THE UNCOUPLER-BINDING PROTEIN IN THE PROTON-PUMPING ATPase FROM BEEF-HEART MITOCHONDRIA

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Received 27 February 1981

1. Introduction

In [1] we reported the isolation from beef-heart mitochondria of both oligomycin-sensitive and -insensitive ATPase complexes. The preparation of the oligomycin-insensitive complex lacks the 30 000 M_r polypeptide and cannot be reconstituted into phospholipid vesicles in such a way that the ATPase reaction induces an energization of the membrane as monitored by the fluorescent dye 9-amino-6-chloro-2-methoxyacridine (ACMA) [2,3]. Since purification of the preparation of the ATPase complex in [4] also resulted not only in removal of a 30 000 M_r polypeptide but also in loss of oligomycin sensitivity, the question arose as to whether the loss of this polypeptide was responsible for the lack of oligomycin sensitivity and reconstitutive capability. Here we show that the 30 000 M_r polypeptide, identified as the uncoupler-binding protein [5], can be removed from the oligomycin-sensitive ATPase complex in such a way that reconstitution of proton pump activity is still possible.

2. Materials and methods

Reconstitution of the ATPase complex into phospholipid vesicles was performed by centrifugation through a small column of Sephadex G-50 medium (cf. [6]), equilibrated with 10 mM Tris–HCl buffer (pH 8.0), 200 mM NaCl, 1 mM dithiothreitol and 10% methanol, of a suspension of ATPase complex (1 mg protein/ml) and sonicated asolectin vesicles (20 mg/ml) in 20 mM Tris–HCl (pH 8.0), 66 mM ammonium sulphate (or 200 mM NaCl), 0.2 mM EDTA and 0.7% sodium cholate.

The quenching of the fluorescence of ACMA upon addition of ATP and $MgCl_2$ was done in a Perkin-Elmer spectrofluorometer as in [1]. ATP–$^{32}P_i$ exchange was measured by incubating the reconstituted vesicles at 0.3 mg protein/ml final conc. in a medium containing 25 mM potassium phosphate, 3 mg bovine serum albumin/ml, 10 mM Tris–HCl buffer, 10 mM ATP, 5 mM $MgCl_2$ and 0.5 mM EDTA (pH 7.4). Before use the $^{32}P_i$ was heated to 100°C for 0.5 h in 3 N HCl and then neutralized. For each experiment 2 μCi was used. The reaction (at 30°C) was stopped after varying times by the addition of 5% trichloroacetic acid. In the protein-free supernatant after centrifugation the amount of $^{32}P_i$ incorporated into ATP was determined after extraction of P_i according to [7] by counting an aliquot of the aqueous layer. No correction was made for the hydrolysis of ATP during the incubation.

Gradient centrifugation was carried out in a Spinco L 50 centrifuge with the SW 50 rotor for 16 h at 35 000 rev./min and 4°C.

Binding of [^{3}H]azido-nitrophenol was performed in cooperation with Dr W. G. Hanstein as described in [8].

Hydrophobic protein was isolated from an impure ATPase complex, containing high amounts of the 30 000 M_r polypeptide. The complex (5 mg/ml) was treated for 5 min at 20°C and 30 min at 0°C with 5 mg lysolecithin/mg protein. The sediment after centrifugation (called hydrophobic protein) contained mainly the 30 000 M_r polypeptide.

ATPase activity was measured spectrophotometrically at 30°C with an ATP-regenerating system, coupled to the oxidation of NADH. Protein was determined with the Lowry method [9] with bovine serum albumin as standard.

3. Results and discussion

The oligomycin-sensitive ATPase, prepared as in [1] was further purified by sucrose-gradient centrifugation in 0.05% Triton X-100, 10 mM Tris–HCl buffer (pH 8.0), 50 mM NaCl and 10% methanol. Sucrose was 18–50% (w/v). The results of a gradient, run in the absence of methanol, are presented in fig.1,2. The ATPase activity (20 μmol P_i formed . min^{-1} . mg^{-1}) is recovered in a colourless fraction and the gels of fig.2 suggest that specifically the 30 000 M_r polypeptide has been removed. The fraction recovered near the bottom of the tube and containing the same polypeptides as the original preparation, is inactive. No separate fraction of the 30 000 M_r polypeptide is obtained. Since we had found (not shown) that the adenine nucleotide translocator, a polypeptide which can bind N_3-ATP [10] and which is present in some preparations of the oligomycin-sensitive ATPase as an impurity, runs on a SDS gel slightly behind the γ-subunit of F_1, the polypeptide apparently removed on the gradient is not the translocator. Another possible candidate is the uncoupler-binding protein [8]. The results of uncoupler-binding experiments performed in cooperation with Dr W. G. Hanstein are presented in fig.3. They clearly show that after the purification

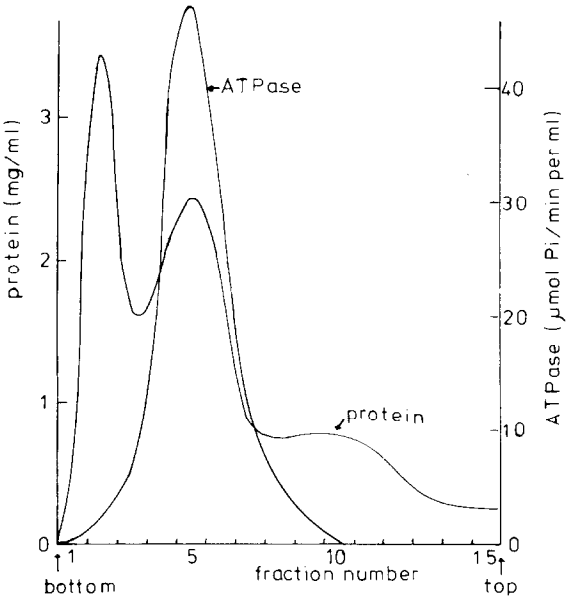

Fig.1. ATPase complex (5 mg) isolated as in [1], was layered on a linear sucrose gradient (5 ml) containing 18–50% sucrose, 50 mM NaCl, 10 mM Tris–HCl buffer (pH 8) and 0.05% Triton X-100. After 16 h centrifugation at 150 000 × g fractions (0.33 ml) were collected and assayed for protein content and ATPase activity.

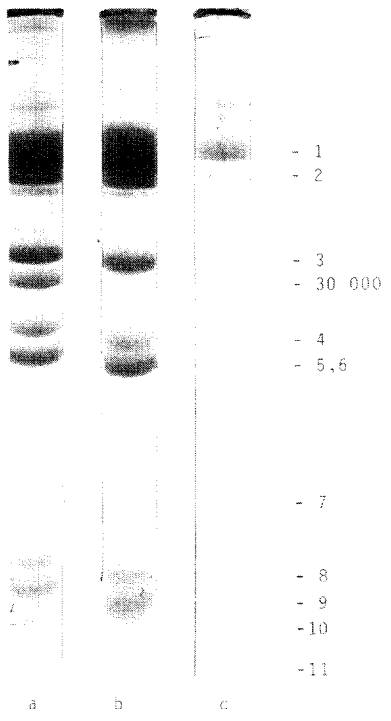

Fig.2. SDS–polyacrylamide gels (12.5%) were run with samples from the fractions 2, 5 and 10 from the gradient in fig.1 (gels a–c, respectively). The method used was that in [1].

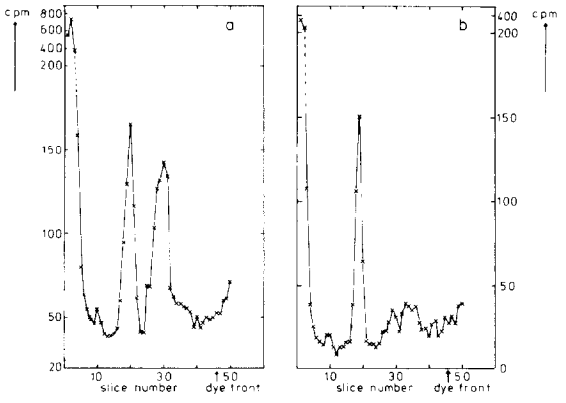

Fig.3. Both unpurified (a) and gradient-purified ATPase (b) complex were labelled with 30 μM 2-[^{3}H]azido-4-nitrophenol as in [8]. After precipitation with 50% saturated ammonium sulphate, samples were run on 10% SDS–polyacrylamide gels. After staining and destaining the gels were sliced and the radioactivity of each slice counted. The slices 20 and 19 in (a) and (b), respectively, correspond to the $\alpha + \beta$ band on the gels, slice 30 in (a) to the 30 000 M_r region.

Table 1
Reconstitution of the ATPase complex into phospholipid vesicles

Preparation used for reconstitution	Oligomycin-sensitivity of the ATPase activity (%)	Quenching of ACMA-fluorescence (%)	ATP–$^{32}P_i$ exchange (nmol/mg/min at 30°C)
Crude ATPase complex	70–80	75–80	25–30
Purified ATPase complex (–M)	20–25	0–10	0
Purified ATPase complex (–M) + hydrophobic protein (1 mg/mg, preincubation 10 min at 20°)	35–40	30–40	n.d.
Purified ATPase complex (–M) + bovine serum albumin (2 mg/mg, preincubation 10 min at 20°)	35–40	35–40	n.d.
Purified ATPase complex (+M)	70	60–70	20–25

Reconstitution was done as in section 2. The crude ATPase complex was purified on a gradient as in section 3, in the presence (+M) or absence (–M) of 10% methanol

over the gradient only specific binding to the α-subunit of F_1 [8] is left and the uncoupler-binding polypeptide is virtually absent.

Reconstitution of proton pump activity of the ATPase complex was followed by measuring the ATP-induced quenching of the fluorescence of ACMA and the ATP–$^{32}P_i$ exchange activity. From the data in table 1 it is clear that conservation of the proton pump activity and the oligomycin sensitivity of the ATPase activity is greatly influenced by the presence of methanol during the purification of the complex on the gradient, although the purification itself is not sensitive to the presence of methanol. Comparison of the data for crude ATPase complex with those for the purified complex leads to the conclusion that the 30 000 M_r polypeptide is not essential for a functional ATPase complex. The need for methanol to preserve the functionality on removal of the 30 000 M_r polypeptide suggests that the conformational stability of the enzyme is lowered by removal of this polypeptide. To test this latter idea we incubated the ATPase complex, obtained by purification in the absence of methanol, not only with phospholipid but also with hydrophobic protein (mainly consisting of the 30 000 M_r polypeptide) before reconstitution was carried out. As shown in tables 1 and 2 such an incubation at room temperature indeed resulted in an increase of the ATP-induced quenching of the fluorescence of ACMA, reaching a maximum at 1 mg hydrophobic protein/mg ATPase complex protein. These results may be compared with [11]. Reconstitution of the ATP–$^{32}P_i$ exchange activity was reported with the 28 000 M_r polypeptide [11]. The specificity of this reconstitution, however, was not investigated and in our reconstitution systems we found, as shown in table 1, that the 30 000 M_r polypeptide can be replaced by bovine serum albumin, again strongly in favour of the conclusion that the 30 000 M_r polypeptide is not an integral part of the ATPase complex.

Our new method for incorporation of the ATPase complex into phospholipid vesicles, which is much more rapid than the more classical dialysis method used in [1], results in vesicles with the same ATP–$^{32}P_i$ exchange activity and the same ATP-induced fluorescence quenching of ACMA as measured for vesicles prepared according to the dialysis method.

Table 2
Effect of hydrophobic protein on the reconstitution of ATPase complex into phospholipid vesicles

Pre-treatment of the ATPase complex	Quenching of ACMA-fluorescence (%)
No pre-treatment	0–10
Preincubation with phospholipid (PL) at 20°C, 10 min	15–20
Preincubation with PL and hydrophobic protein (HP) at 0°C, 10 min	0–10
Preincubation with PL and HP at 20°C, 10 min	
0.5 mg HP/mg ATPase	25–30
1.0 mg HP/mg ATPase	30–40
1.5 mg HP/mg ATPase	20–30
2.0 mg HP/mg ATPase	10–15

ATPase complex, purified on a gradient in the absence of methanol, was reconstituted after various pre-treatments and the ATP-induced quenching of the fluorescence of ACMA was measured

From the reported evidence for the non-essential role of the uncoupler-binding protein for the proton pumping activity of the ATPase complex (cf. [12]), we may conclude that the number of polypeptides belonging to an integral ATPase complex, is maximally 11 (fig.2): 5 belonging to F_1 (bands 1,2,3,7,11); 2 involved in the binding of F_1 to the F_0 part of the complex (Fc_1 and Fc_2, bands 5 and 9, respectively); and maximally 4 components of F_0 similar to the composition of the chloroplast F_0 [13]. The polypeptide of band 8 could be factor B [14], band 10 represents the protein that binds dicyclohexylcarbodiimide (evidence was obtained from the binding of [^{14}C]DCCD), but of the polypeptides represented by the bands 4 and 6 not much is known. Of the polypeptide of band 4 we have determined the amino acid composition (45% polar residues). Band 6 often moves together with band 5, but sometimes these bands are well separated [1]. The polypeptide of band 6 is possibly a product of the mitochondrial genetic system [15].

Acknowledgements

We thank Professor E. C. Slater for his continuous interest and advice and Professor W. G. Hanstein for doing the experiments with [^{3}H]azido–nitrophenol. This work was supported by a grant from the Netherlands Organization for the Advancement of Pure Research (ZWO) under the auspices of the Netherlands Foundation for Chemical Research (SON).

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