PURIFICATION OF A FACTOR FOR BOTH AEROBIC-DRIVEN AND ATP-DRIVEN ENERGY-DEPENDENT TRANSHYDROGENASES OF *ESCHERICHIA COLI*

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1. Introduction

We previously reported that a soluble protein fraction from *Escherichia coli* extracts would enhance the ATP-driven energy-dependent transhydrogenase of this organism [1]. Subsequently, Fisher et al. [2] stimulated the aerobic-driven reaction in this organism with a factor prepared from rat liver. In this paper we report the isolation from *E. coli* of a factor in near homogeneous form which stimulates both the aerobic- and the ATP-driven energy-dependent transhydrogenase reactions.

2. Experimental

2.1. *Purification of coupling factor*

All procedures were carried out at 0°–4°. Respiratory particles were prepared as previously described from 35 g late exponential phase *E. coli* (strain 482 of the culture collection of the National Research Council of Canada) which had been grown on a minimal salts–glucose (0.4%) medium supplemented with 12 μM ferric citrate [3]. The respiratory particles were suspended in 90 ml 0.05 M Tris-H_2SO_4 buffer, pH 7.8, containing 10 mM $MgCl_2$, and sedimented by centrifugation (120,000 *g*; 2 hr). The washed particles were suspended in 30 ml 1 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA, 0.1 mM dithiothreitol (DTT), and 10% (v/v) glycerol, and dialysed overnight against 1.5 l of the same buffer. The dialysed particle suspension was centrifuged (120,000 *g*; 2 hr) to yield a pellet of "stripped" particles and the supernatant fluid which contained the crude coupling factor. The stripped particles were stored at 4°, for not over 5 days, for subsequent assay of coupling factor activity. The supernatant fluid (29.5 ml) was diluted to 40 ml with the same buffer and then recentrifuged as before to removal residual particulate material. The upper, clear part of the supernatant fluid (29.5 ml) was concentrated to 4.3 ml by ultrafiltration through a PM 10 membrane (Amicon Corp.). The concentrated liquid (4.0 ml) was applied to a column of DEAE-cellulose (DE-52) (2 × 5 cm) equilibrated with 2 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM DTT and 10% (v/v) glycerol. The column was eluted successively with 15 ml portions of 2 mM, 100 mM, 150 mM, 200 mM, 250 mM and 500 mM Tris-HCl buffer, pH 7.2, containing 0.1 mM DTT and 10% glycerol to give fractions 1 to 6, respectively. Fractions 4 and 5, which contained most of the coupling factor activity (fig. 1A), were pooled and concentrated by ultrafiltration to 3.3 ml. The concentrated fraction (3.0 ml) was applied to a column of Sepharose 6B (2.5 × 35.5 cm) equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.5 mM EDTA, 0.1 mM DTT, and 10% glycerol, and elution carried out with the same buffer. Fractions of 5.0 ml were collected. Fractions 20–22, which contained most of the coupling factor activity (fig. 1B), were pooled and concentrated by ultrafiltration to 0.6 ml. Part of this solution (0.4 ml) was placed over a discontinuous sucrose gradient prepared from successive 1 ml layers of 25%, 22.5%, 20%, 17.5% and 15% (w/v) sucrose in 0.05 M Tris-H_2SO_4 buffer, pH 7.8, containing 0.5 mM EDTA and 0.1 mM DTT. The solution was centrifuged in a Beckman SW 50L rotor for 15.5 hr at 45,000 rpm. Fractions (0.4 ml) were collected by puncturing a hole in the bottom of the tube. The coupling factor activity was found in fractions 3 and 4 only (fig. 1C).

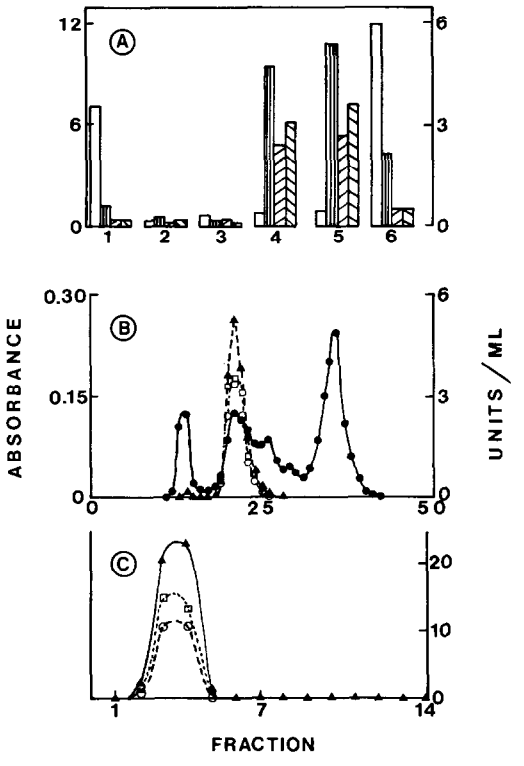


Fig. 1. Purification of coupling factor on (A) column of DEAE-cellulose, (B) column of Sepharose 6B, (C) sucrose gradient. See Experimental for details of methods. The absorbance was measured at 280 nm using cells with 1 cm path length. The units are expressed in μmoles substrate converted per ml of fraction. In (A) the four bars in each fraction represent from the left, absorbance, ATPase, aerobic-driven and ATP-driven transhydrogenase activities, respectively. In (B) and (C) absorbance, ATPase, aerobic-driven and ATP-driven transhydrogenase activities are represented by ●, ▲, ○ and □, respectively.

2.2. *Assay methods*

Protein, Ca^{2+}-activated ATPase, aerobic- and ATP-driven energy-dependent transhydrogenase activities were measured as described previously [3]. The stripped particles were suspended in the medium used before [3]. When coupling factor was added to the stripped particles it was included during the preincubation period. The rate of $NADP^+$ reduction in the presence of coupling factor was corrected for the rate of $NADP^+$ reduction in its absence. One unit of coupling factor activity is defined as that amount of coupling factor which causes a net increase in the rate of $NADP^+$ reduction of 1 μmole per min.

2.3. *Gel electrophoresis*

Disc gel electrophoresis was performed on 5% polyacrylamide gels prepared and run in 0.05 M Tris-glycine buffer, pH 8.7. The gels were prerun for 20 min at 2.5 mA per tube before the sample was applied. For electrophoresis on 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS), the protein sample was depolymerized at 100° in 4 M urea–1% SDS–1% mercaptoethanol solution, and run in system 1 as described previously [4]. The protein bands were stained with Coomassie blue.

3. Results and discussion

Stripped particles did not carry out either the aerobic- or the ATP-driven energy-dependent transhydrogenase reaction unless they were supplemented with the soluble proteins which had been removed previously from the particles (fig. 2). The soluble proteins were fractionated to purify the protein(s) responsible for the restoration of the energy-dependent transhydrogenase activity. The coupling factor activities for both the aerobic-driven and ATP-driven transhydrogenase, and the Ca^{2+}-dependent ATPase activities fractionated together during successive purification steps involving ion-exchange on DEAE-cellulose, gel-filtration on Sepharose 6B, and centrifugation through a sucrose gradient (fig. 1). The purification sequence is summarized in table 1. The coupling factor activity was difficult to quantitate since it was dependent also on the activity of the stripped particle fraction. The amount of factor required to obtain the maximum rate of transhydrogenase activity varied somewhat with different preparations of the stripped particles. The fractions from the sucrose gradient having both coupling factor and ATPase activity consisted mainly of one protein (fig. 3). Thus, a single protein stimulates both the aerobic- and the ATP-driven transhydrogenase activities as well as having ATPase activity.

The coincidence of ATPase activity with the ability to stimulate the ATP-driven transhydrogenase is expected from the results of Cox et al. [5] and Kanner and Gutnick [6] which showed that the latter reaction was not present in cells of mutants which lacked the ATPase. However, the aerobic-driven transhydrogenase persisted in these mutants [6]. These

Table 1
Purification of coupling factor.

Fraction	Volume (ml)	Protein (mg)	ATPase		Aerobic-TH		ATP-TH	
			Units	Specific activity	Units	Specific activity	Units	Specific activity
On DE-52	4	51	145	2.8	9.6	0.19	14.4	0.28
DE-52: fractions 4 + 5	30	6.5	151	23.4	7.5	1.18	10.1	1.57
Sepharose 6B: fractions 20–22	15	2.1	75	38.5	4.8	2.50	5.9	3.50
Sucrose gradient: fractions 3 + 4	0.8	0.94	33	35.9	1.6	1.74	2.1	2.52

Aerobic-TH, aerobic-driven transhydrogenase; ATP-TH, ATP-driven transhydrogenase; specific activity is in μmoles reacted/min/mg protein.

results suggest that different factors might be required for the aerobic-driven and the ATP-driven transhydrogenase reactions. Our finding that one protein stimulated both activities can be explained if this protein contained more than one polypeptide. The mutant would be expected either to lack one of the polypeptides or for the polypeptide to be modified.

In agreement with this hypothesis our coupling factor protein contained at least five polypeptides (fig. 3). The two major polypeptides were resolved on electrophoresis only when low concentrations of protein were used. Preliminary estimates of the molecular weights of the five polypeptides gave: 56,500–57,000; 50,800–52,700; 29,900–31,000; 18,900–

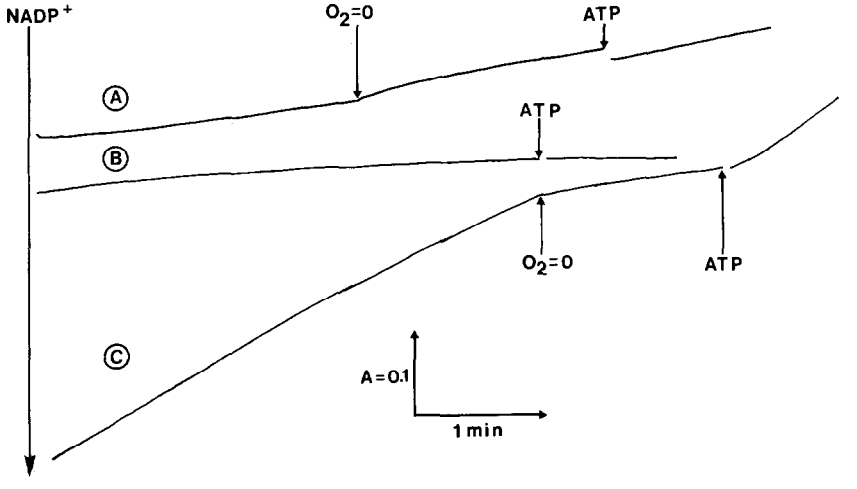
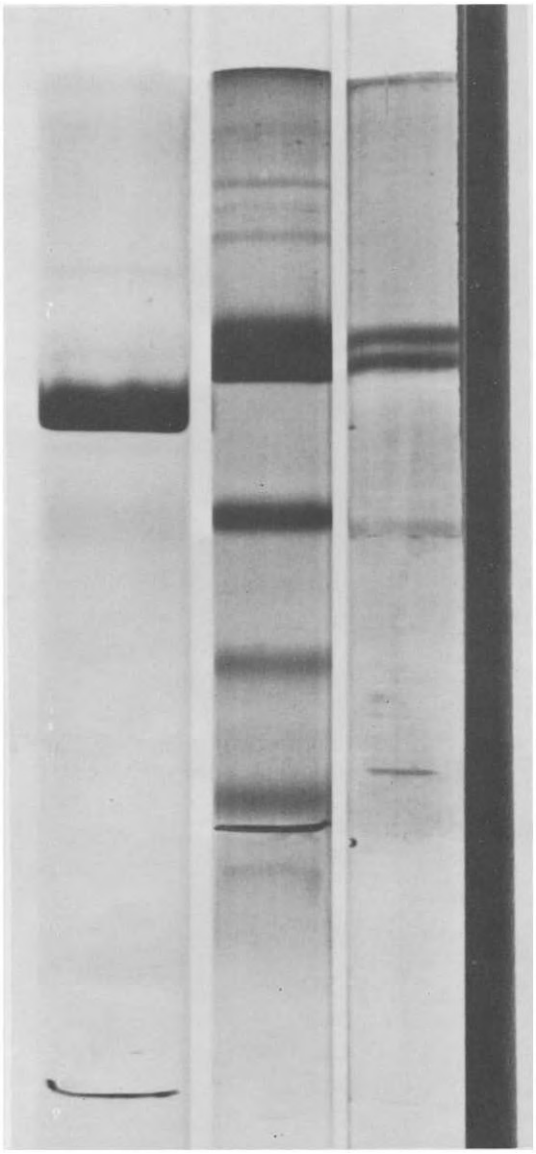


Fig. 2. Aerobic- and ATP-driven transhydrogenase activities in (A) stripped particles, (B) sucrose gradient fraction, (C) stripped particles plus sucrose gradient fraction. The cuvette contained 1.08 ml 0.05 M Tris-H_2SO_4 buffer, pH 7.8, containing 0.1% bovine serum albumin, 0.1 mM DTT, 0.7 M sucrose, 10 mM $MgCl_2$, 0.2 mg alcohol dehydrogenase (Calbiochem), 0.075 mM NAD^+, 5 μl ethanol, and stripped particles (0.64 mg protein) or sucrose gradient fraction (9.1 μg protein). The absorbance at 340 nm was measured following additions of 50 μl 16.3 mM $NADP^+$ and 10 μl 60 mM ATP as indicated in the figure.



a b c

Fig. 3. Electrophoresis of purified coupling factor on polyacrylamide gels. The gels from left to right are (a) sucrose gradient fraction 3, (b) depolymerized sucrose gradient fraction 3 (23 μg protein), and (c) depolymerized sucrose gradient fraction 3 (11.5 μg protein). Gel (a) was run with Tris-glycine buffer, whereas gels (b) and (c) were run with phosphate–0.1% SDS buffer as described in Experimental. The position of migration of bromophenol blue was marked with India ink.

23,000; and 10,000–13,000. Soluble mitochondrial ATPase (F_1) had a similar size distribution of polypeptides, although the molecular weight values for the smaller chains were somewhat different [7]. In contrast, the ATPase from *Streptococcus faecalis* was made up of two non-identical subunits with a molecular weight of 33,000 [8].

Acknowledgements

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