IDENTITY BETWEEN THE MAJOR PROTEIN LOCATED AT THE OUTER FACE OF THE INNER MITOCHONDRIAL MEMBRANE AND CARBOXYATRACTYLATE BINDING PROTEIN

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1. Introduction

Lactoperoxidase catalysed radio-iodination of the cytoplasmic surface of the inner membrane of beef heart mitochondria, readily modifies a polypeptide of apparent mol. wt 29 500. This protein constitutes about 10% of the total membrane protein and appears to be the most abundant protein at this surface of the membrane [1]. The putative adenine nucleotide translocator has been identified and isolated from beef heart mitochondria as a complex with the highly specific inhibitor of adenine nucleotide translocation, carboxyatractylate [2,3], shown to contain a polypeptide of mol. wt 29 000 and accounts for 10% of the mitochondrial protein [4]. In this communication, by isolating the CAT binding protein from lactoperoxidase catalysed radioiodinated mitochondria, we demonstrate that these two proteins are identical.

2. Materials and methods

Beef heart mitochondria (10 mg/ml) were iodinated in media containing 10 μM $Na^{125}I$ (12.5 mCi/μmol) using lactoperoxidase essentially as described earlier [1]. Carboxyatractylate (approx. 2 nmol/mg) was bound to mitochondria at saturating amounts following the procedure of Riccio et al. [5]. Polyacrylamide gel electrophoreses in the presence of sodium dodecyl sulphate (SDS) was performed as described by Neville [6]. Protein was measured by a modification of the Lowry technique to eliminate interference by Triton X-100 [3]. ^{125}I was measured in a Beckman 310 automatic gamma counter. Densitometry of Coomassie Blue stained gels was performed on a modified Eppendorf spectrophotometer. Quantitation of ^{125}I incorporated into protein was estimated by summating the radioactivity found in slices of SDS–polyacrylamide gels of the iodinated material. Estimations made from trichloracetic acid insoluble radioactivity invariably resulted in an overestimation of the iodine covalently bound to protein. This presumably results from lipid iodination which also occurs during the reaction.

The isolation of the CAT binding protein was done following the procedure described in reference [3]. The radio-iodinated, CAT loaded mitochondria were homogenised in a medium containing 0.5% Triton X-100, 0.4 M NaCl, 20 mM morpholinopropane sulphonic acid (MOPS), pH 7.2, and centrifuged at 143 000 × g for 1 h. The pellet depleted of soluble and loosely bound membrane proteins, was resuspended into a medium of final composition 4% Triton X-100, 0.5 M NaCl, 10 mM MOPS, pH 7.2, and stood at 0°C for 30 min. The supernatant obtained following centrifugation as above, was applied to a hydroxylapatite column, equilibrated with 0.5% Triton X-100, 0.1 M NaCl, 10 mM MOPS,

Abbreviations: CAT, Carboxyatractylate; MOPS, Morpholinopropane sulphonic acid; SDS, Sodium dodecyl sulphate

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pH 7.2. The non-adsorbed protein fraction, containing the CAT binding protein, was collected, concentrated by vacuum dialysis and further chromatographed on a Sepharose 6B column after which the final preparation is obtained. All operations were performed at 0–4°C.

3. Results and discussion

Mitochondria were iodinated without prior subfraction since it has been shown that the performance of procedures designed to rupture the outer membrane are unnecessary for the iodination of the cytoplasmic of the inner membrane [1]. SDS–gel analysis of the iodinated mitochondria revealed that the majority of the incorporated iodine was associated with the 29 500 mol. wt region of the gel (not shown). Binding of CAT to the mitochondria, which is essential for the satisfactory isolation of the CAT protein by the procedure used here, could be carried out either before or after the iodination leading to no observable difference in the subsequent purification of the protein. Obviously the iodination of the protein does not abolish its specific capability of tightly binding CAT. This is supported by the observation that iodination of this protein is even increased when loaded with CAT as compared to the unloaded form [7].

Extraction of the iodinated mitochondria with 0.5% Triton X-100 in 0.4 M NaCl, conditions which do not solubilise the CAT binding protein [3] removed about 30% of the membrane protein. SDS–gel electrophoresis of the solubilised protein did not reveal a prominent protein staining band of apparent mol. wt 29 500 nor a significant amount of radioactivity in this region of the gel (table 1). However, virtually all the iodinated protein was dispersed in the 4% Triton X-100 medium which is the initial solubilising step for the CAT protein.

Hydroxylapatite chromatography of the 4% Triton X-100 extract produced an iodine rich fraction in the pass through fraction of the column, where the CAT binding protein normally elutes (fig.1a). The combined fraction (H) migrated mainly as a single band of about 30 000 apparent mol. wt in SDS–gels which accounted for virtually all the protein bound radioactivity of this mol. wt (table 1). Furthermore, the great majority, if not all the 29 500 mol. wt polypepetides applied to this column, eluted in the unadsorbed fraction, since SDS–gel analysis of the material eluted subsequently, failed to reveal such components.

Gel filtration of the iodinated protein from the hydroxylapatite column further purified the 29 500 dalton protein (fig.1b). The protein eluted as a single peak which also contained about a third of the total ^{125}I applied to the column. Fractions were combined as indicated (G). The remainder of the radioactivity eluted at the included volume of the column and was not associated with any protein. This included fraction contains free $^{125}I^-$ and probably iodinated lipid (unpublished observation). The results of SDS–gel analysis of fraction G are shown in fig.2(a) and (b). The protein migrated as a single band which accounted for about 85% of the ^{125}I associated with polypeptides of mol. wt 29 500 present in the original mitochondria. A small amount

Table 1
Summary of purification of CAT binding protein from ^{125}I-iodinated mitochondria

Stage	Protein (mg)	^{125}I[a] (cpm)	(^{125}I) cpm/ (mg protein)	% of total incorporated
Mitochondria	26.8	284 000	10 600	100
0.5% Triton X-100 extract	8.0	36 000	4500	12.6
0.5% Triton X-100 residue	18.0	248 000	13 800	87
Hydroxylapatite pass through (H) fraction	4.4	244 000	55 500	86
Sepharose 6B fraction (G)	3.8	241 000	64 300	85

[a]Calculated as the radioactivity associated with the protein of apparent mol. wt 29 500 after SDS–polyacrylamide gel electrophoresis

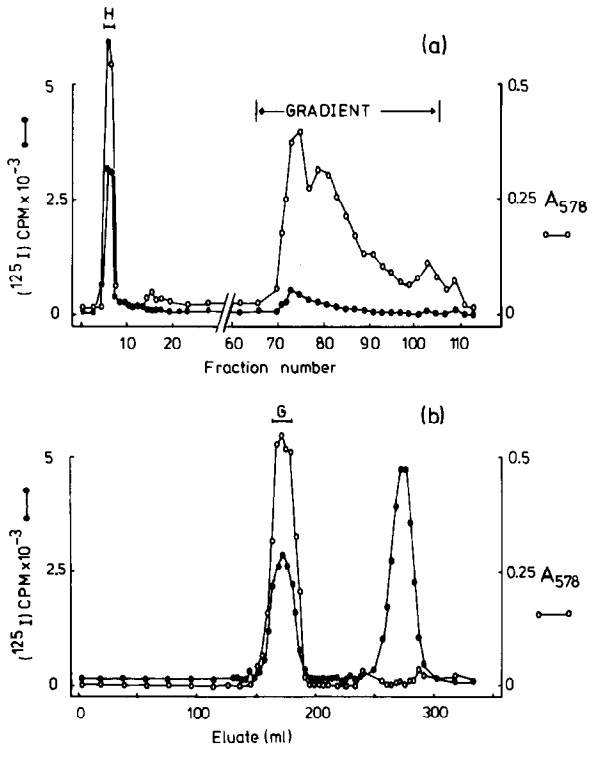

Fig.1. Isolation of CAT binding protein from ^{125}I-iodinated mitochondria. (a) Hydroxylapatite chromatography: 3 ml (5 mg/ml) 4% Triton X-100 solubilised material was applied to a 15 × 1.2 cm hydroxylapatite column equilibrated in 0.5% Triton X-100, 0.1 M NaCl, 10 mM MOPS, pH 7.2. A linear Na-phosphate gradient (0–0.4 M) was passed through the column where indicated. Protein (A_{578}) and ^{125}I were measured in the column eluate. The fractions were combined as indicated (H). (b) Gel filtration: 4 ml (1.1 mg/ml) of the combined fractions from a hydroxylapatite column similar to that described in (a) above, were chromatographed on a Sepharose 6B (85 × 2 cm) column equilibrated in 0.5% Triton X-100, 0.1 M NaCl, 0.05 mM EDTA, 10 mM piperazine-*N,N'*-bis-2-ethane sulphonic acid, pH 7.0. The purified CAT binding protein was obtained by combining the fractions as indicated (G). Protein and radioactivity were measured as in (a) above.

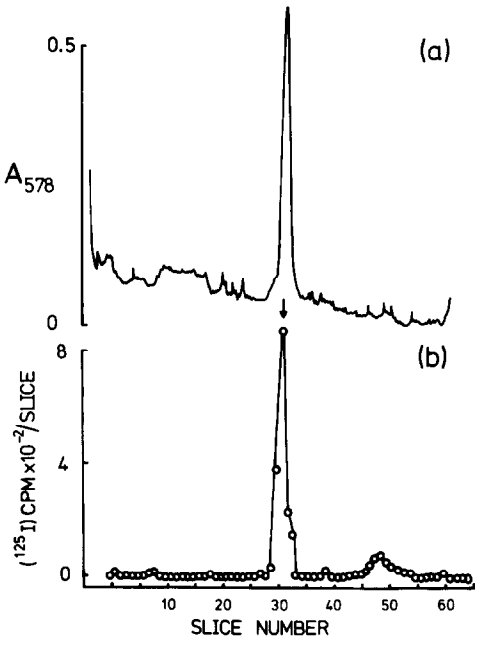

Fig.2. SDS–Polyacrylamide gel analysis of purified CAT binding protein from ^{125}I-iodinated mitochondria. The gel origin in each case is at the left hand side of the diagram. (a) Densitometer trace of a Coomassie Blue stained gel of the CAT protein obtained from the Sepharose 6B column described in fig.1(b). (b) Distribution of ^{125}I in the gel used for the densitometer trace in (a) above. The position of the Coomassie Blue stained band is indicated by the arrow.

of radioactivity was also detected in the lower mol. wt region of the gel which did not correspond to a protein staining band. The nature of this material is unknown.

The iodinated 29 500 dalton polypeptide, therefore, behaved in an identical manner to the CAT binding protein throughout the purification procedure described above. That these proteins are identical was further established by the cross reaction in a double immunodiffusion test, of the iodinated fraction G, with antibodies prepared to purified CAT binding protein [8]. A single precipitin line was formed (data not shown). The fact that the iodinated CAT protein complex also reacts with the highly conformation selective anti-CAT protein indicates that the conformation of the CAT protein is largely retained after iodination.

This work demonstrates that the major protein that can be iodinated at the cytoplasmic surface of the inner membrane of beef heart mitochondria, is identical with the CAT binding protein. This agrees with the about equal protein content estimated by both procedures [1,4] and with the earlier observation that the iodinated protein is absent from the respiratory chain [9,10]. It therefore appears that the

most abundant protein present at the cytoplasmic surface of the inner membrane is the adenine nucleotide translocator.

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