A SECOND FORM OF ENERGY TRANSFER FACTOR B AND A NEW FACTOR (FACTOR C) OF MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION[1]

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SUMMARY

The isolation and characterization of Factor B, a mitochondrial protein involved in oxidative phosphorylation, has been described previously. The occurrence of another form of the factor, designated Factor B', which can be separated from Factor B by CM-cellulose chromatography, has been established. Like Factor B, it is inhibited by p-chloromercuriphenylsulfonate and an antiserum to Factor B. The preparation contains, in addition, another energy transfer factor, Factor C, which stimulates ATP-driven NAD reduction by succinate in a highly depleted particle beyond the stimulation produced by Factor B.

Efforts to resolve the mitochondrial oxidative phosphorylation system into simpler components have led to the isolation of several soluble protein preparations which restore energy linked reactions in appropriately depleted submitochondrial particles (1). One of these, Factor B, is a highly purified protein with a relatively low molecular weight of 29,200 containing two func-

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tionally important thiol groups (2). It restores the activity of AE-particles[5] in oxidative phosphorylation (3) and other energy linked reactions (4). The last step in the purification of Factor B is chromatography on a CM-cellulose column. The sample is placed on the column in 2.0 mM Tris-sulfate. When the column is washed with the same buffer a considerable amount of unadsorbed protein containing factor activity is removed. Factor B can then be eluted in almost pure form using 10 mM Tris-sulfate. The activity in the 2 mM eluate was suspected to be Factor A (3), but the results presented in this paper suggest that it is factor B in a different, undetermined form, which we have designated Factor B'. In addition to Factor B', the preparation contains another energy transfer activity, referred to here as Factor C.

EXPERIMENTAL PROCEDURE

Assay particle and assay. The AE-particle was prepared by sonic disruption of bovine heart mitochondria in 0.25 M sucrose containing 0.6 mM ethylenediaminetetraacetic acid at pH 8.8 (3). The factor activity was assayed by the enhancement that the factor produced in the ATP-driven NAD reduction by succinate when it was added to the AE-particle (2). The reaction was started by the final addition of cyanide as described in the legend to Table I. A unit of activity has been defined as the stimulation of NAD reduction to the extent of 1 μmole per minute above the basal activity of the particle alone. Specific activity is expressed in units per mg of factor protein. The AE-particle can be reactivated by the nearly homogeneous preparation of Factor B (2), or by $F_1 + F_3$ (5). For the experiment described in Table II this particle was further depleted by urea as described in the legend. The urea depleted AEU-particle requires multiple factors for substantial reactivation.

[5]The following abbreviations will be used: F_1 for energy transfer (coupling) factor 1, OSCP for the oligomycin sensitivity conferring protein, AE-particle for the ammonia-EDTA depleted particle and p-CMS for p-chloromercuriphenylsulfonate.

TABLE I

-SH Requirement for Activity in Peak I Fraction with the AE-particle

		nmoles NADH x min^{-1}
1)	AE-particle	15
2)	(1) + 0.3 mg Peak I	45
3)	(1) + 0.3 mg Peak I-pCMS	13
4)	(3) + dithiothreitol	43

The medium containing 0.5 mg AE-particle, 10 µmoles $MgCl_2$, 20 µmoles succinate, 6 µmoles ATP, 1.5 µmoles NAD, 150 µmoles Tris-sulfate buffer at pH 7.8, 2 mg bovine serum albumin and factor as indicated in 2.9 ml was incubated for 2 min at 38°. The reaction was initiated by the addition of 3.0 µmoles of KCN in 0.1 ml and the absorbance at 340 mµ was followed in the Gilford multiple absorbance recorder. For 3, 0.3 ml of Peak I, containing 1 mg protein was mixed with 0.2 ml of 10 mM p-CMS and passed through a 1 x 15 cm column of Sephadex G25. For 4, 3.3 mM dithiothreitol was added to the p-CMS inhibited factor and assayed after standing for 5 min in ice.

TABLE II

Relative Stimulation by Factor B and Peak I Fraction

AE-particle			AEU-particle			
Factor B µg	Peak I µg	nmoles NADH per min	Factor A µg	Factor B µg	Peak I µg	nmoles NADH per min
--	--	12	--	--	--	1
17	--	43	--	42	--	14
28	--	43	--	--	720	8
--	500	41	--	42	720	20
--	775	42	20	--	--	3
			20	42	--	23
			20	--	720	20
			20	42	720	28

The assay for activity in ATP-driven NAD reduction by succinate was carried out as in Table I using 0.3 mg of particle. The AEU-particle was prepared by treating the AE-particle with 3 M urea in 0.125 M sucrose for 10 minutes at 0°. It was then layered on 25% sucrose containing 0.1 mg bovine serum albumin/ml and 1 mM dithiothreitol and centrifuged in a swinging bucket rotor at 100,000 g for 30 min. The factors were used in saturation levels established by assays at several concentrations.

Separation of Factors. All buffers used in the preparation contained 0.1 mM dithiothreitol. The factor activity was extracted from lyophilized acetone-washed mitochondria by 50 mM Tris-sulfate, pH 8.8, then precipitated with 65% saturated ammonium sulfate, as previously described (2). The crude preparation was first chromatographed on a DEAE-cellulose (Whatman microgranular DE-52) equilibrated with 2 mM Tris-sulfate, pH 8.0. Most of the activity was eluted from the column with 100 mM Tris-sulfate, pH 8.0 after the first washing with 50 mM buffer. The specific activity of the factor ranged from 0.5 to 0.8 which is higher than that obtained earlier using DEAE-cellulose from a different source for the separation (3).

The active fraction from the DEAE-cellulose chromatography step was separated into two active fractions on a CM-cellulose column previously equilibrated with 2 mM Tris-sulfate, pH 7.5. Part of the activity was not absorbed on the column and came out in the washings. This protein peak had a specific activity ranging from 0.1 to 0.2 (2) and is hereafter referred to as "peak I". Two small protein peaks were then eluted from the column with 10 mM Tris-sulfate, pH 7.5. The second small peak was active and corresponded to the previously described Factor B. Its specific activity varied from 3 to 6 depending to some extent on the activity of the assay particle.

RESULTS

Activity in peak I: The total activity in the first CM-cellulose chromatography peak amounted to roughly 20% of the activity applied on the column. When a sample of the peak I fraction (37 mg, 4.07 total units, specific activity 0.11) was rechromatographed in a similar manner, over 90% of the activity was again recovered in the 2 mM washings (specific activity 0.075) and only a small amount of activity (0.3 units, specific activity 3.0) was eluted with 10 mM buffer. In another experiment, the peak I fraction was rechromatographed 4 times; each time the activity was recovered mainly in the 2 mM washing, and became negligible in the 10 mM eluate. These results indicate that the activity in

peak I is not due to contamination by free Factor B, and subsequent experiments will show that it is at least partly due to another form of Factor B.

The activity of AE-particles in ATP-driven NAD reduction by succinate is stimulated by F_2, F_3 and F_6 preparations (6,7). The activity in these preparations could also be separated into two active fractions on CM-cellulose in a similar manner to crude Factor B. F_2 and F_6 gave high yield of activity in the 10 mM eluates but F_3 yielded activity predominantly in the 2 mM peak I (unpublished data, M. Kaplay and K.W. Lam).

Effect of thiol inhibitors. In earlier work it was shown that Factor B activity is inhibited by p-CMS[5] and the activity is restored by dithiothreitol. For these experiments, the factor was treated with p-CMS, excess inhibitor was removed on a Sephadex column and the factor activity was determined by its ability to stimulate the AE-particle activity in ATP-driven NAD reduction by succinate. The activity of the peak I fraction was lost on similar treatment with mercurial and the inhibition was reversed by dithiothreitol (Table I). Incubation of 0.2 ml of peak I with 0.01 ml of 2 mM iodine dissolved in 20 mM KI also produced inactivation, and the activity was restored partially by dithiothreitol as in the case of Factor B (2). Thus in terms of sensitivity to thiol binding agents, the CM-cellulose peak I activity is indistinguishable from Factor B activity.

Molecular weight. Sucrose density gradient centrifugation of the peak I fraction, carried out under the conditions previously described for Factor B (2), gave a single activity peak. The molecular weight of the active component, in reference to a hemoglobin marker (MW 64,000) was approximately 45,000 ($S_{20,w}$ 3.47 S). This value is slightly larger than that for Factor B (29,200), but more precise measurements will be needed to be certain of this small difference.

Anti-serum to Factor B. The rabbit antiserum to Factor B prepared as described before (4), inhibited the activity of the CM-cellulose peak I fraction, while normal rabbit serum had no effect (Fig. 1). In immunodiffusion

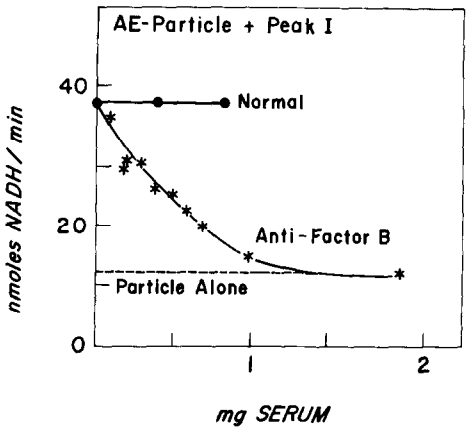

Fig. 1. Inhibition of Peak I Activity by Anti-Factor B Serum. The assay procedure was described in the legend to Table I. ●—● normal serum was added in the assay medium, *—* anti-serum was added in the assay medium. Peak I protein (0.5 mg) was used with 0.5 mg AE-particle. The activity of the particle without factor was 12 nmoles NADH/min.

experiments anti-Factor B gave a single sharp band with Factor B, but gave two bands with peak I; one of these could be due to contaminating Factor B. These data provide strong evidence that the activity in peak I is closely related to Factor B, and hence the tentative designation Factor B'.

Neither Factor B nor the Factor B' preparation gave any immune reaction with anti-F_1, which was kindly provided by Dr. E. Racker and Dr. J. Fessenden-Raden. The cold stable, latent ATPase Factor A gives a single band (8).

A new energy transfer factor (Factor C) in the peak I protein fraction. With the standard AE-particle, Factor B and peak I protein gave the same maximal stimulation of activity (Table II). No additional stimulation was obtained when the two were mixed at saturation or higher levels (data not shown). On the other hand, when the more depleted AEU-particle was used, Factor B consistently gave higher stimulation than peak I. The two together gave a higher stimulation than either alone. When a saturation level of Factor A was present, either Factor B or peak I enhanced the activity by about 20 units. When all three factors were present the activity was highest. With another AE-particle prepared from heart that had been stored overnight (and was even more depleted than AEU-particle), Factor B increased the activity in the presence of saturatior

levels of Factor A from 2.6 to 20 units. Addition of peak I fraction further enhanced the activity to 46, while peak I fraction alone gave a value of only 14. Thus the resulting activity was even greater than the sum of the activities. These results indicate that the peak I fraction contains another energy transfer factor which is different functionally from Factor B, and will be tentatively designated Factor C.

DISCUSSION

The stimulation of the activity of the AE-particle by both Factor B and the peak I fraction from CM-cellulose chromatography in a similar manner, and the sensitivity of both fractions to p-CMS as well as to antiserum immune to Factor B leave little doubt that the peak I fraction has an energy transfer factor (Factor B') which is closely related to Factor B both antigenically and in function. However, the two proteins responsible for the activities are sufficiently different that they can be consistently separated by chromatography on CM-cellulose. The greater magnitude of enhancement given by peak I fraction compared to Factor B with the highly depleted AEU-particle or the AE-particle from aged heart indicates the presence of another energy transfer factor (Factor C) in the preparation. More work will be necessary to determine whether Factor B' and C are tightly associated, as in the case of Factor A·D (8) and F_1 · X (9) or they are both present independently in the peak I fraction.

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