BBA 3983

A SOLUBLE FACTOR FROM MITOCHONDRIA STIMULATING THE RATE OF THE ENERGY-REQUIRING PYRIDINE NUCLEOTIDE REDUCTION

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(Received September 14th, 1962)

SUMMARY

1. The partial purification of a mitochondrial factor which stimulates the energy-requiring reduction of pyridine nucleotides in sonic particles from beef-heart mitochondria is described.

2. Evidence is presented that this factor makes cytochrome *b* more accessible to reducing equivalents when succinate or *p*-phenylenediamine are used as electron donors.

INTRODUCTION

It has been shown by a number of investigators[1-6] that upon mechanical fragmentation of beef-heart mitochondria soluble factors are released, which are necessary for the process of oxidative phosphorylation. Except for the studies reported by SANADI *et al.*[6,7], the experiments have been carried out in terms of enhancement of P:O ratios, *i.e.* measurement of ATP synthesis coupled to the oxidation of a substrate.

This communication deals with the partial purification of a mitochondrial factor which increases the rate of the energy-requiring NAD^+ reduction in submitochondrial particles. The participation of such a factor in this reaction has been described earlier by SANADI *et al.*[6,7].

In agreement with the findings of RACKER[8] and SMITH AND HANSEN[9], it was found that submitochondrial particles prepared in the absence of Mg^{2+} and ATP showed a much lower phosphorylating activity. This is associated, as indicated by the present study, with a decrease in the rate of the succinate-linked NAD^+ reduction. After addition of a factor purified from the 100000 $\times$ *g* supernatant fluid the activity could be partly restored.

In a previous communication a lag phase in the succinate-linked NAD^+ reduction has been described[10]. Evidence is presented here that this purified factor which stimulates the rate of NAD^+ reduction also abolishes this lag phase.

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MATERIALS AND METHODS

Beef-heart mitochondria were prepared according to CRANE, GLENN AND GREEN[11], but modified for small-scale preparation.

Submitochondrial particles were prepared by sonication of a beef-heart mitochondrial suspension in 0.25 M sucrose at pH 7.2–7.5 employing a protein concentration of about 20 mg/ml. A MSE sonic oscillator was used, operating at maximum tuning, at an energy input of 1.5 A, for 2–5 min at 0°. The beef-heart mitochondria were kept frozen for at least 2 weeks before use.

Unbroken mitochondria were removed by centrifugation in a Servall centrifuge, model RC-2, rotor SS34, at 12000 rev./min for 10 min at 0°. The supernatant fluid was decanted and centrifuged for 1 h in Spinco model L centrifuge, rotor 40, at 40000 rev./min at 0°. The resulting supernatant fluid was decanted and stored for the preparation of the mitochondrial factor. The dark-brown precipitate was suspended in 0.25 M sucrose to a final protein concentration of about 40 mg/ml.

The succinate-linked NAD^+ reduction was measured as described previously[10].

The kinetics of cytochrome *b* reduction were studied employing the double-beam spectrophotometric technique as described by CHANCE[12].

In order to avoid the introduction of solubilized substrates and low-molecular-weight cofactors from the active supernatant fluid in the test system, fractions 3 or 4, as described below, were used throughout this study. It was assumed that after ammonium sulfate fractionation and passage through Sephadex G-25 negligible amounts of low-molecular-weight compounds were present in the solution of mitochondrial factor.

Difference spectra were recorded employing the wavelength-scanning recording spectrophotometer described by YANG[13].

Protein concentrations were determined by the biuret method, using bovine serum albumin as a standard. All chemicals used were of analytical grade. The enzymes malate dehydrogenase, lactic dehydrogenase and alcohol dehydrogenase were purchased from Boehringer and Soehne (Mannheim, Germany).

EXPERIMENTAL

Purification of mitochondrial factor

A schematic representation of the purification procedure used for the preparation of the mitochondrial factor which stimulates the rate of the succinate-linked NAD^+ reduction is presented in Fig. 1. This may be briefly described as follows:

(1) *Preparation of crude extract*: The crude extract is the supernatant obtained from the high-speed centrifugation in the preparation of the submitochondrial particles as described above.

(2) *pH fractionation*: The crude extract was adjusted to pH 5.7 by careful addition of 0.1 N HCl, under ice cooling and gentle mechanical stirring. The mixture was centrifuged for 3 min at 10000 rev./min at 0°. The sediment was discarded and the pH of the supernatant immediately readjusted to pH 7.2 by careful addition of 0.1 N KOH. This has to be carried out rather fast. Below pH 5.5 the mitochondrial factor is rapidly inactivated, although not precipitated at this pH. Readjustment of the pH-5.7 supernatant to pH 7.5 has therefore to be done immediately.

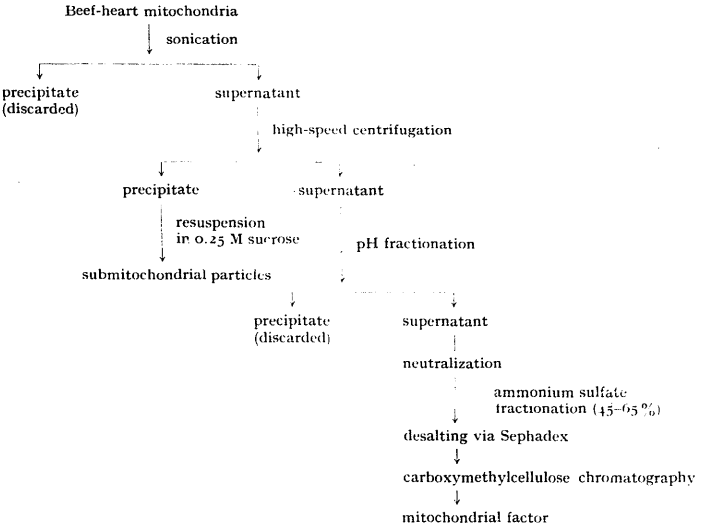

Fig. 1. Scheme for the partial purification of a soluble mitochondrial factor, stimulating the rate of the succinate-linked NAD^+ reduction in submitochondrial particles.

(3) *Ammonium sulfate fractionation*: Solid ammonium sulfate was added (277 mg per ml of the neutralized pH-5.7 supernatant) to give a final ammonium sulfate concentration of 45 % satn. The pH was readjusted by careful addition of 0.1 N KOH to pH 7.0 and the turbid suspension was centrifuged for 10 min at 10000 rev./min at 0°. The supernatant was decanted and brought to 65 % ammonium sulfate by the addition of 134 mg of solid ammonium sulfate per ml of supernatant. The mixture was centrifuged as above and the precipitate redissolved in a minimum amount of 0.25 M sucrose–0.005 M triethanolamine (pH 7.5). For desalting a Sephadex G-25 (1.5 × 10 cm) was used. The Sephadex was previously equilibrated with the sucrose–triethanolamine buffer. The proteins were eluted with the sucrose–triethanolamine buffer.

(4) *Carboxymethylcellulose chromatography*: The Sephadex G-25 eluate was applied to a carboxymethylcellullose column (1 × 12 cm), previously equilibrated with 0.005 M potassium phosphate buffer (pH 7.5). The proteins were eluted with the same buffer at a flow rate of 25 ml/h. Fractions of 5 ml were collected with the aid of an automatic fraction collector. The chromatography was carried out at 0°–4°.

The elution diagram of the carboxymethylcellulose chromatography is given in Fig. 2. Most of the proteins are not absorbed under the given conditions and are eluted at the column volume (Peak 1). The activity is recovered in Peak 2, emerging

from the column just behind Peak I. At this stage the factor is extremely thermolabile: 10 min at room temperature is sufficient to inactivate it completely.

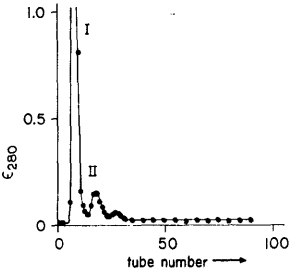

Fig. 2. Chromatography of Sephadex G-25 eluate on carboxymethylcellulose (Whatman 70 powder). Column dimensions 1 × 12 cm. Eluate: 0.005 M potassium phosphate (pH 7.5). Flow rate 25 ml/h, collected in 5-ml fractions.

Definition of unit and specific activity

A unit of mitochondrial factor is defined as that amount of protein which increases the rate of succinate-linked NAD^+ reduction by 1 fluorescence unit per min. Specific activity is defined as units/mg of protein.

A summary of the purification procedure is given in Table I.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

	Volume (ml)	*Protein (mg)*	*Units*	*Specific activity*	*Yield (%)*
1. Crude extract	180	1534	14 400	9.4	(100)
2. pH-5.7 supernatant	190	958	38 000	39.7	264
3. Sephadex eluate	10	167	12 000	71.8	84
4. Carboxymethylcellulose chromatography	14	5.2	8 660	1646	60

RESULTS

As demonstrated earlier, a pronounced lag phase in the succinate-linked NAD^+ reduction was observed with submitochondrial particles[10].

Fig. 3A shows this phenomenon for the submitochondrial particles prepared in the absence of Mg^{2+} and ATP. Addition of the 100000 × *g* supernatant increases the rate more than 2-fold, while the lag phase has almost completely disappeared (Fig. 3B).

Sometimes it was observed that the crude extract did not stimulate the rate of NAD^+ reduction at all, although in those cases no lag phase in the NAD^+ reduction was observable. This may be due to the presence of an inhibitor. The presence of such an inhibitor was demonstrated by heat denaturation. In one series of experiments a partially purified sample of the mitochondrial factor was subjected to heat denatu-

ration by immersing in a constant-temperature water bath at 65°. After varying times of incubation, aliquots were withdrawn and immediately cooled in ice. The samples were centrifuged in the cold to remove insoluble proteins and the supernatant fluid tested for activity. Table II shows the result of such an experiment. A decrease in activity was observed, finally going below the rate of the control. Prolonged heating restored, however, the original activity.

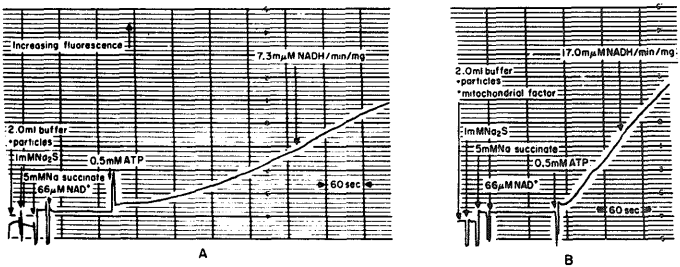

Fig. 3. Kinetics of NAD+ reduction by succinate and ATP in submitochondrial particles. NADH formation was followed fluorometrically. The submitochondrial particles were suspended in 2 ml of buffer (80 mM KCl, 3 mM $MgCl_2$, 10 mM triethanolamine–HCl, pH 7.5). Subsequently Na_2S, sodium succinate, NAD+ solution and ATP were added in the order indicated to final concentrations as indicated in the figure (A). B, the same in the presence of mitochondrial factor.

TABLE II

EFFECT OF HEATING OF A SOLUTION OF THE MITOCHONDRIAL FACTOR ON THE STIMULATION OF THE RATE OF THE SUCCINATE-LINKED NAD+ REDUCTION

Time of incubation at 65 (min)	*Rate of NAD+ reduction (fluorescence units/min)*	*Protein concentration of incubate after centrifugation of insoluble proteins (mg/ml)*
0	16	11.0
2	13	6.6
3	11	5.7
4	12	4.6
5	8.5	3.5
7	6	2.7
10	6	2.6
30	10	0.1
Control, without mitochondrial factor	10	

A definition of unit and specific activity is only meaningful if the relation between the unit and activity is a linear one. Fig. 4 shows that this is true for the above given definition of the unit if suboptimal amounts of mitochondrial factor are used. The units referred to in Table I were measured in the first part of this biphasic curve. As indicated a saturation level is reached at higher levels of mitochondrial factor. This saturation concentration depends on the endogenous amount of factor

retained by the submitochondrial particles. This varies, however, from preparation to preparation. Therefore the units and specific activities do not have absolute significance. The different fractions obtained during the purification were tested, however, on the same day with the same sample of submitochondrial particles. So a direct comparison in a single series of experiments can be made.

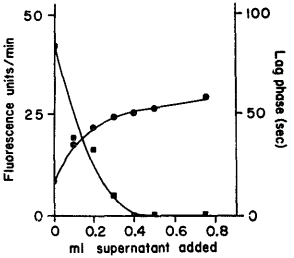

Fig. 4. Relation between the amount of supernatant added and the rate of NAD+ reduction, measured in arbitrary fluorescence units (●—●) and the duration of the lag phase in seconds (■—■). Experimental conditions as in Fig. 3.

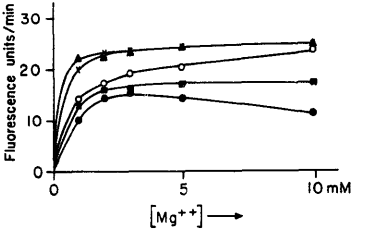

Fig. 5. Relation between the rate of NAD+ reduction measured in arbitrary fluorescence units and the Mg^{2+} concentration, at different concentrations of mitochondrial factor: O—O, without mitochondrial factor; ▲—▲, 5 units added; ×—×, 10 units added; ■—■, 20 units added; ●—●, 30 units added. Experimental conditions as in Fig. 3.

Fig. 4 also shows the decrease in lag phase with increasing concentration of mitochondrial factor. Under conditions in which maximum activity is obtained, the lag phase has completely disappeared. The association of an increase in NAD+ reduction and a decrease in lag period was observed in all fractions obtained in the purification procedure, *i.e.* both activities were purified together. The inverse relationship between the duration of the lag phase and the rate of NAD+ reduction suggests that the same factor is responsible for both effects.

The form of the curve, as shown in Fig. 4, depends, however, on the Mg^{2+} concentration. Fig. 5 shows the rates of the succinate-linked NAD+ reduction as a function of the Mg^{2+} concentration at different concentrations of the mitochondrial factor. It is noteworthy that maximum rates observed in the presence of mitochondrial factor are obtained at a much lower Mg^{2+} to ATP ratio than in the absence of mitochondrial factor (2:1 as compared to 10:1). Furthermore in the presence of excess mitochondrial factor, excess Mg^{2+} inhibit. Mg^{2+} was also found to be inhibitory for the succinate-linked NAD+ reduction in intact pigeon-heart mitochondria[14].

Before accepting this mitochondrial factor as a new entity, one has to prove that it is not identical to a known mitochondrial enzyme. It has been suggested that the succinate-linked NAD+ reduction is not a reversal of oxidative phosphorylation but that it is due to malate dehydrogenase activity[15]. Although conclusive evidence has been given against this suggestion[16–18], this possibility cannot be ruled out in a reconstituted system, the more so as the enzyme malate dehydrogenase is not present in the submitochondrial particles and thus is presumably present in the 100000 × *g* supernatant from which the mitochondrial factor is purified.

It was found that the partly purified mitochondrial factor was still contaminated with malate dehydrogenase. An increase in fluorescence was observed when malate was added to a solution containing supernatant factor and NAD+. The same effect was observed when lactate, methanol, ethanol, propanol-1 or propanediol-1,2 were added. No increase in rate of the succinate-linked NAD+ reduction in the submitochondrial particles was observed, however, when purified malate dehydrogenase, lactate dehydrogenase or alcohol dehydrogenase was added to the system, nor was a decrease in the lag phase observed. The ATP-dependent cytochrome *b* reduction was not affected by these enzymes (see below).

It has been shown that the point of inhibition by Amytal shifts from between pyridine nucleotide and flavin in intact mitochondria to a point after flavin in non-phosphorylating heart-muscle preparations during the oxidation of NAD+-linked substrates[19,20]. In the first case only the NADH peak is seen in the difference spectra, while in the latter case in addition to the NADH peak the flavin trough is observed. Furthermore in the latter case a peak with a maximum at about 430 mμ is often observed. This same pattern was also observed in the submitochondrial particles used here, when Amytal or Rotenone[27,28] were used as inhibitors and NADH or β-hydroxybutyrate and NAD+ were used as electron donor. Addition of the supernatant factor restored the situation as observed in intact mitochondria in that the peak with a maximum at 430 mμ disappeared gradually when increasing amounts of supernatant factor were added[21].

When the same type of experiments were carried out for the reverse reaction, *i.e.* when sulfide and succinate were added to the reference and measuring cuvette in the presence of Rotenone, addition of ATP gave rise to a peak with a maximum at 430 mμ (Fig. 6, curve A). Addition of mitochondrial factor, however, did not show a change in the difference spectrum (Fig. 6, curve B) (*cf* ref. 22).

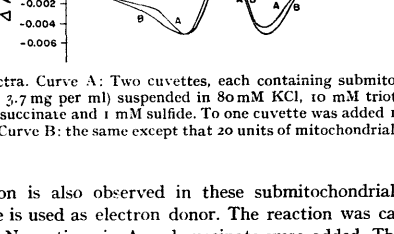

Fig. 6. Difference spectra. Curve A: Two cuvettes, each containing submitochondrial particles (protein concentration 3.7 mg per ml) suspended in 80 mM KCl, 10 mM triethanolamine, 2 mM $MgCl_2$ (pH 7.5), 5 mM succinate and 1 mM sulfide. To one cuvette was added 10 μl 0.1 M ATP, to the other 10 μl buffer. Curve B: the same except that 20 units of mitochondrial factor were added.

NAD+ reduction is also observed in these submitochondrial particles when *p*-phenylenediamine is used as electron donor. The reaction was carried out in the presence of sulfide. No antimycin A and succinate were added. This system differs thus from the systems described by SLATER *et al.*[23] and PACKER AND DENTON[24]. The rates are usually one tenth of the succinate rate, measured under otherwise identical conditions, with a concomitant increase in lag phase. As reported earlier[10], an ATP-dependent cytochrome *b* reduction, as measured at 564–575 mμ, was observed.

It was found that the rate of this ATP-dependent cytochrome *b* reduction was doubled in the presence of mitochondrial factor.

Upon anaerobiosis in succinate oxidation by the submitochondrial particles a certain percentage of cytochrome *b* was reduced, as measured at 564–575 mμ. Addition of mitochondrial factor increased the amount of succinate-reducible cytochrome *b* upon anaerobiosis (Table III) at the expense of the total amount of cytochrome *b* (the total amount of cytochrome *b* is defined here as that amount reducible upon addition of dithionite to the sample). Amytal did not inhibit this increased reduction. The amount of Amytal added was sufficient to inhibit NADH oxidation, as addition of NADH did not give rise to an increase in cytochrome *b* reduction when the system was anaerobic, as was observed when Amytal was omitted from the system.

TABLE III

EFFECT OF MITOCHONDRIAL FACTOR ON THE EXTENT OF CYTOCHROME *b* REDUCTION UPON ANAEROBIOSIS IN SUCCINATE OXIDATION

The submitochondrial particles were suspended in buffer containing 80 mM KCl, 10 mM triethanolamine, 3 mM $MgCl_2$ (pH 7.5) to a final protein concentration of 1.7 mg/ml. Cytochrome *b* was measured at the wavelength pair 564–575 mμ.

	Cytochrome b reduced upon anaerobiosis in succinate oxidation		*Total cytochrome b reduced after addition of* $S_2O_4^{2-}$
	(mμmoles/mg protein)	*(%)*	*(mμmoles/mg protein)*
No addition	0.162	38.5	0.422
+ 4 units mitochondrial factor	0.200	48.1	0.416
+ 8 units mitochondrial factor	0.246	62.3	0.395
+12 units mitochondrial factor	0.289	70.9	0.409
+ 8 units mitochondrial factor +3 mM Amytal	0.243	63.3	0.385

DISCUSSION

The increase in cytochrome *b* reduction upon anaerobiosis in succinate oxidation in the presence of mitochondrial factor suggests that the factor makes cytochrome *b* more accessible to reducing equivalents from succinate. Evidence has been presented earlier that cytochrome *b* is involved in the succinate-linked NAD^+ reduction in these submitochondrial particles[10]. In agreement with this concept is the observed increased rate of the ATP-dependent cytochrome *b* reduction in the presence of mitochondrial factor when *p*-phenylenediamine is used as electron donor. In this case the generation of malate from succinate is completely avoided.

It has been shown by SMITH *et al.*[25] that the properties of cytochrome *b* can be drastically changed when cytochrome *b* complexes with structural protein. However, a comparison of the methods of isolation of structural protein[26] and the mitochondrial factor described here shows some significant differences. Structural protein is precipitated at an ammonium sulfate concentration of 12% satn., while the mitochondrial factor described here is precipitated between 45 and 65% satn. Structural protein seems to be stable at high or low pH values, which is not the case for the mitochondrial factor.

The mitochondrial factor described here is not cold labile, does not precipitate with protamine sulfate and has no ATPase activity. It is therefore unlikely that it is identical to the soluble ATPase described by RACKER *et al.*[3]. A possible identity to one of the factors announced by SMITH AND HANSEN[5] cannot be excluded but has to await until more is known about their methods of purification. The factor may be identical to the factor described by SANADI *et al.*[6].

ACKNOWLEDGEMENTS

The author is deeply indebted to Prof. Dr. B. CHANCE and Dr. R. W. ESTABROOK for many helpful suggestions during the course of experimentation and preparation of the manuscript. Thanks are due to Drs. D.R. SANADI and A. L. FLUHARTY for valuable discussion.

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