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Involvement of a Dithiol Protein in Mitochondrial Energy-Linked Functions and Its Relation to Coupling Factor B[1]

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The coupling factor protein isolated previously in pure form with a molecular weight of 11–12 × 10^3 (K.-S. You and Y. Hatefi, 1976, *Biochim. Biophys. Acta* 423, 398–412) has been shown to restore ATP-induced NAD reduction by succinate, transhydrogenation from NADH to NADP, and ATP–$^{32}P_i$ exchange to submitochondrial particles rendered deficient by extraction with 1 M NH_4OH. The factor also stimulated the oxidative phosphorylation activity of the extracted particles 2.5- to >3-fold. The stimulatory effect of the factor was inhibited by mercurials, Cd^{2+}, phenylarsine oxide, and diamide, indicating that it contains an essential dithiol. Dithiothreitol and dihydrolipoate did not replace the protein factor in stimulating the deficient particles. The purified dithiol-containing protein was precipitated and inhibited by antibody raised against coupling factor B. Since this antibody also inhibits coupling factor F_2, it is concluded that the active principle of coupling factors B and F_2 is the purified dithiol-containing protein of molecular weight 11–12 × 10^3 referred to above.

Lam and co-workers (1) discovered a soluble protein factor, designated factor B, which stimulated certain energy-linked functions of submitochondrial particles treated with 1 M NH_4OH and 0.6 mM EDTA. The molecular weight of factor B was given as 32,000, and subsequently revised to 29,200 (2). More recently, another preparation with a molecular weight of 47,000 was described by this group, which exhibited a somewhat higher factor B activity (3). The former preparation moved as a single protein peak upon ultracentrifugation, but the latter preparation was stated to exhibit three polypeptides upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (3). The authors concluded from these studies that "Factor B activity is heterogeneous in terms of its fractionation properties" (3). In other studies, Racker, Sanadi, and their colleagues showed that antibody raised against Sanadi's factor B inhibited the factor B-like activity of coupling factor F_2 of Racker's group (4). Thus, it was concluded that factors B and F_2 are "identical" (4). This conclusion was based only on enzymatic and immunological tests, not on relative preparation purity (e.g., sodium dodecyl sulfate–acrylamide gel electrophoresis patterns), molecular weights, amino acid composition, etc. In the absence of such evidence, the only possible conclusion is that both factor B and F_2 contained the same "active" component at about the same concentration or specific activity.

In 1976, You and Hatefi (5) purified a soluble protein from beef heart mitochondria, which exhibited very high factor B-like activity (six times that of Sanadi's factor B preparation in stimulating ATP-driven electron transfer from succinate to NAD as catalyzed by ammonia-EDTA particles). The preparation moved as a single protein band when filtered through Sephadex G-100 or electrophoresed on polyacrylamide gels. It also moved as a single band upon sodium dodecyl sulfate–acrylamide gel electrophoresis in the presence of 2-mercaptoethanol

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but was very faintly stained with Coomassie blue under the conditions employed. Its molecular weight as determined by gel filtration and sedimentation equilibrium studies was estimated to be 11–12 × 10^3. The purpose of the present communication is to show that the purified protein of You and Hatefi appears to contain an essential dithiol and that the factor B preparation of Sanadi and co-workers appears to contain the same active principle as the purified protein of You and Hatefi.

METHODS AND MATERIALS

The coupling protein factor of You and Hatefi and submitochondrial particles treated with NH_4OH and EDTA (AE-particles)[5] were prepared essentially according to the published procedures of these authors (5). The factor was stored at −70°C in a buffer composed of 50 mM Tris–sulfate, 1 mM EDTA, and 2 mM 1,4-dithiothreitol. The activity was quite stable to storage at −70°C and to repeated freeze–thawing. After repeated freeze–thawing and storage at −70°C for 3 months, the potency of the factor diminished only about 30%. Dodecyl sulfate–acrylamide gel electrophoresis was performed on 12.5% gels according to Weber and Osborn (6). ATP-Driven reverse electron transfer from succinate to NAD (1), ATP-driven transhydrogenation from NADH to 3-acetylpyridine adenine dinucleotide phosphate (AcPyADP) (7), ATP–$^{32}P_i$ exchange (8), and oxidative phosphorylation in the presence of $^{32}P_i$ (9) were carried out according to the references given. Double diffusion experiments were performed according to Ouchterlony and Nilsson (10).

Nicotinamide nucleotides were obtained from P-L Biochemicals, *p*-chloromercuribenzoate (*p*CMB) and ATP from Sigma, [^{32}P]phosphate from the New England Nuclear, phenylarsine oxide from Aldrich Chemical Company, and diamide (diazenedicarboxylic acid-bisdimethylamide) from Calbiochem. Dibutylchloromethyltin chloride was a gift of Dr. D. E. Griffiths, University of Warwick, Coventry.

RESULTS

Figure 1 shows the electrophoretic pattern of the factor preparation on 12.5% sodium dodecyl sulfate–acrylamide gels in the presence of 2-mercaptoethanol, using the procedure of Weber and Osborn (6). Sanadi and co-workers (1) and You and Hatefi (5) had stated that their respective preparations did not stain well with Coomassie blue when treated with sodium dodecyl sulfate, and the latter authors published the results of the gel electrophoresis of their preparation in the absence of sodium dodecyl sulfate. However, it is seen that even in the presence of the detergent, the factor preparation still moves as a single protein band with a mobility corresponding to M_r of 11–12 × 10^3, which is in agreement with the data of You and Hatefi (5). The staining problem of You and Hatefi appeared to be due to the use of sulfosalisylic acid in the fixation of protein on the gels. When the gels were prefixed in methanol/acetic acid, then stained with Coomassie blue in methanol/acetic acid, and destained in the fixing solution, the protein was stained as much as comparable amounts of other proteins with similar M_r.

Figure 2 shows the results of reactivation of AE-particles by the factor preparation for three different energy-linked reactions, namely ATP-driven electron transfer from succinate to NAD, ATP–P_i exchange, and ATP-driven transhydrogenation from NADH to the NADP analog 3-acetylpyridine adenine dinucleotide phosphate (AcPyADP). It is seen that all three reactions were stimulated by the factor essentially to the activity level of phosphorylating submitochondrial particles before extraction with NH_4OH and EDTA. The reduction of NAD by succinate was stimulated 15-fold, ATP–P_i exchange about eightfold, and transhydrogenation only 2.4-fold, because the residual transhydrogenase activity of AE-particles is high. This is due in part to nonenergy-linked transhydrogenation from NADH to AcPyNADP, which would not be expected to be diminished in AE-particles. Another reason is that stimulation of energy-linked transhydrogenation appears to require less energy than, for example, electron transfer from succinate to NAD (11–13). In addition to the experiments of Fig. 2, the ability of the factor to improve phosphorylation in AE-particles (in the absence of added F_1-ATPase) was tested with 3-hydroxybutyrate + NAD, succinate, and

[5] Abbreviations used: AE-particles, submitochondrial particles treated with NH_4OH and EDTA; AcPyADP, 3-acetylpyridine adenine dinucleotide phosphate; *p*CMB, p-chloromercuribenzoate; DBCT, dibutylchloromethyltin chloride.

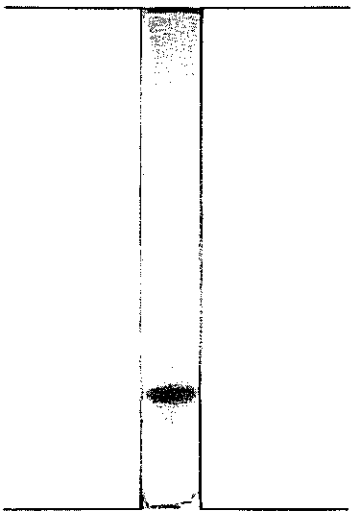

FIG. 1. Polyacrylamide gel electrophoresis of the purified factor in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. The factor (18 μg) was applied to a gel containing 12.5% acrylamide.

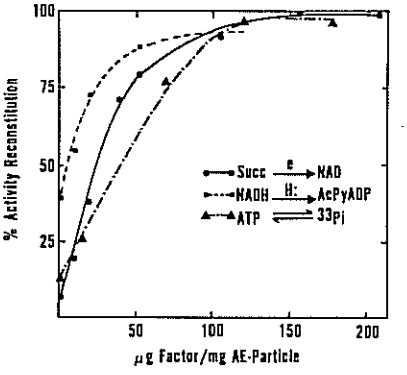

FIG. 2. Effect of the factor on the energy-linked activities of AE-particles. The activities was assayed according to the references indicated under Methods and Materials. The ordinate gives the percentage of the respective activities of phosphorylating submitochondrial particles (ETP_H) not treated with NH_4OH + EDTA. The activities of ETP_H taken as 100% were as follows: ATP-driven succinate reduction of NAD (●), 420 nmol/min/mg; ATP-driven transhydrogenation from NADH to AcPyADP (■), 160 nmol/min/mg; ATP–$^{32}P_i$ exchange (▲), 120 nmol/min/mg.

NADPH as substrates. In all cases, P/O ratios were increased upon addition of the factor preparation from about 2.5-fold in the case of succinate to more than threefold in the case of NADPH as substrate.

As shown in Fig. 3, and in agreement with the previous results of Shankaran *et al.* (3) and You and Hatefi (5), the factor contains essential thiols, since its activity is abolished by mercurials. The mercurial inhibition was shown elsewhere (5) to be reversible when the inhibited preparation was treated with dithiothreitol. In addition, Fig. 3 shows that the factor is also inhibited by treatment with Cd^{2+}, phenylarsine oxide, and diamide (diazinedicarboxylic acid bisdimethylamide). Cd^{2+} and phenylarsine oxide are known inhibitors of proteins containing vicinal dithiols, and diamide is a reagent which oxidizes dithiols to disfulfides (4). In agreement with these results, it was shown by You and Hatefi (5) that the factor loses activity when aged under aerobic conditions, and that treatment with dithiothreitol results in reactivation.

Higashiyama *et al.* (15) have reported the isolation of a preparation (F_B) with factor B-like activity, a molecular weight of 360,000, subunit molecular weight of 43,500, and an amino acid composition different from that of Sanadi's factor B preparation. F_B was prepared twice in our laboratory according to the procedure of Higashiyama *et al.*, and both preparations were found to be completely ineffective in stimulating the energy-linked reactions of our AE-particles. Sanadi and co-workers have concluded from enzymatic and immunological studies that the low stimulatory effect of F_B is unrelated to factor B and appears to arise from the presence of dithiothreitol in F_B preparations (16). In our experience, stimulation by dithiothreitol was found to be related to the state of the AE-particles. If the AE-particles were prepared such that they exhibited high residual activities for the various energy-linked functions described above, then addition of dithiols such as dithiothreitol resulted in partial stimulation of activity. However, when the extraction of submitochondrial particles with NH_4OH and EDTA was repeated until the energy-linked activities were diminished to very low levels, then various dithiol compounds exhibited no stimulatory effect (Table I). Another point that should be added in this

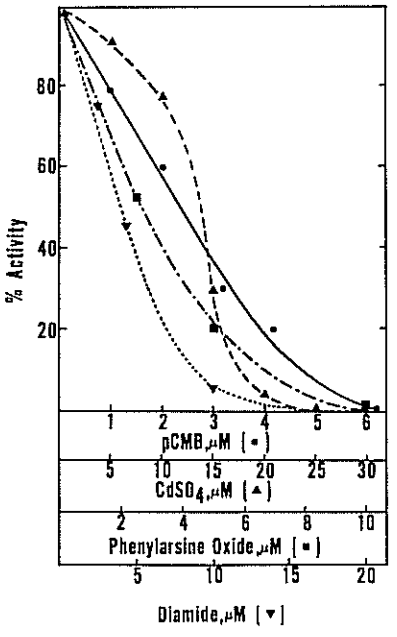

FIG. 3. Effects of *p*-chloromercuribenzoate (*p*CMB), $CdSO_4$, phenylarsine oxide, and diamide on the stimulation by the factor of ATP-driven NAD reduction by succinate as catalyzed by AE-particles. *p*CMB experiment: 3.7 μg factor (5 μl) was incubated in a 1-ml cuvette at room temperature with the indicated amounts of *p*CMB in ethanol for 1 min, then buffer (containing succinate, $MgCl_2$ and bovine serum albumin), ATP, KCN, and 110 μg AE-particle were added, and incubated for 2 min at 38°C. Reaction was started by the addition of NAD. $CdSO_4$ experiment: 3.7 μg factor was incubated with the indicated amounts of $CdSO_4$ for 3 min at 0°C, then 15 s at 38°C before other additions and initiation of the reaction as above. Phenylarsine oxide experiment: 20 μl factor (22.4 μg protein) was incubated at 0°C with 0.5 to 2 μl of 20 mM inhibitor dissolved in dimethylformamide. Then a volume containing 5 μg factor was added to a reaction mixture as above and incubated for an additional 1 min at 38°C before starting the reaction by additon of NAD. Diamide experiment: 20 μl factor (22.4 μg protein) was incubated at 0°C with 0.5 to 4 μl of 20 mM diamide in ethanol, then a volume containing 5 μg factor was used for assay as in the preceding experiment. Controls with ethanol or dimethylformamide added to the factor and incubated as above, or with inhibitor added at the corresponding diluted concentrations to the reaction mixture containing AE-particles were also performed. Any inhibitory effects, which were marginal, and the residual activity of AE-particles were substrated from the results shown in the figure.

regard is the effect of dithiol compounds on reversing the inhibition of mitochondrial energy-linked functions caused by dibutylchloromethyltin chloride (DBCT). According to Cain *et al.* (17), the inhibition by DBCT is specifically relieved by dihydrolipoate. Our results showed that while dihydrolipoate was a highly effective agent in reversing the inhibition of ATP-driven NAD reduction by succinate and transhydrogenation from NADH to AcPyADP, dihydrolipoamide and dithiothreitol were also effective. These results are consistent with the possibility that DBCT reacts with the dithiol protein discussed above, and reversal by dithiol compounds is due to transfer of DBCT to the added dithiols.

The similar coupling factor properties of our preparation and Sanadi's factor B prompted a comparison of their immunologic responses. Figure 4 shows immunoprecipitation of our dithiol protein by serum containing factor B antibody (kindly supplied by Dr. Sanadi), and Table II shows data on the inhibition by factor B antibody of ATP-driven NAD reduction by succinate as catalyzed by AE-particles in the presence of our factor preparation. It is seen that serum containing factor B antibody resulted in considerable inhibition of activity, while normal serum at comparable and higher levels was completely without effect.

TABLE I

EFFECTS OF DITHIOL COMPOUNDS ON THE ATP-DRIVEN REDUCTION OF NAD BY SUCCINATE AS CATALYZED BY AE-PARTICLES

Addition	Concentration in assay	Specific activity (nmol/min/mg)
None		32.2
Dithiothreitol	0.5 mM	31.3
Dihydrolipoate	1.0 mM	19.0
Purified factor	3.7 μg/ml	277

[a] Assay mixtures contained 0.11 mg AE-particle in 1.0 ml. Reactions were started with the addition of NAD after all other components had incubated together for 2 min at 38°C. Specific activity is defined as in Fig. 2.

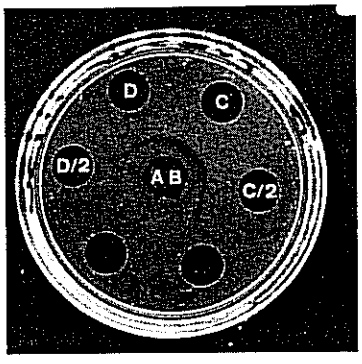

FIG. 4. Ouchterlony double diffusion plate of the purified factor and antifactor B antibody. AB, 100 μl of 10 mg/ml anti-factor B serum; C and C/2, 100 and 50 μl of 0.2 mg/ml purified factor; D and D/2 100 and 50 μl of 0.7 mg/ml factor at the penultimate stage of purification. The gel was treated with Coomassie blue to stain the proteins.

TABLE II

EFFECT OF ANTI-FACTOR B ANTIBODY ON STIMULATION BY PURIFIED FACTOR OF ATP-DRIVEN ELECTRON TRANSFER FROM SUCCINATE TO NAD

Conditions	Activity[a]	Inhibition (%)
AE-Particles	14.4	—
AE-Particles + factor (2-min incubation)	151	0
AE-Particles + factor + 50 μg anti-factor B (2-min incubation)	86	43
AE-Particles + factor + 100 μg anti-factor B (2-min incubation)	68.5	55
Factor + 100 μg anti-factor B (1-min incubation) + AE-particles (1-min incubation)	56.2	63

[a] Activity is expressed as in Fig. 2. Incubations as indicated were carried out at 38°C in 1 ml spectrophotometric cells in the presence of buffer and other components of the assay mixture (see Ref. (1)), except NAD. The amounts of AE-particle and factor in the reaction mixtures were, respectively, 110 and 4 μg. Reaction was started by addition of NAD.

The following criteria lead, therefore, to the conclusion that the active principle in factors F_2 and B, but not in F_B of Higashiyama *et al.* (15), is the protein purified by You and Hatefi with a molecular weight of $11-12 \times 10^3$: (a) Factors F_2 and B, and the present preparation stimulate the energy-linked reactions of AE-particles; (b) factors F_2 and B, and the present preparation are inactivated by anti-factor B serum; and (c) factor B and the present preparation are inhibited by thiol reagents.

DISCUSSION

Previous studies from this laboratory had shown that a water-soluble protein of molecular weight $11-12 \times 10^3$ purified from beef heart mitochondria restores various energy-linked functions to submitochondrial particles rendered deficient by extraction with 1 M NH_4OH and 0.6 mM EDTA (5). Sephadex gel filtration and electrophoresis on polyacrylamide (in the absence of sodium dodecyl sulfate) indicated that the protein preparation was essentially pure. In addition, it was shown that the activity of the protein is reversibly inhibited by mercurials, and irreversibly by *N*-ethylmaleimide. These results were confirmed and extended in the present studies, using a preparation which appeared to be >95% pure when subjected to acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol.

Except for the lower molecular weight and the higher activity of the above preparation, its characteristics are similar to two factor B preparations from Sanadi's laboratory with reported molecular weights of 29,200 and 47,000, and to coupling factor F_2 of Racker's group (18). The results shown above suggest that factors B, F_2, and the purified protein of You and Hatefi are all related, because antibody raised against factor B by Sanadi inhibits F_2 and precipitates and inhibits our preparation. Thus, it appears that the above coupling factor preparations contain the same active component. Furthermore, the lower molecular weight, higher activity, and the high purity of the preparation of You and Hatefi

(5) suggest that this is the active component in the preparations designated factors B and F_2.

The finding in the present study that our factor preparation is inhibited by Cd^{2+}, phenylarsine oxide, and diamide suggests that the protein contains an essential dithiol. Since the molecular weight of our preparation is close to half that of Sanadi's factor B, the presence of a dithiol could be interpreted in two ways. One possibility is that the factor in solution is dimeric, each molecule has one essential thiol, and in the dimer the respective monothiols of each molecule are located close to each other. This interpretation agrees with the view of Sanadi and his colleagues. They state that their original factor B has one tryptophan per 14,600 daltons, a molecular weight of 29,200 (estimated only by sucrose density gradient centrifugation), and two thiols per mole. If this is so, then their new factor B preparation with a molecular weight of $46-47 \times 10^3$, again estimated by sucrose density gradient centrifugation against a single marker (3), could be a trimer, with each monomer being comparable to our preparation.

A second possibility is that the dithiol is located on the same molecule. This is more in agreement with the results of You and Hatefi on the molecular weight of their factor preparation. Both sedimentation equilibrium and gel filtration (Sephadex G-100) studies indicated a molecular weight of $11-12 \times 10^3$ (5). If the latter interpretation is correct, then one must ask whether the dithiol arises from two cysteine residues of the protein or from an unknown dithiol compound attached to the protein. Recent publications (19–21) regarding the involvement of a dithiol compound (specifically dihydrolipoate) in oxidative phosphorylation have not been confirmed by others (22, 23). However, there is earlier indication in the literature regarding the apparent uncoupling of oxidative phosphorylation in rat liver mitochondria by the dithiol reagents γ-(*p*-arsenophenyl)-*n*-butyrate and Cd^{2+} (24, 25). Whether the essential dithiol of our protein is due to cysteine residues or otherwise, it is clear that a vicinal dithiol associated with a water-soluble, low molecular weight protein is involved in energy transfer to and from ATP. Results presented here and previously (5) also indicate that in the process of energy transfer to and from ATP the dithiol does not undergo cyclic reduction and oxidation, because its oxidized form is inactive. This consideration makes the possible conversion of the thiols during energy transfer to sulfenyl (RS+) group(s) not very attractive.[6] However, as nucleophiles (RS^-) they might very well be involved in oxidative phosphorylation, for example, in proton transfer or possibly even in the formation of labile structures with other components of the ATP synthetase system. Finally, it might be added that McKinney *et al.* (26, 27) have shown that reduced thioredoxin isolated from chloroplasts stimulates the activity of soluble chloroplast F_1-ATPase by twofold, and that the isolated δ subunit of the ATPase has a similar effect on the activity of heat-activated chloroplast F_1. Whether the isolated δ subunit of F_1-ATPase from beef heart mitochondria might activate factor B-deficient particles remains to be investigated. However, our preparation of the factor has no effect on the ATPase activity of AE-particles (5), and the available data show no (28, 29) cysteine in the δ subunit of beef heart F_1-ATPase. Furthermore, the molecular weight of this subunit is higher than that of our factor, and the two polypeptides exhibit clearly different mobilities on sodium dodecyl sulfate–acrylamide gels.

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[6] One of us (R.K.) has investigated the possible involvement of sulfenyl groups in oxidative phosphorylation, using the membrane-permeable sulfenyl reagent *N*-*n*-nonyl-*N'*-phenylthiourea. This compound has no effect on our dithiol-containing protein, but when added to mitochondria or Complex V it interacts with the uncoupler binding site and acts as a weak uncoupler.

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