondria is also observed here for the solubilized protein [8,9]. It is expected

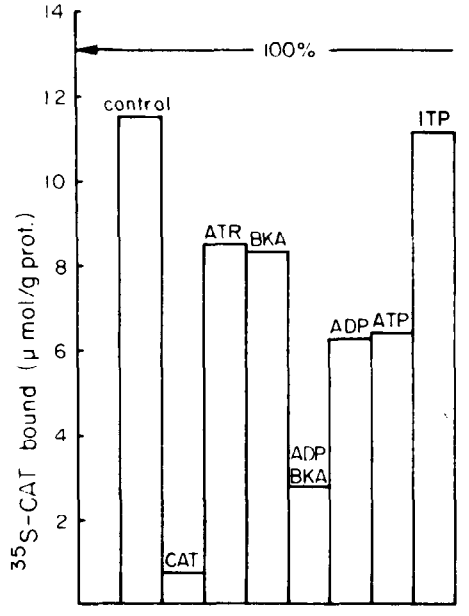


Fig.4. Demonstration of the specificity of ligand interaction with the CAT-binding protein as determined by measuring displacement of bound ^{35}S-CAT. CAT-protein (of about 70% purity) in Triton X-100 solution was added to the indicated ligands and subjected to equilibrium dialysis at room temperature for 5 hr. 300 μg/ml CAT–protein; ligands were added at 430 μM in 0.5% Triton X-100, 100 mM NaCl, 10 mM MOPS, pH 7.2

that in all these cases such as shown for CAT, an exchange against the ligand takes place. However, as controlled with [^{3}H] ATP, this does not result in a binding of ATP to the protein. Obviously the intermediate ATP-carrier complex is labile in solution. All these results do not contradict the contention that there is a single binding site for all these ligands [10].

4. Discussion

The isolation of the CAT loaded protein from the total extract of mitochondria after solubilization by Triton X-100 is surprisingly simple with the new technique. The procedure profits from an unexpected additional benefit of CAT binding to the protein: when loaded with CAT, the protein is separated with more purity in the pass-through of hydroxylapatite column. It can be purified from the solubilized mitochondria up to 7-fold in a first step. This indicated further that CAT confers a certain conformational state to the protein.

The mol. wt in SDS of $29 \cdot 10^3$ might suggest that the 'hydrophobic' protein fraction of Capaldi [11], to which no function was attributed, is a denatured form of our CAT binding protein. A difference between the molecular weight in SDS, as determined by PAGE, and in Triton X-100, might be suggested from agarose gel filtration indicating that the CAT binding protein exists as a micelle with a maximum mol. wt close to $65 \cdot 10^3$.

The phospholipid content which accounts for about $10 \cdot 10^3$ of the $65 \cdot 10^3$ mol. wt of the CAT–protein–phospholipid complex in Triton X-100 must also be taken into account. The unusually high isoelectric point of 9.8 demonstrates the cationic nature of this protein.

An average binding of value of 18 μmol CAT/g protein is obtained instead of 34 μmol CAT/g protein as to be expected for 1 mol CAT/29 000 mol. wt protein. This is interpreted to be due to some release of CAT during the purification procedure. The other possibility that there is 1 mol CAT bound per 2 mol of the 29 000 mol. wt protein in a dimer, must also be considered and is under investigation.

From the assumption that 1 mol CAT binds per 29 000 mol. wt it can be estimated that the carrier accounts for about 5% of the total protein of beef heart mitochondria, considering that about 1.8 CAT μmol/g protein binds to the mitochondria. This agrees with the high yield of this protein and with the function of this protein in the powerful ADP,ATP transport through the mitochondrial membrane. Evidence for the identity of the CAT binding protein with the ADP,ATP carrier comes from the specific displacement of CAT from the protein by ATP or ADP (fig.4).

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