COMPARATIVE STUDIES ON MITOCHONDRIAL COUPLING FACTOR B AND F_B

Shanta Sharma and D. Rao Sanadi

Department of Cell Physiology, Boston Biomedical Research Institute
20 Staniford Street, Boston, Massachusetts 02114
and
Department of Biological Chemistry, Harvard Medical School

Received May 5,1976

SUMMARY: Beef heart mitochondrial protein factor F_B [Higashiyama et al, Biochemistry 14, 4117-4121 (1975)] was purified and its properties were compared with those of coupling factor B. Both proteins stimulated ATP-driven NAD^+ reduction in ammonia and EDTA-treated (AE-) submitochondrial particles, but the extent of stimulation (maximum activity of particles) was very low with F_B. F_B was found to be ineffective in stimulating P_i-ATP exchange in either AE-particles or reconstituted oligomycin-sensitive ATPase vesicles. Furthermore, F_B failed to stimulate ATP-driven NAD^+ reduction activity of AE-particles in the presence of saturating amounts of dithiothreitol (DTT). DTT alone stimulates the particle activity extensively as reported earlier. Rabbit antiserum to F_B did not show a precipitin band with purified Factor B, nor did the antibody inhibit Factor B stimulated activity of the AE-particles. The data suggest that F_B and Factor B are two different molecular species with different functions and fail to provide evidence that F_B is a coupling factor.

Isolation, purification and characterization of Factor B from beef heart mitochondria have been carried out in this laboratory (1-3). These studies revealed that Factor B is a protein with a molecular weight of 29,200, which stimulates the AE-particle activity in ATP-driven NAD^+ reduction by succinate, in net phosphorylation coupled to NADH or succinate oxidation and in P_i-ATP exchange. Immunological experiments confirmed that it was a homogeneous protein (4). It contains functional, reactive -SH groups and is inhibited by mercurials. The inhibition is reversible, and can be prevented by the addition of excess dithiothreitol (DTT) during preincubation.

Recently, Higashiyama et al (5) have described a highly purified mito-

chondrial protein preparation with unusually efficient B-type coupling factor activity. This protein, denoted as F_B[1], has been reported to be an octomer with a molecular weight of approximately 330,000, with 44,000 dalton subunits. F_B also stimulates ATP-driven reduction of NAD^+ by succinate in A-particles and has reactive -SH groups. However, the reported specific activity of F_B is much higher (1000-2000 µmoles NAD reduced X min^{-1} X mg^{-1} of factor protein at 38°C) than that of Lam's Factor B (2-6 µmoles under similar conditions). In spite of its extraordinarily high specific activity, the magnitude of net stimulation brought about by F_B is only 20% above the basal activity of A-particles, whereas Factor B stimulation is three- to four-fold. In the present investigation, an attempt was made to examine the role of F_B and compare its properties with those of Factor B using additional functional parameters conventionally used for the characterization of coupling factors.

MATERIALS AND METHODS:

F_B and A-particles were prepared essentially as described by Higashiyama et al (5). The method of Lam et al (1 and 3) was followed for the preparation of AE-particles and Factor B respectively. Besides a few minor differences in the early preparative stages of the two factors, the significant differences included the elution of F_B protein from DEAE-cellulose (Whatman DE-52) column with 50mM Tris at pH 8.0, whereas Factor B was collected mainly in the 100mM eluate fractions. The final purification of F_B was achieved by removing salts and DTT, followed by the aerobic oxidation of the suspension for 2 hours at 0°C, resulting in the inter-molecular disulfide formation and subsequent precipitation of F_B protein.

Two assay procedures for the determination of F_B and Factor B activities were used for the reasons to be given in the section on results. Procedure H was identical to the method used by Higashiyama et al (5), and procedure L was that reported by Lam et al (3) carried out in the presence of 2 µmoles of DTT in the 3 ml reaction medium.

Protein was determined in submitochondrial particles and oligomycin-sensitive ATPase preparations by the modified Biuret (6) and by the Lowry method (7) in F_B and Factor B preparations.

P_i-ATP exchange in AE-particles was measured by th modified method of Andreoli et al (8) (refer to Table 1 for details). Reconstitution of vesicles capable of catalyzing the P_i-ATP exchange reaction from the oligomycin-sensitive ATPase (OSATPase) of Tzagoloff et al. (11) was carried out in the presence of partially purified asolectin and coupling factor 1 (F_1), as described by Joshi et al. (12). Coupling factors, when used for the stimulation studies, were added after the formation of the active vesicles. Production

---

1 F_B for Higashiyama's B-type factor preparation.

of rabbit antiserum against F_B and its effect on ATP-driven reduction of NAD^+ by succinate were carried out as reported earlier (4).

RESULTS AND DISCUSSION:

Figure 1 summarizes the effect of low and higher F_B levels on the ATP-

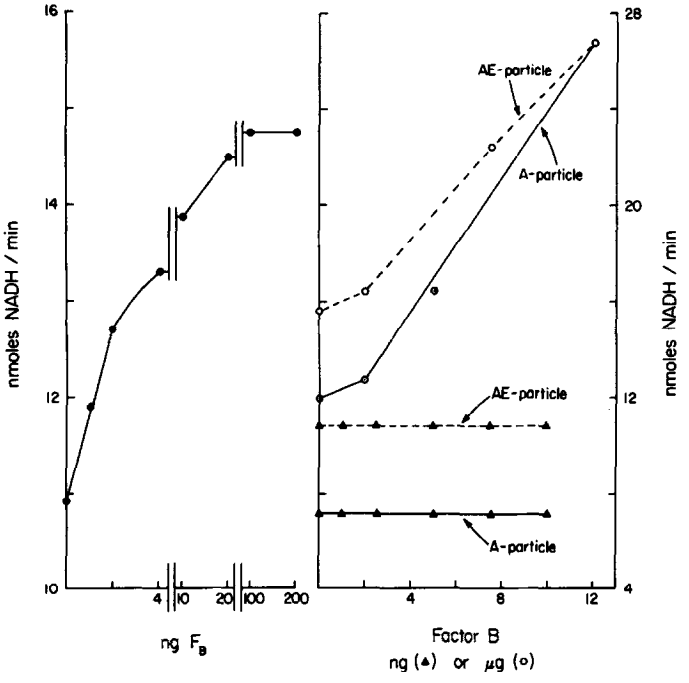

Figure 1: Effect of F_B and Factor B on the activity of A- and AE-particles.

(Left) Assay procedure H was used for these experiments. A 50 µl sample of A-particle containing 0.5 mg of protein was incubated with F_B solution at 38°C for 1 minute and then mixed with 3 ml of assay mixture (at 38°C) containing 50 mM Tris-sulfate, pH 7.8, 3.3 mM $MgCl_2$, 6.7 mM succinate, 2 mg bovine serum albumin per milliliter, 2 mM ATP, 1 mM NAD^+, 3.3 mM KCN (added last). Increase in absorbance at 340 mM was measured. The specific activity of F_B (expressed in µmoles of NAD reduced X min^{-1} X mg^{-1} of F_B) was about 600 from the linear part of the curve.

(Right) Assay procedure L was followed for o—and o--- and assay procedure H for ▲--- and ▲—, For o— and o---, 0.5 mg AE-particles were mixed with Factor B (DEAE-cellulose stage) and the reaction mixture containing 150 µmoles Tris-sulfate, pH 7.8, 10 µmoles $MgCl_2$, 20 µmoles succinate, 6 µmoles ATP, 2 mg bovine serum albumin, 3 µmoles NAD, and 2 µmoles DTT in a volume of 2.9 ml was added. Contents were incubated for 2 minutes at 38°C. The reaction was started by the addition of 3 µmoles KCN in 0.1 ml. Increase in the absorbance at 340 nm was measured. The specific activity of Factor B is expressed as µmoles NAD reduced X min^{-1} X mg^{-1} protein.

driven NAD^+ reduction activity of A-particles in assay procedure H. The stimulation is fairly linear with low levels of F_B (1-4 ng) and falls off rapidly with the increasing amounts. Figure 1 also shows the effect of Factor B on the two types of ammonia-treated submitochondrial particles, using assay procedure L. The specific activity of Factor B is quite comparable with both types of particles. The stimulation of particle activity by very low levels of oligomycin, which is a criterion in testing the suitability of the particles (13), was also essentially the same. In all cases, more than two-fold stimulation of activity was produced by oligomycin (data not shown).

In contrast to the activation by F_B, there was no detectable activation by nanogram levels of Factor B with either type of particle using assay procedure H or procedure L. Similarly, an increase in the level of F_B protein in procedure H did not stimulate activity of A-particles any more than 20%, which is extremely low compared to the several-fold stimulation brought about by Factor B.

It is interesting to compare the basal activity of A- and AE- particles in assay procedures H and L (Fig. 1). In the absence of added DTT (assay procedure

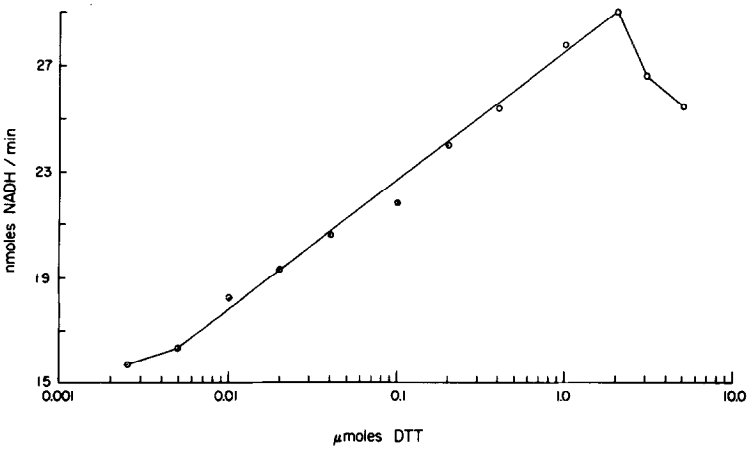

Figure 2: Effect of DTT on ATP-driven NAD^+ reduction in A-particles.

DTT (in 10-50 μl volumes) was first added to A-particles, followed by preincubation at 38°C for 1 minute. Order of addition of other reagents was as described in the legend for Fig. 1.

H), A-particles showed activity of 7.2 nmoles NAD reduced X min^{-1} X mg^{-1}, which is roughly 60% of the activity shown with procedure L. Consistently, AE-particles showed a decrease of about 40% in the absence of DTT. It was therefore found necessary to titrate the particles for their DTT dependency. Figure 2 shows the effect of externally added DTT on the activity of A-particles. As we had noted earlier (3), there was linear increase in the activity of A-particles with increasing levels of DTT over the range of 0.005 to 2 µmoles in the 3 ml assay medium. Further increase in the DTT concentration was slightly inhibitory.

The above observation has raised an important question, i.e., is the low stimulation, although with very high apparent specific activity, shown by F_B actually caused by the DTT present in F_B preparations? In none of the experiments was the extent of stimulation by F_B more than that observed with DTT; furthermore, as shown in Fig. 3, F_B over a wide range of concentration did not

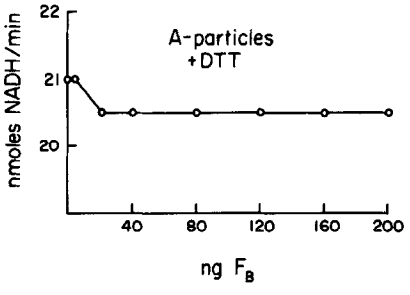

Figure 3: Effect of F_B on the activity of A-particles in the presence of DTT.

Two µmoles DTT and the desired levels of F_B were added to A-particles followed by preincubation at 38°C for 1 minute. Activity was assayed by procedure H, as described in Fig. 1.

produce stimulation of ATP-driven NAD^+ reduction activity of A-particles in the presence of 2 µmoles of DTT. The effect without DTT (see Fig. 1) was in agreement with the data of Higashiyama.

Table 1 shows the effect of both factors on the P_i-ATP exchange activity

of AE-particles and of the reconstituted OSATPase vesicles capable of catalyzing the above reaction. With the latter system, the exchange activity is roughly 60% higher in the presence of DTT, and addition of Factor B further stimulates its activity over two-fold. Very low (ng) levels of Factor B failed to produce any significant stimulation. Both low and high levels of F_B were ineffective in catalyzing P_i-ATP exchange in the above system.

TABLE 1

Effect of F_B and Factor B on P_i-ATP Exchange Activity of A-Particles and Oligomycin-Sensitive ATPase Vesicles

	A-particles*	OSATPase vesicles+	
Factor		+DTT	-DTT
None	61.2	102	63.6
5.7 ng F_B	62.0	84	63.4
11.4 ng F_B	56.0	104	72.4
57 µg F_B	58.9	101	70.6
114 µg	59.8	104	65.8
5 ng Factor B.	60.4	98	70.9
10 ng Factor B	74.4	101	74.8
50 µg Factor B	179.9	241	168.0

*nmoles P_i exchanged X min^{-1} X mg^{-1} A-particles.

+nmoles P_i exchanged X 15 min^{-1} X mg^{-1} OSATPase protein. 0.5 mg A-particles were incubated at 37°C with 100 µmoles Tris-sulfate, pH 7.8, 10 µmoles phosphate, 30 µmoles $MgCl_2$, 1.5 µmoles EDTA and 300,000 cpm ^{32}P. After 5 minutes the reaction was started (total volume 1.0 ml) by the addition of 24 µmoles ATP. The reaction was terminated after 5 minutes with 0.5 ml 20% TCA. Extraction of unesterified phosphate was carried out by the procedure of Avron et al. (9). P_i was measured by the method of Fiske and SubbaRow (10).

For the exchange assay, 1 mg OSATPase was dialyzed in the presence of 2 mg cholate, 5 µmoles phospholipid phosphorus, 10 µmoles ammonium sulfate and 1 µmole DTT where indicated. After dialysis for 16-18 hours factor proteins were added and incubated for 10 minutes at room temperature. P_i-ATP exchange activity was measured as described earlier. Factor B was used at the CM-cellulose stage of purification.

The endogenous P_i-ATP exchange activity of AE-particles was independent of the addition of DTT. Factor B stimulated this activity over two-fold but F_B showed no such activating effect. Both F_B and Factor B preparations had no detectable P_i-ATP exchange activity by themselves.

Rabbit antiserum to F_B gave a single precipitin band against this factor on immunodiffusion in agarose gel, whereas it failed to react with the purified Factor B under the identical experimental conditions. In agreement with the above observation, the purified F_B antibody protein did not inhibit either the Factor B-stimulated activity of the AE-particles, or the intrinsic ATP-driven NAD^+ reduction activity of AE particles or ETP_H.

In our experiments, F_B protein has none of the activities exhibited by Factor B and shows none of the typical properties expected of mitochondrial coupling factors.

ACKNOWLEDGMENTS:

This investigation was supported by grant no. 5-R01-GM13641 from the National Institutes of Health.

REFERENCES:

1. Lam, K.W., Warshaw, J.B., and Sanadi, D.R. (1967) Arch. Biochem. Biophys. 119, 477-484.
2. Sanadi, D.R., Lam, K. W., and Kurup, C.K.R. (1968) Proc. Natl Acad. Sci. U.S. 61, 277-283.
3. Lam, K. W., Swann, D., and Elzinga, M. (1969) Arch. Biochem. Biophys. 130, 175-182.
4. Lam, K.W., and Yang, S.S. (1969) Arch. Biochem. Biophys. 133, 366-372.
5. Higashiyama, T., Steinmeier, R.C., Serriane, B.C., Knoll, S.L., and Wang, J.H. (1975) Biochemistry 14, 4117-4121.
6. Jacobs, E.E., Jacob, M., Sanadi, D.R., and Bradley, L.B. (1956) J Biol. Chem. 223, 147-156.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
8. Andreoli, T.E., Lam, K. W., and Sanadi, D.R. (1965) J. Biol. Chem. 240, 2644-2653.
9. Avron, M. (1960) Biochim. Biophys. Acta 40, 257-272.
10. Fiske, C.H., and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375-400.
11. Tzagoloff, A., Byington, K.H., and MacLennan, D.H. (1968) J. Biol. Chem. 243, 2405-2412.
12. Joshi, S., Shaikh, F., and Sanadi, D.R. (1975) Biochem. Biophys. Res. Commun. 65, 1371-1377.
13. Lam, K.W., Warshaw, J.B., and Sanadi, D.R. (1966) Arch. Biochem. Biophys. 117, 594-598.