IS THE UNCOUPLER-BINDING PROTEIN AN INTEGRAL PART OF COMPLEX V?

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1. INTRODUCTION

Complex V has been isolated as the segment of the mitochondrial oxidative phosporylation system which is concerned with ATP synthesis and hydrolysis. In the isolated state, complex V catalyzes oligomycin-sensitive hydrolysis of ATP, oligomycin and uncoupler-sensitive ATP-P_i-exchange [1], shows oligomycin and uncoupler-sensitive energy dependent oxonol VI response but no energy dependent ACMA-quenching [2]. The uncoupler sensitivity of complex V suggested that the specific uncoupler binding site of mitochondria might be located in complex V. As in mitochondria [3] a polypeptide designated UBP, the α-subunit of F_1 and a small polypeptide are labeled by 2-azido-4-nitrophenol (NPA) [4]. It could be further shown that complex V contained UBP, but not the CAT-sensitive nucleotide carrier [5]. When chromatographed on Agarose columns in the presence of Triton X-100 UBP could be removed. The preparation had cold stable ATPase activity comparable to that of purified complex V, but lacked oligomycin sensitivity and ATP-P_i-exchange [5]. A similar complex was obtained [6,7] which, after reconstitution into vesicles, showed ATP-induced quenching of the fluorescence of ACMA and oligomycin sensitivity in the presence of methanol. From these data, it was concluded that UBP was not an integral subunit of the proton pumping ATPase. However, ATP-P_i-exchange activity of this complex was very low and could not be improved even by incorporation into vesicles [7]. Therefore, the conclusion reached for un-

controlled (uncoupled) H^+-pumping ATPase activity may not be applicable for other energy linked reactions (oxonol VI response, ATP-P_i-exchange). Also, the possibility that UBP could be important for stability of the ATPase has not been ruled out by the experiments with BSA [6].

We now wish to present new data which allow the clarification of some of the questions arising from different ATPase preparations: Is the 30 000 M_r UBP of complex V important for controlled (coupled) energy-linked functions of the ATPase? Is it necessary for the stability of this complex, and should it therefore be considered to be a subunit of the membrane part (F_o)?

2. MATERIALS AND METHODS

Submitochondrial particles (ETP_H) were isolated as in [8]. Complex V has been prepared from this ETP_H as described in [1] with modifications of step 3, i.e., the cholate-ammoniumsulfate fractionation after sephadex gel filtration.

Hydrophobic protein (HP) was isolated from the red pellet of the dialysate (step 2) applying step 3 for complex I-III [9] and the procedure for complex I preparation [10] but using a potassium cholate concentration of 4 mg/mg protein instead of 0.4 mg/mg protein. The UV-spectra showed a maximum at 273 nm and a shoulder at 289 nm ($273/289 \approx 2,2$) in SDS-solution. The protein precipitated upon dilution into buffers.

The 30 000 M_r hydrophobic protein of complex V could be removed by treatment with lysolecithin as in [6,11].

Protein was determined by the method of Lowry et al. [12]. ATP-P_i-exchange activity was measured at 30°C according to published procedures [13]. ATPase activity was measured spectrophotometrically at 30°C [14], and energy linked oxonol VI response as in [2]. 12.5% SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol was performed as in [15]. Oxonol VI was synthesized as described in [16].

3. RESULTS AND DISCUSSION

To prepare complex V as described in [1,2] it is important to perform step 3 of the procedure [1] exactly as described. If, after adding potassium cholate to the suspended material obtained by the initial 42% $AmSO_4$ precipitation, the next addition of ammonium sulfate is given after ~~a~~ too long an interval, potassium cholate incubation changes the properties of the ATPase obtained by the following $AmSO_4$ fractionation: UBP fractionates exclusively into the 25% $AmSO_4$-pellet, and at 42% $AmSO_4$ there ist no precipitation at all. However, an UBP-free complex does appear at 70% $AmSO_4$ or higher concentrations (up to 100%) (Fig. 1). The properties of these fractions are listed in Table 1.

Most of the protein obtained after Sephadex G-25 filtration and cholate treatment appears in the 25%-pellet. This fraction contains the same polypeptides as the following fractions and, in addition, some impurities and high amounts of hydrophobic protein (HP). Treatment of the fraction with lysolecithin results in a complex almost free of HP and up to five times the original ATPase activity. High oligomycin sensitivity can be obtained if cardiolipin is included in the resolution procedure. The yield of protein obtained from SMP as starting material is, after the Sephadex G-25 step, in the range of 20% and consists of 80-90% OS-ATPase-protein (Fig. 2, for the initial 42% $AmSO_4$ precipitate).

It should be noted that the OS-ATPase activity can only be elicited by proper treatment (disaggregation) of the protein fraction obtained in an obviously aggregated form. Another similar procedure for ATPase preparation from SMP yields also about 16-20% protein which can be activated by cardiolipin/lysolecithin treatment (not shown). In this as in other cases, yields of OS-ATPase expressed in units of activity are obviously not meaningful.

The ATPase preparation obtained at 70% $AmSO_4$ has high oligomycin sensitivity and shows high ATP-P_i-exchange activity as well as energy linked oxonol VI response without any further treatment. The main differences from the normal 42% complex V as described [1,2] are the absence of UBP, the down to 45% lowered DCCD-sensitivity and the cold lability: all activities are almost totally lost after freezing in liquid N_2. The ATPase activity after 2 hrs at 0^oC is decreased to about 50%. This fraction has therefore much more the properties of soluble F_1 than those of membrane bound F_1 ($F_1 \cdot F_o$). The energy linked oxonol VI response can be abolished by titration of the complex with hydrophobic protein (HP) (Fig. 3). Since this effect by HP could be due to depletion of phospholipids in the ATPase preparation by HP,titration has also been performed in the presence of sonicated asolectin, and the same results were obtained. ATP-P_i-exchange is lowered in the presence of high amounts of hydrophobic protein, which tends to aggregate and preci-

pitate the soluble OS-ATPase. As seen in Table I, the 25% fraction which has high amounts of 30 000 M_r protein is unable to perform ATP-P_i-exchange or energy linked oxonol VI response.

The 100% $AmSO_4$ pellet has almost the same polypeptide composition as the 70% preparation (Fig. 1) but, as the 25% pellet, no exchange and oxonol VI response. Dislocation of one or more of the subunits or missing of a low M_r polypeptide may be reason for this.

The data show quite clear that UBP is not involved in oligomycin sensitivity, ATP-P_i-exchange and energy linked oxonol VI response. On the other hand it seems to be essential for maximal DCCD sensitivity and cold stability of the OS-ATPase in the absence of methanol or Triton.

From the properties of the different ATPase fractions reported here it is suggested that UBP is an integral part of a stable ATPase complex. UBP may be a neighbor of one of the membrane proteins, M_r 22-24 000 [5,17] and of the DCCD-binding proteolipid [17,18] and stabilize structure as well as confer maximal DCCD sensitivity. Whether UBP controls the energy linked functions of the ATPase complex, and whether it has any relationship to other known hydrophobic 30 000 M_r proteins, including phosphate carriers and proteins of the respiratory chain, is an open question. Research on isolated 30 000 M_r proteins is in progress and should be able to clearify these questions.

ACKNOWLEDGEMENTS

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Table 1

Properties of the $AmSO_4$-fractions after cholate treatment

Fraction	ATPase µmol/min per mg	OS-ATPase %	DS[1]ATPase %	ATP ⇄ P_i µmoles/min per mg	Energy linked Oxonol VI response %
25%-Pellet	0.8 - 1	80 - 86	85 - 87	0	0
a) Lysolecithin treated (1.7 mg/mg protein)	3.1 - 3.4	32 - 35	n.d.	0	0
b) Lysolecithin treated/plus cardiolipin (2 mg/mg protein)	2.4 - 2.6	83 - 85	n.d.	0	0
70%-Pellet	4 - 4.6	94 - 97	40 - 45	170 - 206	100[2]
plus 0.5 mg HP[3]/mg ATPase	(cold labile) n.d.	n.d.	n.d.	n.d.	0
100%-Pellet	4 - 4.3	95	40 - 45	0	0

[1] DS = DCCD sensitive ATPase, [2] 100% ≙ $\Delta OD_{630-602}$ = 0.034, obtained in the best preparations reported in ref. [2],

[3] HP = hydrophobic protein

Fig. 1

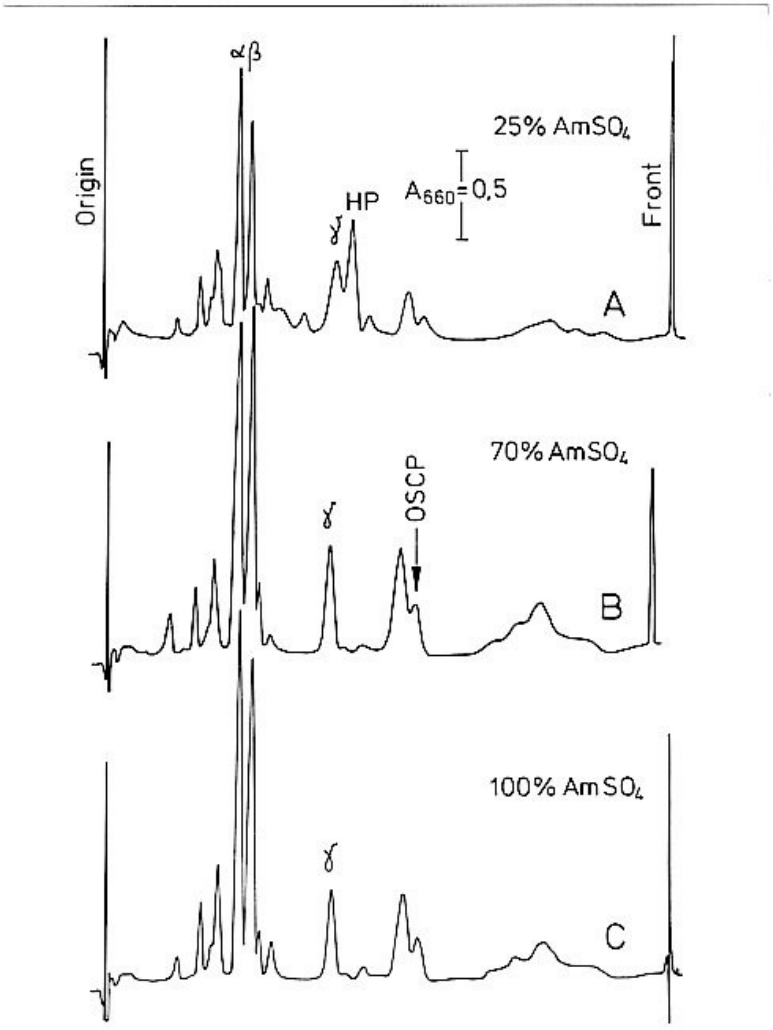

αβ
Origin
25% AmSO4
A660=0,5
HP
γ
Front
A
70% AmSO4
γ
OSCP
B
100% AmSO4
γ
C

Fig. 2

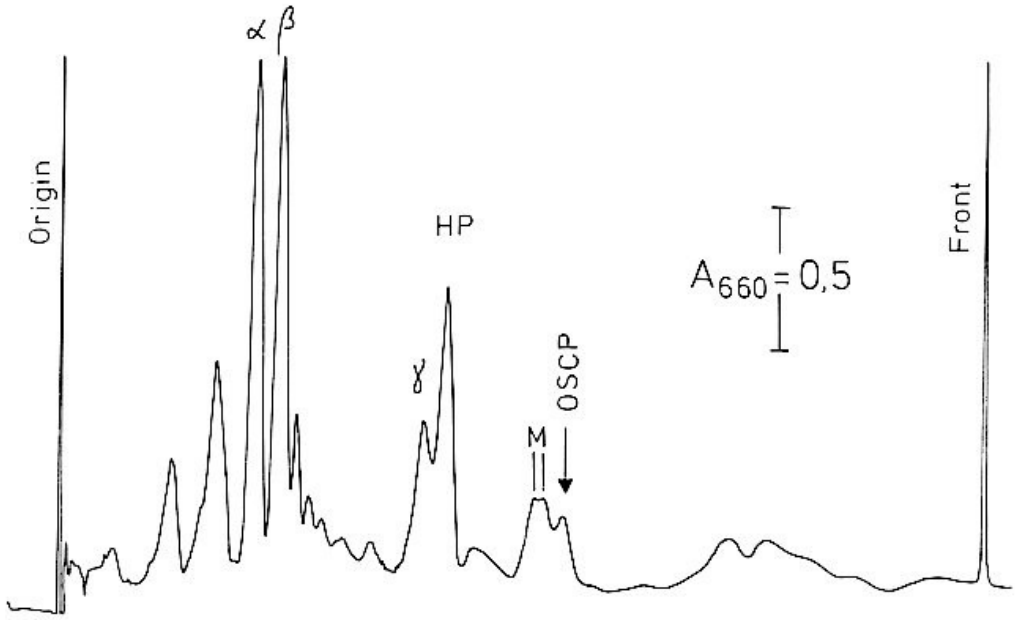
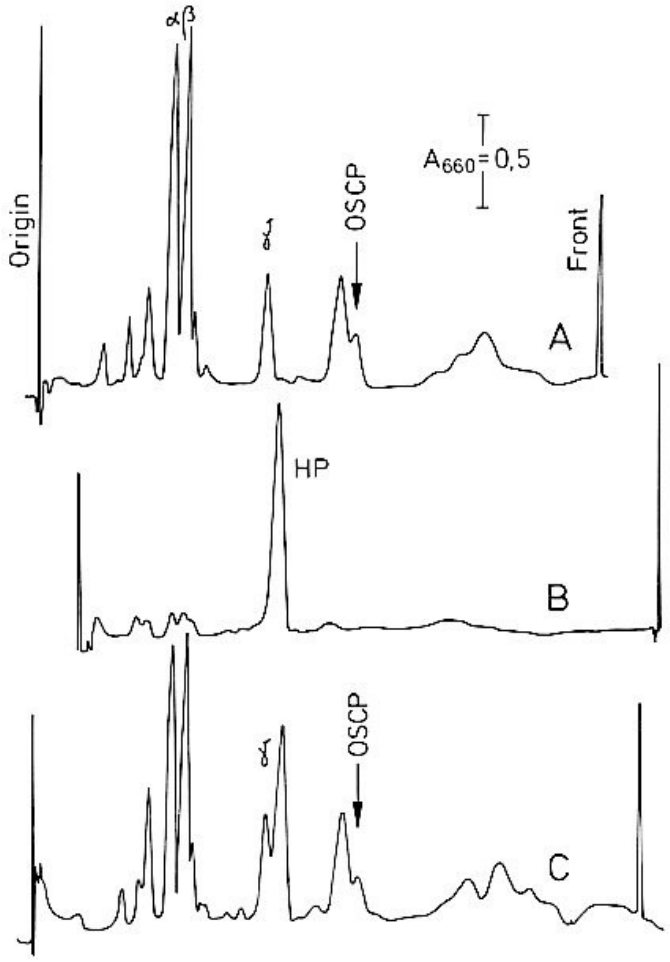


Fig. 3



LEGENDS

Fig. 1 SDS-polyacrylamide gel electrophoresis of fractions obtained with cholate-ammoniumsulfate after sephadex gel filtration. 0.38 mg K-cholat/mg protein was used before $AmSO_4$ precipition. Fractions obtained with 25% $AmSO_4$ (A), 70% $AmSO_4$ (B) and solid $AmSO_4$ to 100% saturation (C). HP = hydrophobic proteins.

Fig. 2 SDS-polyacrylamide gel electrophoresis of the 42% $AmSO_4$ precipitate after sephadex G-25 gel filtration and before cholate-$AmSO_4$ fractioning. M = membrane proteins. Energy linked oxonol VI response = 0, ATPase activity = 5.5 µmol/min per mg, 76% oligomycin sensitive.

Fig. 3 SDS-polyacrylamide gel electrophoresis of the 70% $AmSO_4$-fraction (A), HP (B) and the reconstituted complex: 70% $AmSO_4$-fraction plus HP (C).

FORM C 1

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The authors try to convince us that the uncoupler binding protein (UBP) is an integral part of the ATPase complex. The work presented is unconvincing and, at best, very preliminary. The experiments described rely on an assay thet is not well understood (oxonol-VI) and the results (Table 1) contain internal contradictions. Also, it is not clear what the relationship between uncoupler binding protein and ATPase may be. What is the role of the protein in the enzyme comples supposed to be?

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