Biochimica et Biophysica Acta, 423 (1976) 398–412

BBA 47056

PURIFICATION AND PROPERTIES OF A LOW MOLECULAR WEIGHT PROTEIN FACTOR OF MITOCHONDRIAL ENERGY-LINKED FUNCTIONS

KWAN-SA YOU* and YOUSSEF HATEFI

Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, Calif. 92037 (U.S.A.)

(Received August 12th, 1975)

SUMMARY

1. A soluble protein with a molecular weight of $11–12 \cdot 10^3$ has been isolated from bovine-heart mitochondria, which stimulates the following ATP-dependent reactions of submitochondrial particles treated with 0.6 mM EDTA and 1 M NH_4 OH: reverse electron transfer from succinate to NAD, transhydrogenation from NADH to NADP, and ATP · P_i exchange. The factor has no effect on the NADH oxidase, succinate oxidase and ATPase activities of the particles.

2. The stimulatory effect of the factor in the ATP-dependent reduction of NAD by succinate is 12 μmol · min^{-1} · mg^{-1} of the factor protein. However, the NH_4OH · EDTA treated particles are saturated for maximal activation of the above reaction by very small amounts of the factor (about 20–40 μg factor per mg particle).

3. Electrophoresis of the factor preparation on polyacrylamide gels showed a single protein band plus a nonprotein material which moved at the dye front and was weakly stained with Coomassie Blue. The protein was shown to be required for activation of the particles; whether the fast-moving, nonprotein material is also required is not known.

4. The factor is inhibited by mercurials and *N*-ethylmaleimide. The former, but not the latter, inhibition is completely reversed by 1,4-dithiothreitol.

5. The NH_4OH · EDTA treated particles are also stimulated by rutamycin up to about 0.1 nmol of rutamycin per mg particle; higher rutamycin concentrations inhibit. Depending on the particle preparation, the factor stimulates up to about 3 nmol per mg particle, but does not inhibit at higher concentrations. In addition, under certain conditions in which appropriate concentrations of rutamycin fail to stimulate the particles, the factor still does.

Abbreviations: EDTA, ethylenediaminetetraacetate; BSA, bovine serum albumin; *p*-$ClHgBzSO_3$, *p*-chloromercuriphenylsulfonate.

* Present address: Department of Chemistry, University of California, San Diego La Jolla, Calif. 92037 (U.S.A.)

INTRODUCTION

One of the approaches to the study of oxidative phosphorylation has been the isolation of protein factors from mitochondria which are required for the energy-linked functions of particles apparently rendered deficient with respect to the factor(s) under study. A large number of such factors have been described (for reviews, see refs 1–3); however, very few have been adequately purified. The pure "coupling" factors described to date are F_1 (ATPase), the oligomycin sensitivity-conferring protein (OSCP), and possibly a factor isolated by Sanadi and coworkers, designated factor B [4]. The latter factor is considered by Sanadi et al. [5] to be located between the electron transport system and the site at which oligomycin inhibits ATPase activity. Operationally, factor B may be defined as a water-soluble, colorless protein, which contains active thiols and which stimulates the energy-linked functions of submitochondrial particles treated with EDTA [1] and 1 M NH_4OH.

Presumably, this treatment renders the particles deficient with respect to factor B. Several preparations with factor B-like properties have been reported. These are as follows. (a) Factor B: This is the original preparation of Sanadi and coworkers [4, 5] with a molecular weight of 32 000 (subsequently revised to 29 200 [6]). (b) Factor B′: This is a second fraction with factor B-like activity, which was isolated by the above workers during purification of factor B on CM-cellulose [4]. (c) F_2: This is one of the partially purified coupling factors of Racker's laboratory. In collaborative studies with Sanadi's group, it was shown that the active component of F_2 is apparently factor B [7]. (d) Factor F_B: This is a preparation isolated by Wang and his colleagues [8]. It is reported to have a molecular weight of 360 000, and to contain 8 identical subunits of approximate molecular weights of 43 500. The amino acid composition of F_B is stated to be different from that of factor B. (e) A new preparation from Sanadi's laboratory, which is twice as active as factor B in stimulation of NH_4OH · EDTA-treated particles for ATP-driven reverse electron transfer from succinate to NAD [9]. The molecular weight of the new factor, as estimated by sucrose density gradient centrifugation, is given as 47 000. However, it is stated that sodium dodecyl sulfate-gel electrophoresis of this preparation shows three bands, which are stained for protein with nearly equal intensity.

These results suggest either that mitochondria contain multiple forms of factor B or that this interesting factor occurs in the above preparations along with variable amounts of other proteins. Another possibility is that NH_4OH · EDTA-treated particles respond in a similar manner to a variety of unrelated proteins of mitochondrial origin. This lack of specificity of NH_4OH · EDTA-treated particles is not very likely, since the particles are stimulated and saturated by very minute quantities of the purified factor (see below).

The present paper describes the isolation and purification from beef-heart mitochondria of a water-soluble preparation which has factor B-like properties, and appears to contain a single protein with a molecular weight of $11–12 \cdot 10^3$. This preparation is 6 times as active as factor B in stimulating ATP-driven reverse electron transfer by NH_4OH · EDTA-treated particles.

METHODS AND MATERIALS

Acetone-dried bovine heart mitochondria were prepared and suspended in

50 mM Tris · SO_4, pH 8.8, essentially according to Lam et al. [4], except that the buffer also contained 1 mM EDTA and 2 mM dithiothreitol. The suspension was centrifuged at 144 000 × *g* for 60 min, and the supernatant was dialyzed overnight against 5 mM Tris · SO_4, pH 7.5, containing 7.2 mM β-mercaptoethanol. The dialyzed crude extract was applied to a DEAE-52 column, which had been equilibrated with the same buffer mixture used for dialysis. The ratio of bed-height to diameter of the column was 12 : 1, and a bed volume of 60 ml was used per gram of protein in the crude extract. After application of the extract, the column was washed with the equilibration buffer until A_{280} of the effluent was lower than 0.4. Then the column was eluted once with 30 mM Tris · SO_4 and a second time with 100 mM Tris · SO_4, both at pH 7.5 and containing 7.2 mM mercaptoethanol. Most of the factor activity was found in the first eluate. The protein fractions having the factor activity in the first eluate were pooled and precipitated by dialysis against saturated ammonium sulfate, containing 50 mM Tris · SO_4, pH 7.5, 2 mM dithiothreitol, and 1 mM EDTA. The precipitate was dissolved in a minimum volume of 50 mM Tris · SO_4, pH 7.5, containing 2 mM dithiothreitol and 1 mM EDTA, applied to a Sephadex G-100 column, and eluted with the same buffer mixture. Other details of the purification of the factor are described under Results.

Treatment of the factor with *p*-$ClHgBzSO_3$ was carried out as follows. 90 μg of the factor, which had been prepared in the absence of thiol compounds at the Sephadex step and dissolved in 5 ml of 50 mM Tris · SO_4, pH 7.5, was incubated for 60 min at 0 °C with 1.0 μmol of *p*-$ClHgBzSO_3$. The free *p*-$ClHgBzSO_3$ was then removed by filtering the mixture through a Sephadex G-25 column (1.4 × 22 cm). To determine the reversibility of *p*-$ClHgBzSO_3$ inhibition, the mercurial-treated factor was incubated for 40 min at 0 °C with 5 μmol of dithiothreitol, then filtered through a similar Sephadex column.

Depleted particles for recombination with the factor were prepared according to the procedure of Lam et al. [4] for the preparation of NH_4OH · EDTA-treated particles. Thus, heavy beef-heart mitochondria suspended in 0.25 M sucrose, containing 0.6 mM EDTA, were treated with 1 M NH_4OH, sonicated, and centrifuged at 100 000 × *g* for 40 min. The pellets were suspended in the same medium as above, and treated once more with 1 M NH_4OH. The suspension was centrifuged at 24 000 × *g* for 10 min to remove large particles. The supernatant, containing submitochondrial particles, was centrifuged at 100 000 × *g* for 40 min, and the pellet was suspended in 0.25 M sucrose, containing 10 mM Tris · SO_4, pH 7.2. This constituted the preparation of NH_4OH · EDTA-treated particles. Submitochondrial particles were prepared in the same manner, except that the treatment with NH_4OH was omitted.

The assay for stimulation by the factor of ATP-induced reverse electron transfer from succinate to NAD was performed at pH 7.5 according to procedure A of Lam et al. [4]. Specific activity of the factor is given as μmol or nmol of NAD reduced per min by one mg of the factor minus the residual activity of the depleted particles used. The concentration of NH_4OH · EDTA-treated particle in each 3 ml assay was 0.2 to 0.25 mg protein. However, since under these conditions 1 mg of factor was far in excess of the amount needed for maximal activity, a saturation curve was obtained from a series of assays performed at various levels of the factor. Then the linear portion of the curve was extrapolated to 1 mg of the factor protein to obtain the numerical value of the factor specific activity.

ATPase activity was assayed by the method of Nishimura et al. [10], using the reaction mixture described by Mitchell and Moyle [11]. Polyacrylamide gel electrophoresis was carried out by the method of Davis [12], and the gels were stained with Coomassie Blue (1.25 g dissolved in a mixture of 454 ml of 50 % methanol and 46 ml of glacial acetic acid). Molecular weight of the factor was estimated by filtration through a Sephadex-G100 column (2.5×84 cm), which had been equilibrated at 4 °C with 50 mM Tris · SO_4, pH 7.5, containing 0.1 M KCl. The column was calibrated with BSA, ovalbumin, α-chymotrypsinogen A, whale myoglobin, and horse heart cytochrome *c*. The factor was located by assaying the effluent for enzymatic activity as described above. The molecular weight of the factor was also estimated by sedimentation equilibrium centrifugation as described by Yphantis [13]. The factor (0.15 mg/ml) was dialyzed against 50 mM Tris · SO_4, pH 7.5, containing 0.1 M NaCl, and was centrifuged at 28 500 rev./min and 14.7 °C for 16 h. Protein was determined by the biuret method [14] and, when the solution was too dilute, it was determined by the method of Lowry et al. [15]. Rutamycin and dicyclohexylcarbodiimide were used as ethanolic solutions.

Chemicals were obtained from the following sources: protein calibration kit from Boehringer-Mannheim; DEAE-52 and CM-52 from Reeve Angel; rutamycin from Eli Lilly and Co; dicyclohexylcarbodiimide from K and K; ATP and NAD from P-L Biochemicals; succinate and dithiothreitol from CalBiochem; soybean trypsin inhibitor from Worthington; Sephadex G-100 from Pharmacia; ingredients for polyacrylamide gels from Eastman Kodak; and *p*-ClHgBzSO$_3$, *N*-ethylmaleimide and trypsin (Type II) from Sigma.

RESULTS

1. Purification of the factor

As mentioned above, the factor stimulates energy-dependent reverse electron transfer from succinate to NAD when it is added to "depleted" particles prepared by treatment of sonicated mitochondria with NH_4OH and EDTA. It is a water-soluble

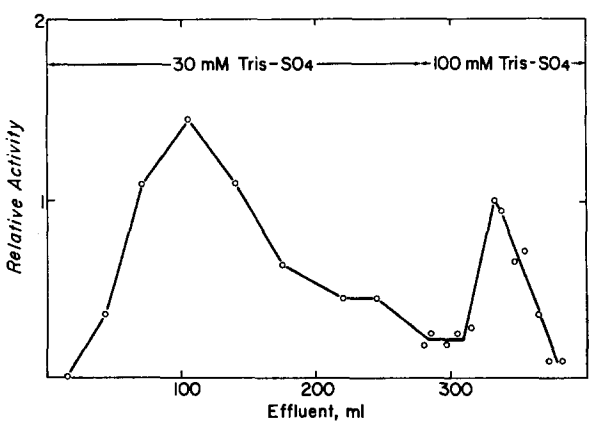

Fig. 1. Chromatography of the crude extract of acetone-dried mitochondria on DEAE-52 column. The column was loaded with 480 mg protein in 20 ml of buffer. For details, see text.

protein, which is extracted from acetone-dried mitochondria by 50 mM Tris · SO_4, pH 8.8, containing 1 mM EDTA and 2 mM dithiothreitol. Chromatography of the extract of acetone-dried mitochondria on DEAE-52 resulted in two active fractions. As shown in Fig. 1, approximately 80 % of the activity of the crude extract was eluted

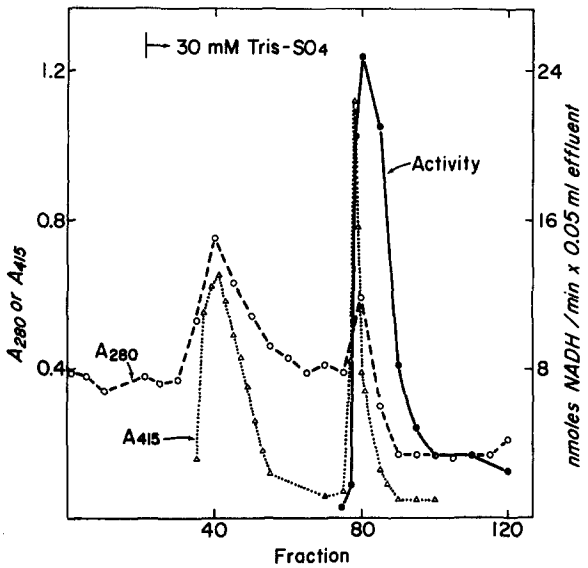

Fig. 2. Elution pattern of protein (○–○), the pigment (△ ... △) and activity (●–●) from DEAE-52 column by 30 mM Tris · SO_4, pH 7.5.

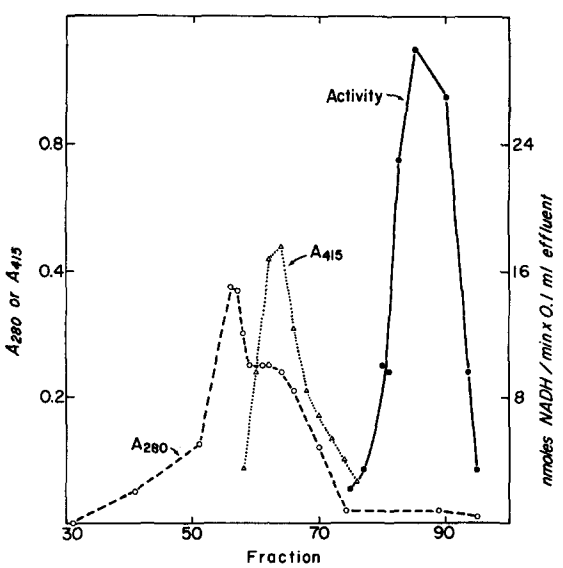

Fig. 3. Purification of the factor by filtration through Sephadex G-100 column. The lines have the same significance as in Fig. 2. Column dimensions, 3.5 × 50 cm; fraction volume, 4.7 ml. For other details, see text.

TABLE I

SUMMARY OF THE PURIFICATION OF THE FACTOR

The crude extract was obtained from 20 g of acetone-dried mitochondria. 0.25 mg NH_4OH · EDTA treated particle protein was used for each assay. As stated under Methods and Materials, specific activity (i.e., per mg factor) is a computed value, not a value that is experimentally obtained. This is because the amount of particles used in each assay is saturated with very small amounts of the factor (see Fig. 4). The loss of activity units between the second and the third steps shown below occurred mainly at the stage of factor concentration by dialysis against saturated ammonium sulfate (see Methods and Materials). A similar activity loss occurred when the protein was concentrated by ultrafiltration.

Steps	Protein (mg)	Protein recovery (%)	Spec. act. (μmol·mg^{-1}·min^{-1})	Total activity (units)	Activity recovery (%)
Crude Extract	1170	100	0.06	70.2	100
DEAE-52	34	2.9	1.6	54.4	77
Sephadex G-100	0.9	0.08	11.8	10.6	15

with 30 mM Tris · SO_4. The elution of this portion of activity required approximately 3 bed volumes of the eluant. The bulk of the protein, plus a small amount of activity, remained on the column, and could be eluted with 100 mM Tris · SO_4. Fig. 2 shows the distribution of protein and activity during elution with 30 mM Tris · SO_4. Also shown in this figure is the elution pattern of a pigment with hemoglobin-like peaks at 580, 540 and a Soret-like peak at 415 nm. This pigment elutes in two bands, the second of which is sharp and marks the onset of the elution of the factor. Thus, the

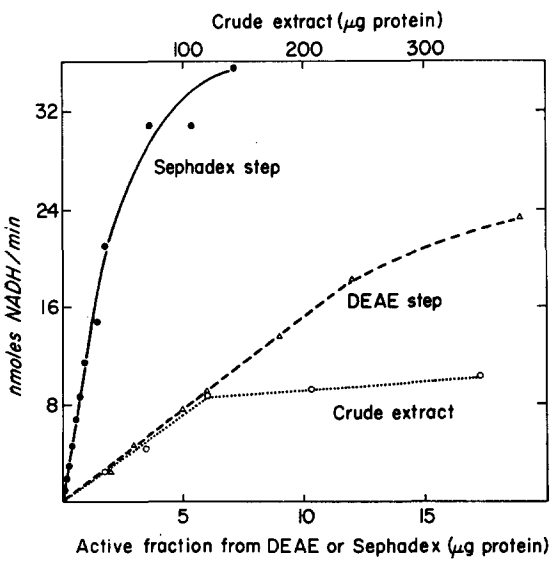

Fig. 4. Activation of NH_4OH · EDTA-treated particles for ATP-dependent reverse electron transfer from succinate to NAD by various amounts of the crude extract and the active fractions from DEAE and Sephadex columns. For details, see Table I.

elution of the factor can be monitored by eye by following the elution of the red pigment. The two proteins are then separated by Sephadex G-100 filtration as shown in Fig. 3. It is seen in Fig. 3 that the activity peak is associated with very little protein. The reason that the protein elution pattern, as measured by 280 nm absorption, has an unusual shape under the red pigment peak is probably in part because the pigment also absorbs at 280 nm.

Table I summarizes the purification procedure in terms of protein and activity. It is seen that only about 0.1 % of the protein of the crude extract is recovered as the final active material, the specific activity of which is 200 times that of the crude extract. Fig. 4 shows the manner in which increasing concentrations of the crude extract, the material eluted from DEAE-52 column, and the purified factor stimulate the energy-dependent reverse electron transfer activity of NH_4OH · EDTA-treated

Fig. 5. Electrophoresis of the purified factor on polyacrylamide gel showing a protein band and a sharp band at the dye front stained with Coomassie Blue.

particles. It is seen that the stimulatory effects of the intermediate fractions, especially that of the crude extract, reach a limit long before maximum stimulation as achieved by the purified factor can be reached. This suggests that the crude fraction might contain an inhibitor (Pullman-Monroy inhibitor?), which has a different titer from the factor under consideration, and is removed during purification (e.g., by Sephadex).

The purified factor is a colorless protein with a maximum absorption at about 278 nm. The absorbance ratio A_{280}/A_{260} at pH 7.5 is 1.26. When it is subjected to electrophoresis on polyacrylamide gels in the absence of sodium dodecyl sulfate, the purified factor shows two bands, which are stained by Coomassie Blue (Fig. 5), a single protein band and a sharp band, which was weakly stained with Coomassie Blue and which had the same electrophoretic mobility as the bromphenol blue dye front (this latter band cannot be visualized on sodium dodecyl sulfate-acrylamide gels). The mobility and staining property of the latter band raised the question as to whether it was a protein. This band was, therefore, cut from 50 gels, extracted, hydrolyzed in 6 M HCl for 24 h at 110 °C, and subjected to analysis by the Beckman automatic amino acid analyzer. The result showed that only negligible amounts of amino acids (i.e., other than the glycine used as buffer) were present in the acid hydrolyzate. Fessenden-Raden [16] has reported, however, that unsaturated *cis*-fatty acids show similar mobility and staining characteristics when subjected to electrophoresis on polyacrylamide gels. They move as a sharp band close to the dye front, and are weakly stained with Coomassie Blue. Whether the above fast-moving component is required for activity of the factor is not known at this time.

2. Molecular weight

Except for the nonprotein component discussed above, the protein band seen on the gel of Fig. 5 is the fastest moving band in the crude extract. Chromatography

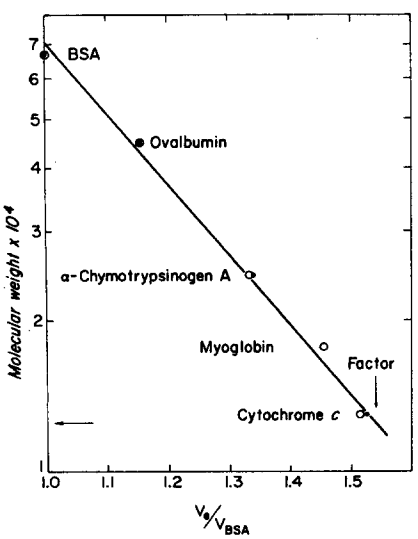

Fig. 6. Estimation of the molecular weight of the factor by filtration on Sephadex G-100. For details, see text.

on Sephadex G-100 showed that the factor had an elution volume slightly larger than that of horse heart cytochrome *c*. The gel filtration data of Fig. 6 suggested a molecular weight of approximately 12 500 for the factor. Sedimentation equilibrium experiments showed that the material was monodisperse, and indicated a molecular weight of 11 000, when $\bar{v}$ was assumed to be 0.74.

Two proteins of comparable molecular weight have been described previously to be associated with the mitochondrial energy transfer system. These are the dicyclohexylcarbodiimide binding protein [17] and the Pullman-Monroy inhibitor [18]. The possibility that the factor described above might be the dicyclohexylcarbodiimide binding protein was ruled out by the following experiments. In the first experiment, the factor was incubated for 2 h at 0 °C with dicyclohexylcarbodiimide in a ratio of 2 nmol of dicyclohexylcarbodiimide per mg of the factor. The dicyclohexylcarbodiimide-treated factor retained full stimulatory activity when assayed in conjunction with NH_4OH · EDTA-treated particles. In the second experiment, NH_4OH · EDTA-treated particles were treated with dicyclohexylcarbodiimide. This treatment resulted in complete loss of the residual activity of the particles, and addition of the factor did not restore the activity of the dicyclohexylcarbodiimide-treated particles. These results suggested that the dicyclohexylcarbodiimide binding site is not associated with the factor, and resides in NH_4OH · EDTA-treated particles. In addition, the factor is a completely water-soluble protein, whereas the dicyclohexylcarbodiimide binding protein appears to be hydrophobic in nature [3].

The factor does not appear to be related to the Pullman-Monroy inhibitor, because (a) it stimulates, rather than inhibits, ATP-induced reverse electron transfer, and (b) it does not inhibit the ATPase activity of the particles. Furthermore, the Pullman-Monroy inhibitor resisted heat inactivation by incubation for 8 min at 90 °C [18], whereas, by contrast, the factor under consideration was very sensitive to heat. It is also unlikely that this factor is related to the oligomycin sensitivity-conferring protein, because the latter has a molecular weight of 18 000, is not inhibitable by mercurials (see below) and, so far as known, its removal from particles also leads to the removal of F_1 (ATPase) [19, 20].

3. Activities

NH_4OH · EDTA-treated particles treated twice with NH_4OH have a residual ATP-dependent reverse electron transfer activity from succinate to NAD of about 5 to 6 nmol NAD reduced · min^{-1} · 0.25 mg^{-1} particle protein, which is the amount usually used for assaying the activity of the factor. This residual activity can be decreased by further treatment of the particles with NH_4OH. However, such treatment also affects the extent to which activity can be restored by the factor, thus suggesting damage to the particles by excessive treatment with NH_4OH. As shown in Fig. 4, addition of the factor to particles twice treated with NH_4OH results in a considerable increase of energy-dependent reverse electron transfer activity from 5 to about 40 nmol NAD reduced · min^{-1} · 0.25 mg^{-1} particle or 8–10 μg factor. Fig. 4 and the calculated specific activity of Table I also show the dramatic activation of ATP-dependent reverse electron transfer that is achieved by extremely small amounts of the purified factor. This is also true for other ATP-dependent reactions catalyzed by NH_4OH · EDTA-treated particles, such as ATP-P_i exchange and transhydrogenation from NADH to NADP. However, NADH oxidase, succinate oxidase, and the

TABLE II

REACTIVATION OF STORED FACTOR WITH DITHIOTHREITOL

Conditions: 0.25 mg NH_4OH · EDTA-treated particle, 3.5 μg factor, 4 mM dithiothreitol. The activity of 0.25 mg NH_4OH · EDTA-treated particle by itself was 5.1 nmol · min^{-1}, which was subtracted from the overall activity to obtain the factor-stimulated activities shown in the Table. The factor was stored in 50 mM Tris · SO_4, pH 7.5.

Hours at −20 °C	nmol NADH · min^{-1}	
	−dithiothreitol	+dithiothreitol
0	25.9	25.9
8	7.0	25.8
80	1.2	12.4

ATPase activities of NH_4OH · EDTA-treated particles are not stimulated by addition of the factor.

The activity of the factor is lost completely by 1 min incubation at 65 °C, or 10 min incubation at 30 °C in the presence of trypsin. The latter effect was prevented when trypsin was treated with soybean trypsin inhibitor. However, venom phospholipase did not destroy the activity of the factor. As seen in Table II, the factor also loses activity upon storage at −20 °C. Addition of dithiothreitol to the factor restored the lost activity completely after several hours of storage, and partially after prolonged storage when nearly all the activity had disappeared (Table II). These observations suggested thiol involvement. Other experiments shown in Table III indicated that the activity of the factor could be inhibited by *p*-$ClHgBzSO_3$ addition to the factor, and restored by treating the *p*-$ClHgBzSO_3$-inhibited factor with dithiothreitol (for details see Methods). Addition of *N*-ethylmaleimide to the factor also resulted in inhibition, but as expected the inhibition by *N*-ethylmaleimide could not be relieved upon subsequent treatment of the factor with dithiothreitol. The stimulatory effect of the factor upon the reverse electron transfer activity of NH_4OH · EDTA-treated particles and its behavior toward thiol inhibitors suggested similarities with factor B of Sanadi and his colleagues [4, 9]. However, these two factors differ in molecular weight and chromatographic properties. As stated above, Sanadi and his colleagues have described

TABLE III

INHIBITION OF FACTOR ACTIVITY BY *p* CHLOROMERCURIPHENYLSULFONATE

For the preparation of *p*-$ClHgBzSO_3$-treated factor and its subsequent treatment with dithiothreitol, see Methods and Materials.

System	Activity (nmol NADH · min^{-1})
NH_4OH · EDTA-treated particle	5.8
NH_4OH · EDTA-treated particle + Factor	22.2
NH_4OH · EDTA-treated particle + *p*-$ClHgBzSO_3$-Factor	7.7
NH_4OH · EDTA-treated particle + *p*-$ClHgBzSO_3$-Factor + dithiothreitol	19.3

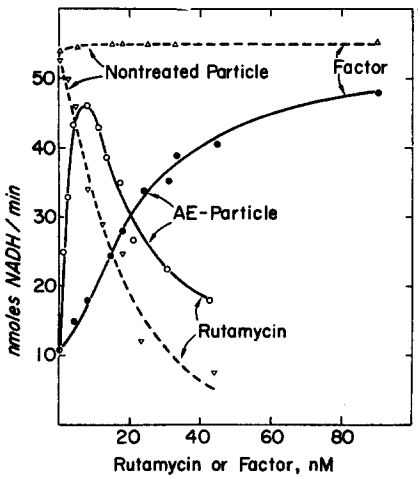

Fig. 7. Effects of rutamycin and the factor on the ATP-dependent reduction of NAD by succinate as catalyzed by nontreated and NH_4OH · EDTA-treated particles (AE-particle). 0.5 mg particle protein was used per assay. The molecular weights of rutamycin and the factor used for computation of the abscissa values were, respectively, 776 and 11 000.

two preparations of factor B. The original factor B had a molecular weight of 29 200 [6]. A more recent preparation is stated to be less pure, but more active than factor B, and its main protein component has a molecular weight of 47 000 [9]. Both preparations of Sanadi bind to CM-52 columns equilibrated with 2.5 mM Tris · SO_4, pH 7.5, while our preparation does not. Another difference is that Sanadi's best preparation has a specific activity of 4 μmol NAD reduced · min^{-1} · mg^{-1} factor protein, while the specific activity of the present preparation is 3 times as much.

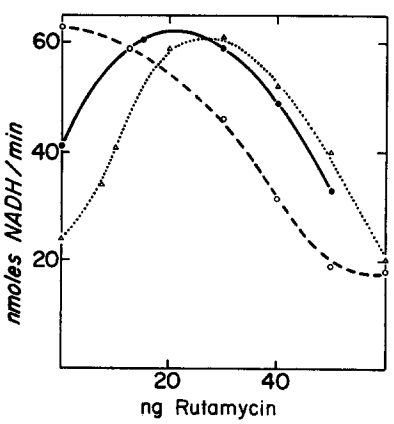

Fig. 8. Effect of rutamycin on the ATP-dependent reverse electron transfer activity of NH_4OH · EDTA-treated particles (△ ... △), NH_4OH · EDTA-treated particles treated with 1.5 μg factor (●–●), and NH_4OH · EDTA-treated particles treated with 15 μg factor (○– – –○). 0.42 mg of NH_4OH · EDTA-treated particle protein was used in each experiment.

As shown originally by Lee and Ernster [21], the ATP-driven reactions of NH_4OH · EDTA-treated particles are stimulated by oligomycin. This stimulatory effect appears to be somewhat similar to that of factor B [4] or the present factor. As shown in Fig. 7, rutamycin inhibits ATP-dependent NAD reduction by succinate as catalyzed by submitochondrial particles, which had not been treated with NH_4OH. However, when rutamycin is added to NH_4OH · EDTA-treated particles, it stimulates the above activity at low concentrations, then inhibits at higher levels. The factor also stimulates ATP-dependent reverse electron transfer when added to NH_4OH · EDTA-treated particles, but it inhibits neither the NH_4OH · EDTA-treated particles nor the non-treated particles. The results of Fig. 7 suggest, therefore, that the effects of rutamycin and the factor are different. However, NH_4OH · EDTA-treated particles activated with saturating amounts of the factor behave like non-treated particles toward rutamycin (Fig. 8). They cannot be stimulated further by addition of low levels of rutamycin, but are still inhibitable by about the same concentrations of rutamycin as shown in Fig. 7. Furthermore, when NH_4OH · EDTA-treated particles are treated with a sub-saturating amount of the factor, the stimulatory effects of the factor and rutamycin become additive (Fig. 8). Thus, at low concentrations, rutamycin augments the activity of the partially stimulated system up to the maximum level possible by either the factor or rutamycin alone, then at higher concentrations it inhibits as before. These results suggest that (a) the NH_4OH · EDTA-treated particles appear to be functionally depleted with respect to the factor, and (b) that in its stimulatory effect rutamycin mimics the role of the factor. However, it can be shown that rutamycin does not possess the full potential of the factor for stimulating NH_4OH ·

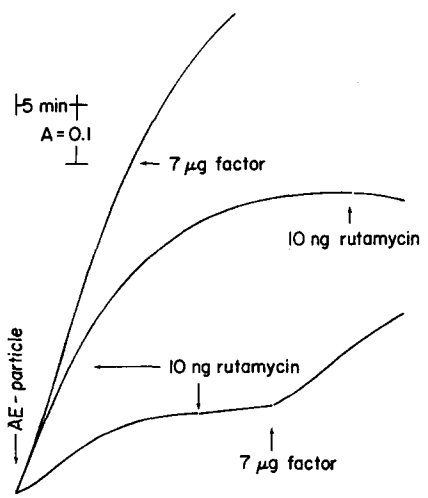

Fig. 9. Effects of rutamycin and the factor on stimulation of ATP-dependent NAD reduction by succinate as catalyzed by NH_4OH · EDTA-treated particles. Top trace: 7 μg factor added to the reaction mixture immediately after addition of NH_4OH · EDTA-treated particles (AE-particle). Middle trace: 10 ng rutamycin added to the reaction mixture immediately after addition of NH_4OH · EDTA-treated particles and once again after reaction reached a plateau. Bottom trace: the residual activity of NH_4OH · EDTA-treated particles; at the points shown, 10 ng rutamycin and 7 μg factor were added. Upward deflection of the traces indicates NADH production as measured spectrophotometrically at 340 nm. 0.25 mg NH_4OH · EDTA-treated particle protein was used in each experiment.

EDTA-treated particles. As seen in Fig. 9, the residual reverse electron transfer activity of NH_4OH-EDTA-treated particles decays to near zero after about 10 to 15 min in the reaction mixture. At the onset of the experiment, addition of either rutamycin or the factor resulted in stimulation. However, after the residual activity of NH_4OH · EDTA-treated particles had decayed to near zero, then only the factor could restore reverse electron transfer activity, and rutamycin had little or no effect on the particles after the disappearance of their residual activity. These results suggest that the stimulatory effect of rutamycin depends on the presence of a labile component in NH_4OH · EDTA-treated particles, whereas the stimulatory effect of the factor does not require the presence of this component in active form.

DISCUSSION

Since the ATPase, NADH oxidase and succinate oxidase activities of NH_4OH · EDTA-treated particles are unaffected by addition of the factor described above, but various energy-linked functions of the particles are stimulated, it may be concluded that the factor is an energy-transfer factor operating between the electron transport system and ATPase. As stated above, the NH_4OH · EDTA-treated particles treated twice with NH_4OH and EDTA exhibit a residual reverse electron transfer activity of about 5 nmol NADH formed · min^{-1} · 0.25 mg^{-1} particle protein. When a freshly made preparation of the factor is used, the above residual activity of NH_4OH · EDTA-treated particles is increased to about 40, and maximal activation is achieved with the addition of about 8–10 μg factor per 0.25 mg of NH_4OH · EDTA-treated particle protein. These data indicate (a) that the particles are about 90 % depleted with respect to the factor, and (b) that maximal activation is achieved by the addition of approximately 3 nmol of the factor per mg of particle protein. The value of 3 nmol is about 10 times the molar concentration of F_1 [22] or cytochrome c_1 [23, 24], and only about 4 times the molar concentration of cytochrome a or a_3 (23, 24) in 1 mg of particles. At these low levels, the amount of the factor actually rebound to NH_4OH · EDTA-treated particles could not be determined, even though activated particles could be recovered after NH_4OH · EDTA-treated particles were mixed with the factor and centrifuged at 144 000 $\times g$. Considering that the factor is completely water-soluble and would very likely equilibrate between the particles and the aqueous phase, it seems possible, therefore, that the amount of the factor taken up by the depleted particles could be roughly in the range of the concentration of other components of the electron transport/oxidative phosphorylation system.

The phenomena discussed in this paper could be formally represented by the following minimal equations:

$$ATP + H_2O + X \rightleftharpoons ADP + P_i + X^* \quad (1)$$

$$X^* + Y \rightleftharpoons X + Y^* \quad (2)$$

$$Y^* + \text{deenergized electron transport} \rightleftharpoons Y + \text{energized electron transport} \quad (3)$$

$$X^* \rightarrow X + \text{heat} \quad (4)$$

$$X + \text{rutamycin} \rightarrow X\text{-rutamycin} \quad (5)$$

Thus, ATP hydrolysis would result from reactions (1)+(4); oxidative phosphorylation or reverse electron transfer from reactions (1)+(2)+(3); ATP · P_i exchange from reaction (1) or more likely from reactions (1)+(2)* (see below); rutamycin inhibition of ATP hydrolysis from reactions (1)+(5); and rutamycin-induced respiratory control in EDTA-particles [27] from reactions (3)+(2)+(5), because upon interaction of rutamycin with X, energy dissipation through reactions (2)+(4) would be prevented. Since the present factor is concerned with energy transfer, but very likely is not the receptor of rutamycin, it might be equivalent to Y. Particles depleted with respect to Y would not be able to couple the energy of ATP hydrolysis to reverse electron transfer, hence the energized X* would dissipate the energy through reaction [4].

The activation of NH_4OH · EDTA-treated particles at low concentrations of rutamycin could be explained by the reasonable assumption that rutamycin preferentially interacts with X in Y-deficient systems, thus inhibiting energy dissipation through reaction [4] and allowing the energy to be funnelled from reaction (1) to reactions (2) and (3). At higher concentrations then, rutamycin would also react with X in Y-containing systems and cause inhibition of ATP-dependent reactions. These considerations do not explain, however, the observation that at low levels rutamycin results in a near maximal activation of NH_4OH · EDTA-treated particles (Fig. 7 and 8). The reason for this is probably based on the fact that, as compared to ATP synthesis and ATP · P_i exchange, ATP-dependent reverse electron transfer is a slow process. Thus in the formal scheme shown above, reaction (3) in the forward direction (marked by broken arrow) would be slower than in the reverse direction. This reaction could also be relatively insensitive to the concentration of Y, both of which possibilities would favor efficient energy transfer in the direction of ATP synthesis. Thus, once energy dissipation is inhibited at low levels of rutamycin as discussed above, the deficient particles having residual reverse electron transfer activity could show maximal activation since reactions (1) and (2) would turn over faster than reaction (3) in the direction of the broken arrow. These considerations require that rutamycin should activate the systems which contain a small amount of Y in active form and exhibit a residual reverse electron transfer activity, but not when this residual activity is lost. However, under the latter conditions, addition of Y to the system should still cause activation. These requirements are borne out by the results shown in Fig. 9.

The relative position of the present factor (plausibly Y in the above scheme) in the mitochondrial energy transfer reactions can be further delineated by considering the position of the mitochondrial uncoupler-binding site [28–30] in the above scheme. Both ATP-P_i exchange (reactions 1+2) and rutamycin-induced respiratory control in EDTA-particles (reactions 3+5) are susceptible to uncouplers. Therefore, the uncoupler-binding site would have to be located at the level of reaction (2). Other studies have shown that the principal uncoupler-binding site of mitochondria is a polypeptide of molecular weight 30 000±10 %, and that rutamycin does not affect uncoupler binding to the particles [28–30]. Thus, the mitochondrial uncoupler-binding component appears to be different from the rutamycin binding site (X in the scheme) and the factor described in this paper (Y in the scheme), but might be located

* As shown with complex V, the mitochondrial ATP · P_i exchange complex [25], and yeast promitochondria [26], the ATP · P_i exchange reaction does not require the participation of the electron transport system.

between these two components. These considerations lead to the working hypothesis that the present factor is functionally located between the electron transport system and the mitochondrial uncoupler-binding site.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. C. Muñoz for the preparation of beef-heart mitochondria. K.-S.Y. is a postdoctoral trainee, USPHS Training Grant AM5458. The work was supported by USPHS grant AM08126.

REFERENCES

1 Lardy, H. A. and Ferguson, S. M. (1969) Annu. Rev. Biochem. 38, 991–1034
2 Kagawa, Y. (1972) Biochim. Biophys. Acta 265, 297–338
3 Beechey, R. B. and Cattel, K. J. (1973) in "Current Topics in Bioenergetics" (D. R. Sanadi and L. Packer, eds.), Vol. 5, pp. 305–357, Academic Press, New York
4 Lam, K. W., Warshaw, J. B. and Sanadi, D. R. (1967) Arch. Biochem. Biophys. 199, 477–484
5 Sanadi, D. R., Lam, K. W. and Kurup, C. K. P. (1968) Proc. Natl. Acad. Sci. U.S. 61, 277–283
6 Lam, K. W., Swann, D. and Elzinga, M. (1969) Arch. Biochem. Biophys. 130, 175–182
7 Racker, E., Fessenden-Raden, J. M., Kandrach, M. A., Lam, K. W. and Sanadi, D. R. (1971) Biochem. Biophys. Res. Commun. 41, 1474–1479
8 Higashiyama, T., Saunders, D. R., Serrianne, B. C., Steinmeier, R. C. and Wang, J. H. (1975) Fed. Proc. 34, 596
9 Shankaran, R., Sani, B. P. and Sanadi, D. R. (1975) Arch. Biochem. Biophys. 168, 394–402
10 Nishimura, M., Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177–182
11 Mitchell, P. and Moyle, J. (1971) Bioenergetics 2, 1–11
12 Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427
13 Yphantis, D. A. (1964) Biochemistry 3, 297–317
14 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751–766
15 Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
16 Fessenden-Raden, J. M. (1972) Biochem. Biophys. Res. Commun. 46, 1347–1353
17 Cattel, K. J., Lindop, C. R., Knight, I. G. and Beechey, R. B. (1971) Biochem. J. 125, 169–177
18 Pullman, M. E. and Monroy, G. C. (1963) J. Biol. Chem. 238, 3762–3769
19 MacLennan, D. H. and Tzagoloff, A. (1968) Biochemistry 7, 1603–1610
20 Ernster, L., Nordenbrand, K., Chude, O. and Juntti, K. (1974) in "Membrane Proteins in Transport and Phosphorylation" (G. F. Azzone, M. E. Klingenberg, E. Quagliariello and N. Siliprandi, eds), pp. 29–41, North-Holland Publishing Co., Amsterdam
21 Lee, C.-P. and Ernster, L. (1965) Biochem. Biophys. Res. Commun. 18, 523–529
22 Bertina, R. M., Schrier, P. I. and Slater, E. C. (1973) Biochim. Biophys. Acta 305, 503–518
23 Green, D. E. and Wharton, D. C. (1963) Biochem. Z. 338, 335–348
24 Klingenberg, M. (1968) in "Biological Oxidations" (T. P. Singer, ed.), pp. 3–54, Interscience Publishers, New York
25 Hatefi, Y., Stiggall, D. L., Galante, Y. and Hanstein, W. G. (1974) Biochem. Biophys. Res. Commun. 61, 313–321
26 Groot, G. S. P., Kováč, L. and Schatz, G. (1971) Proc. Natl. Acad. Sci. U.S. 68, 308–311
27 Lee, C.-P. and Ernster, L. (1968) Eur. J. Biochem. 3, 391–400
28 Hanstein, W. G. and Hatefi, Y. (1974) J. Biol. Chem. 249, 1356–1362
29 Hatefi, Y. and Hanstein, W. G. (1974) in "Membrane Proteins in Transport and Phosphorylation" (G. F. Azzone, M. E. Klingenberg, E. Quagliariello and N. Siliprandi, eds.), pp. 187–200, North-Holland Publishing Co., Amsterdam
30 Hatefi, Y. (1975) J. Supramol. Struct. 3, 201–213