FURTHER OBSERVATIONS ON COUPLING FACTOR A

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1. Introduction

The isolation, purification and characterization of coupling factor A from beef heart mitochondria has been reported [1–3]. Its subunit composition is similar to that of F_1-ATPase [4]. These studies revealed that factor A is a different form of mitochondrial F_1-ATPase [5]. It stimulates the activity of urea-depleted submitochondrial particles in oxidative phosphorylation coupled to NADH or succinate oxidation, ATP-supported reversed electron flow, and P_i–ATP exchange. It differs from F_1-ATPase in that it is cold-stable, and the ATPase specific activity is latent, although it can be enhanced to match F_1-ATPase activity by exposing it to elevated temperatures.

It has been suggested in the past that the lower ATPase activity of factor A may result from the presence of mitochondrial ATPase inhibitor [6] in amounts sufficient to mask most of the enzyme activity [7,8]. Analysis of factor A and F_1-ATPase for inhibitor content showed that both had roughly the same inhibitor content (0.4 mol/340 000 g ATPase protein) but only F_1 had high ATPase activity [3]. However, there remained the possibility that the inhibitor was bound to a non-specific, non-functional site in F_1, but it was located at its proper site in factor A leading to masking of the ATPase activity. The only way to overcome this criticism is to prepare factor A in a form that had zero inhibitor content.

It was found [9] that membrane bound, as well as soluble F_1 and F_1-X [10] preparations form a fluorescent complex with the antibiotic inhibitor, aurovertin. They reported that a factor A preparation did not form such a complex with aurovertin and further proposed that it was either dissociated into subunits or combined with other proteins so as to prevent interaction with aurovertin. They also recognized that since the preparation had been left in prolonged storage before testing, it might have lost activity. It was unfortunate that it was not tested for catalytic activity before carrying out the aurovertin-binding experiments.

In the light of the above uncertainties, it was considered necessary to re-examine factor A and compare it with F_1-ATPase with special reference to the inhibitor content and aurovertin binding in order to settle the long standing controversy on the relationship of factor A to F_1-ATPase.

2. Materials and methods

Urea-depleted particles were prepared by a modification of the procedure in [1,2] as described in table 1. F_1-ATPase [11] and ATPase inhibitor [5] were prepared by published procedures. Factor A was prepared by a modification of the procedure in [12]. Fraction S_3 containing factor A activity was desalted on a 2X20 cm Sephadex G-25 column equilibrated with 10 mM phosphate buffer (pH 7.5) containing 10% glycerol and 0.1 mM DTT. The Sephadex eluate was applied to a 2X12 cm calcium phosphate gel column (10 mg Sigma C1125 calcium phosphate gel.mg^{-1} S_3 on wet wt basis, mixed with 3 parts cellulose) and eluted sequentially with 10 mM, 25 mM and 125 mM phosphate buffers (pH 7.5) containing 10% glycerol and 0.1 mM DTT. The coupling factor activity was eluted in the 125 mM buffer. The active fractions were pooled and concentrated to a small volume in a high pressure ultra-

Table 1
Purification of factor A

Preparation	Protein (mg)	Reverse electron flow activity			ATPase activity	
		Spec. act. (μmol NAD red. $min^{-1} . mg^{-1}$)	Total units	% recovery	Spec. act. (μmol NADH oxid. $. min^{-1} . mg^{-1}$)	Units
Sonic supernatant	673	0.24	161			
DEAE-cellulose fraction	31	6.7	206	127	1.2	37.2
Ammonium sulfate fraction (35-55%)	23	6.1	140.3	87	1.5	34.5
Calcium phosphate gel fraction	4.4	15.4	67	42		
Sepharose 4B fraction	1.5	15.2	22	14	5.3	7.95

Beef heart mitochondrial protein (10 g) was processed to obtain factor A. The urea particles for reverse electron flow activity assay were made by a modification of the method in [1]. ETP_H was extracted with 0.25 M sucrose–2.0 M urea–1 mM ATP at 0°C for 30 min, washed with 0.25 M sucrose and suspended in the assay medium for storage. The assay medium contained 0.15 M Tris–sulfate at pH 7.8, 5 mM ATP, 20 mM succinate, 10 mM $MgCl_2$ and 2 mg/ml bovine serum albumin. The activities of the particle and the stimulation provided by factor A were higher than with the system reported in [1]. The Sepharose filtration step is necessary to remove significant amount of inactive, contaminating proteins and some ATPase inhibitor. The specific activity in the reversed electron flow assay, however, shows no increase because of concomitant partial loss during concentration of the active fractions from calcium phosphate eluate. In [17] we reported that some preparations of F_1-ATPase contained factor B, as shown by immunoelectrophoresis against anti-factor B serum. The gel filtration procedure described here, besides removing Pullman inhibitor, also removes contaminating factor B from F_1. Preparations of factor A are free of factor B contamination

filtration cell with PM 10 membrane, and further purified on a 2×100 cm Sepharose 4B column equilibrated with 25 mM Tris–SO_4 buffer (pH 7.5) 10% glycerol, 10 mM phosphate, 0.1 mM DTT and 0.5 mM EDTA. All the steps were carried out at or near 0°C. The results are shown in table 1. This preparation was free of propionyl CoA carboxylase activity [13].

The coupling activity was assayed by ATP-dependent NAD reduction by succinate [1] using urea-depleted particles. The ATPase activity was assayed spectrophotometrically by measuring NADH oxidation as in [5]. The enzymatic assays for ATPase inhibitor were carried out strictly by the procedure in [6]. Propionyl CoA carboxylase activity was measured by the procedure in [13].

Protein was determined in submitochondrial particles by the modified Biuret method [14] and by Lowry's method [15] in factor A and F_1-ATPase preparations. SDS–PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) was carried out as in [16] using 12% polyacrylamide gel at pH 7.0 in K-phosphate buffer.

3. Results

3.1. *Purification of factor A*

Introduction of the calcium phosphate gel chromatography step was primarily for the purpose of removing propionyl CoA carboxylase contamination [12]. It can be eliminated for routine preparations without significant change in specific activity. The overall purification of factor A, as measured by its ability to stimulate the urea particle in NAD reduction, is > 600-fold (table 1). Half-maximal stimulation of the urea particle activity was obtained at ~2–3 μg factor A/mg particle. The purification steps were selected to maintain the ATPase activity low. ATPase followed the coupling factor activity during the purification procedures.

3.2. *Properties*

The final preparation obtained after gel filtration on Sepharose 4B had an ATPase activity of 5–10 μmol.min^{-1}.mg^{-1}. The stimulation of reversed electron flow activity was 15–20 μmol.min^{-1}.mg^{-1}. The ATPase as well as the coupling factor activities were cold-stable. The ATPase activity was insensitive to oligomycin, DCCD or mercurials.

3.3. *Inhibitor analysis*

For the assay of Pullman inhibitor [6] factor A was subjected to heat treatment at 75°C for 4 min as in [3,18], then allowed to cool to room temperature. It was centrifuged to separate the supernatant containing the inhibitor. Aliquots of the supernatant were then incubated with F_1 and assayed [6] using authentic inhibitor for comparison. The inhibitor activity was 1.8 units.mg^{-1} factor A (table 2). Assuming a one-to-one binding of inhibitor to factor A and mol. wt 340 000, the expected recovery of functional inhibitor according to the data in [6] would be ~115 units.mg^{-1} factor A. Our experimental observations would then indicate an inhibitor content of 0.015 mol.mol^{-1} factor A. Parallel analysis carried out on two samples of F_1-ATPase prepared by the procedure in [11] showed an inhibitor content of 3.6 and 10.2 units.mg^{-1} (table 2). For confirmation, the supernatant fractions obtained from factor A and F_1 were analyzed on SDS–PAGE. It is evident that the heating releases mainly only one polypeptide in the supernatant with a molecular weight indistinguishable from the Pullman inhibitor (fig.1). The high molecular weight peptide seen in fig.1B and fig.1C was observed only occasionally and seemed to depend upon the heating conditions. It appears to be the γ subunit of F_1. The inhibitor content of the samples was computed from the area under the curves, using pure inhibitor as standard (table 2). These results indicate that the ATPase inhibitor content of factor A prepared by the above procedure is < 0.03 mol/mol factor A as compared to 0.40 mol obtained in our preparation [3], is lower than that of F_1-ATPase, and yet it has lower ATPase activity. Evidently, the low ATPase activity and the cold stability of factor A cannot be attributed to higher inhibitor content.

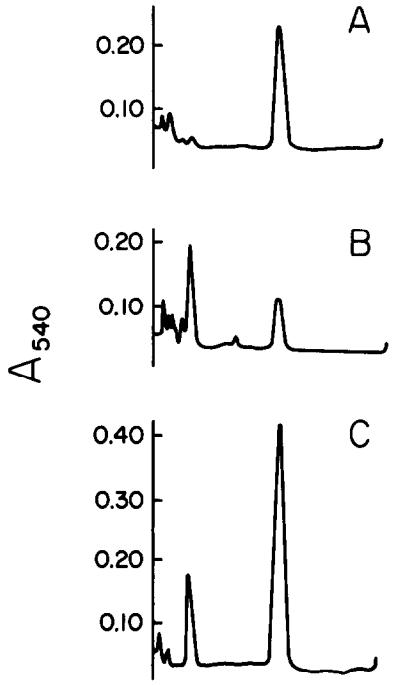

Fig.1. SDS–PAGE tracings of the supernatant fraction from the heat-treated ATPase. The electrophoresis was conducted in 12% polyacrylamide gels by the method in [16]. (A) Supernatant fraction obtained from 150 μg F_1 after heat treatment. (B) Same, from 150 μg factor A. (C) Supernatant fraction from factor A and 1 μg Pullman inhibitor.

Table 2
ATPase inhibitor content of factor A and F_1-ATPase

ATPase activity (μmol.min^{-1}.mg^{-1})		Inhibitor content (mol/340 000 g factor)	
		Enzymatic analysis	SDS–PAGE scan
Factor A	5.0	0.015	0.026
F_1	70.0	0.030	0.063
F_1	70.0	0.085	0.184

The enzymatic assays for ATPase and ATPase inhibitor activities were carried out by the procedure in [6]

3.4. *Aurovertin binding*

For the aurovertin binding experiments, a preparation of factor A was purified on a glycerol gradient (1.4–6.9 M) [12] instead of Sepharose filtration. A constant amount of protein was incubated with increasing amounts of aurovertin. The fluorescence

of aurovertin was measured using excitation at 370 nm and emission at 490 nm. The reference had similar concentration of aurovertin and enough glycogen to parallel the scattering effect due to addition of the protein sample in the experimental cuvette. Aurovertin binding was determined by the fluorescence enhancement with fixed factor A and variable aurovertin concentrations. Two experiments indicated 1.8 and 2.54 (mean of 2.17) nmol binding sites $.mg^{-1}$ factor A and K_d 4.34–5.15 × 10^{-7} M, in 50 mM Tris–SO_4 buffer (pH 8.0). Based on mol. wt 340 000 for factor A, the aurovertin binding would amount to 0.74 mol/mol. This value is in the range reported for aurovertin binding to F_1 [19], although a value of two binding sites has also been reported [20]. These results would show that, in contrast to [9], factor A does bind aurovertin in amounts similar to that bound by F_1.

4. Discussion

Bovine heart F_1 containing 6 subunits including the ATPase inhibitor and having low ATPase activity has been isolated [21]. It can be converted to other electrophoretically-distinct forms (or allomorphs) by altering the solvent medium. The absence of ATPase inhibitor in factor A prepared by the revised procedure described here, would distinguish it from the 6 subunits preparation, although the ATPase activity is latent in both.

From [1,2], we had attributed the low ATPase activity of factor A, in contrast to F_1, to a conformational difference between the two preparations. Similar polymorphism has now been seen with *E. coli* F_1 which appears to exist in two allomorphs, one with low and other with high ATPase activity [22]. The change from the low to high activity form is brought about by preincubation at higher temperatures, just as in the case of conversion of factor A to F_1 [1,2]. The most interesting finding, however, is that the 4 subunit *E. coli* enzyme containing the ϵ or inhibitor subunit, as well as the three and two subunit forms without the inhibitor, showed the same type of transition from the low to the high activity form as we had reported earlier for factor A [1,2]. The results in [21] establishing polymorphism in F_1 preparations and in [22] showing transition of *E. coli* F_1 from a low ATPase activity form to a high activity form are fully consistent with our conclusions that factor A and F_1 probably differ only in conformational state.

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