# A COMPLEX OF MITOCHONDRIAL FACTOR A AND A NEW FACTOR INVOLVED IN OXIDATIVE PHOSPHORYLATION[1]

B.P. Sani, K.W. Lam[2] and D. Rao Sanadi[3]

Department of Bioenergetics Research, Retina Foundation
20 Staniford Street, Boston, Massachusetts 02114

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SUMMARY

In a revised procedure for the preparation of the mitochondrial energy transfer Factor A, three additional active protein peaks were recovered from DEAE-cellulose column chromatography. The peak eluted with 150 mM phosphate buffer has been characterized as Factor A·D, a tightly associated form of Factor A with a new coupling factor, Factor D. Factor D obtained after dissociation and precipitation of Factor A following urea-protamine treatment activated both urea particles and AE-particles in the ATP driven NAD reduction by succinate but Factor A·D stimulated only the urea particle. Factor D had no activity in the assay for the 'oligomycin sensitivity conferring protein'.

Some success has been achieved in resolving the mitochondrial oxidative phosphorylation system into electron transport particles deficient in phosphorylation and several types of soluble protein preparations which restore phosphorylation activity to the particle (1). Four of these, $F_1$ (2), Factor A (3), Factor B (4), and OSCP[4](5), have been purified highly and characterized by physical, chemical and immunochemical methods. Much evidence has been presented that $F_1$ and Factor A are functionally indistinguishable and their physical properties are also similar (6). The only known differences between them are the cold stability of Factor A in contrast to the lability of $F_1$ and the

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[2]Recipient of a Research Career Development Award (HE 24,202) from the U.S. Public Health Service.

[3]Also at the Department of Biological Chemistry, Harvard Medical School.

[4]The following abbreviations are used: $F_1$ for coupling factor 1, OSCP for the oligomycin sensitivity conferring protein, ETPH for phosphorylating electron transport particles from heavy layer mitochondria, AE-particles for submitochondrial particles depleted by exposing to ammonia and EDTA, S.A. for specific activity (units/mg) (3,4,5).

presence of ATPase activity in $F_1$; ATPase activity can be induced in Factor A by exposing it to elevated temperatures (3,6). Both have the same amount of the ATPase inhibitor described by Pullman (6). More recently, the occurrence of a third form of the factor, $F_1 \cdot X$ has been reported from Slater's laboratory (7,8). Evidence has been presented suggesting that X may be identical with OSCP (9).

This communication reports the purification of Factor A·D, which appears to be a complex of Factor A and a new factor designated Factor D. No OSCP activity has been found in Factor D either before or after dissociation from Factor A.

## EXPERIMENTAL PROCEDURE

Preparation of Factor A and Factor A·D. The method is similar to that previously described for Factor A preparation (6) except for the presence of phosphate buffer during activation and fractionation. The preparation was chromatographed on DEAE-cellulose and four active peaks (Peaks I to IV) were eluted with 10, 50, 80 and 150 mM phosphate buffer, pH 7.5. The 80 mM protein peak corresponded to Factor A.

The energy transfer activity of the protein factors was assessed by their stimulatory effect on the initial rate of ATP-dependent NAD reduction by succinate when added to the deficient particles (4). Urea particles (3) were used for the assay of Factor A, and AE-particles (10) for Factor B. ATPase and OSCP activities were measured as described before (6,11).

Immunodiffusion studies were carried out using antiserum to Factor B (4) and to $F_1$ in diffusion plates containing 2% agarose. Anti-$F_1$ was kindly supplied by Dr. J. Fessenden-Raden and Dr. E. Racker.

## RESULTS

Peaks I and II are distinct in their OSCP activity (S.A. - 16 and 9.2 respectively); they also activate the urea and AE-particles slightly (S.A. - <1.0). Peak III, which is Factor A, stimulates urea particle strongly (S.A.-

4.5) and AE-particle to a lesser degree (S.A.-<1.0). The magnitude of stimulation of the AE-particle with a saturation level of Factor A (Peak III) is low as in previous experiments (10). The specific activity of Factor A prepared by this procedure is sometimes considerably higher than that of previous preparations (4.5 in this experiment compared to 1.8). Peak IV is quite active with the urea particle (S.A.-2.0) but shows only little activity with the AE-particle (S.A.-0.1).

The stimulation of the ATP dependent NAD reduction activity of the urea particle by Factor A (Peak III) and Peak IV are shown in Fig. 1. Factor A stimulated activity reaches a maximum of 31 while Peak IV stimulation goes higher to 48. When the particle is supplemented with a saturation level of Factor A, further stimulation is obtained with Peak IV indicating the presence of an additional component, which is designated Factor D. On heating Peak IV at 60°C, its activity declines to the level of Factor A activity, suggesting that Factor D has been inactivated.

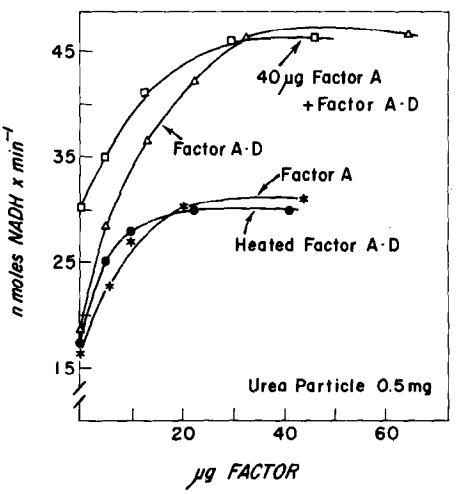


Fig. 1. The Greater Stimulation of Reversed Electron Flow by Factor A·D Compared to Factor A. Energy transfer factors were preincubated in 2.9 ml with 0.5 mg of the urea particle, 10 µmoles $MgCl_2$, 20 µmoles succinate, 6 µmoles ATP, 150 µmoles Tris-sulfate, 2 mg bovine serum albumin, 1.5 µmoles NAD for 2 min at 38°C and the reaction initiated by the addition of 3 µmoles KCN in 0.1 ml. The Factor A had a specific activity of 1.4 µmoles NADH/min/mg and the Factor A·D (Peak IV) had a specific activity of 2 µmoles NADH/min/mg. Factor A·D was heated at 64°C for 2 min in sucrose-Tris-EDTA medium (pH 7.5) containing 20 mM ATP.

Peak IV breaks down ATP (ATPase) at the rate of 10 μmoles $P_i$ released x $min^{-1}$ x $mg^{-1}$ protein. The activity is insensitive to oligomycin. On heating as described under Fig. 1, the activity reaches 90, which is in the same range as the ATPase activity of $F_1$ (2). The appearance of an ATPase site on heating Factor A has been previously documented (6).

When Peak IV protein is exposed to 2 M urea at 0°C for 1 hour and the urea is removed by dialysis, approximately 60% of the protein is precipitated on addition of protamine. If the urea treatment is omitted, only a small precipitate is obtained containing about 15% of the protein. It should be recalled that $F_1$ is precipitated by protamine. The protein remaining in the supernatant following urea and protamine treatment has little ATPase activity (less than 1 μmole x $min^{-1}$ x $mg^{-1}$), but it stimulates the ATP driven NAD reduction activity of the AE-particle much more strongly than the original Peak IV protein (Fig. 2). The results indicate that urea-protamine treatment results in separation and precipitation of Factor A leaving Factor D behind. Free Factor D is apparently active with the AE-particle while its activity with this particle is masked by association with Factor A. On the other hand,

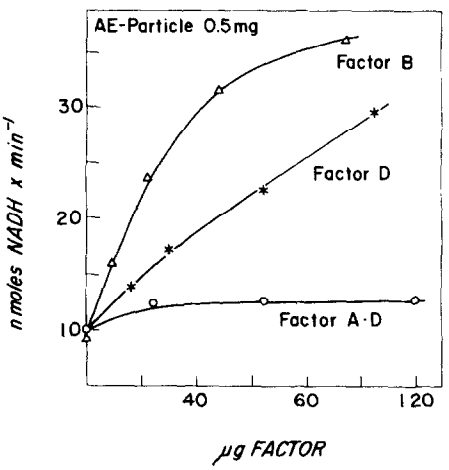


Fig. 2. Stimulation of Reversed Electron Flow by Factor B and Factor D. The assay conditions were the same as under Fig. 1 except that 0.5 mg AE-particles were used. Factor A·D was exposed to 2 M urea at 0°C for 1 hr and the urea was removed by dialysis. Protamine sulfate (1%) was added to precipitate $F_1$-like protein. Factor D was present in the supernatant after centrifugation.

both Factor A and Factor A·D seem to be active with the urea particle (Fig. 1), and the dissociated Factor D from Factor A·D is also quite active with the same particle (S.A. averages 1.1 μmoles NADH x $min^{-1}$ x $mg^{-1}$).

It should be noted that Factor D obtained as above stimulates the activity of the AE-particles in much the same manner as Factor B does. However, the two are distinct since the antiserum to Factor B does not inhibit Factor D activity, nor do they form a precipitin band in immunodiffusion.

Anti-$F_1$ forms a major and a minor band with Peak IV protein (Factor A·D) but a single band with Factor A. After urea protamine treatment, the isolated Factor D preparation gives only a faint band with anti-$F_1$ suggesting that the bulk of Factor A has been removed.

No OSCP activity has been detected in Factor A·D or in Factor D after dissociation.

## DISCUSSION

Multiple forms of the same energy transfer factor have been recognized for some time. They are Factor A, $F_1$, $F_1$·X and now Factor A·D. The properties of the terminal ADP phosphorylation enzyme change depending upon the method of isolation. The results presented here also show that when two factors are in association, the activities of both can be expressed in some particles (e.g., Factor A·D is more active than Factor A with urea particle, Fig. 1), but can be shielded in different types of particles (e.g., Factor A·D is inactive with AE-particle, Fig. 2, while Factor A has some activity). On dissociation and separation, one factor can show activity that was absent before separation (e.g., separated Factor D becomes highly active with the AE-particle). Beechey *et al.* (12) have drawn attention to a basic coupling factor activity whose behaviour on CM-cellulose seems to be altered by prior chromatography on DEAE-cellulose. However, since no rigorous characterization of the protein has been completed, the significance of the findings remains to be clarified.

The relationship of X in $F_1$·X to D in Factor A·D needs further exploration. Groot and Meyer (9) found OSCP activity in the $F_1$·X preparation and

concluded that X is identical with OSCP. The OSCP activity prevailed even after cold inactivation of $F_1$ of $F_1 \cdot X$. Vallejos (13) has presented evidence that X may be $F_3$ which also has OSCP activity. However, $F_3$ is a mixture of energy transfer factors containing Factor B, (M. Kaplay and K.W. Lam, unpublished data). Factor D obtained from Factor A·D after urea-protamine treatment showed no OSCP activity but stimulated the ATP-driven reduction of NAD by succinate in the AE-particle and in the urea particle. One possibility is that X is a larger protein comprising Factor D and OSCP.

These results also indicate that the mitochondrial energy transfer factors can be obtained in tightly associated complexes containing more than one protein in much the same manner as the complexes of the electron transport system. If the interaction and association is not a secondary phenomenon subsequent to detachment from the membrane, analysis of the components would allow determination of their sequence in the energy transfer process. Such sequence determination is not possible at the present time since all assays, other than ATPase activity involve a host of components integrated on a membranous structure.

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