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EVIDENCE SUPPORTING THE IDENTITY OF BEEF HEART MITOCHONDRIAL CHLOROFORM-RELEASED ADENOSINE TRIPHOSPHATASE (ATPase) WITH COUPLING FACTOR I

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SUMMARY

Highly purified mitochondrial chloroform-released beef heart ATPase had molecular weight 330 000, five bands ($\alpha, \beta, \gamma, \delta, \varepsilon$) in sodium dodecyl sulfate gel electrophoresis and could restore the oxidative-phosphorylation function of A particles. Maximal inhibition (90 %) of the enzyme by *N,N'*-dicyclohexylcarbodiimide was achieved at a molar ratio of inhibitor to protein of 30 : 1. Chloroform introduced into an aqueous solution of beef heart coupling factor I protected it from cold inactivation.

INTRODUCTION

Recently, Beechey et al. [1] reported a simple and rapid procedure for extraction of bovine heart mitochondrial ATPase from submitochondrial electron transport particles by treating them with chloroform. This enzyme had some properties resembling other preparations of beef heart coupling factor I (sedimentation coefficient, inhibition by aurovertin B, nonsensitivity to oligomycin), but it had other properties which were different (cold stability before precipitation by ammonium sulfate, low specific activity, three bands instead of five in sodium dodecyl sulfate (SDS) gel electrophoresis, and inhibition by comparatively low concentration of *N,N'*-dicyclohexylcarbodiimide (DCCD)). Naturally, these differences raised some questions about the molecular nature of the chloroform-released enzyme, partly discussed by Beechey et al. [1]. Using a small amount of starting material Barrett and Selwyn [2] demonstrated the possibility of preparation of mitochondrial chloroform-released ox heart ATPase with specific activity 50–80 units/mg protein by chromatography on the DEAE-Sephadex A-50, but they did not report the macromolecular properties of the isolated enzyme. In the present report, we provide evidence that highly purified

Abbreviations: A particles, submitochondrial particles prepared by sonication of beef heart mitochondria in an ammonia solution at pH 9.2; DCCD, *N,N'*-dicyclohexylcarbodiimide.

mitochondrial chloroform-released ATPase and coupling factor I are, in fact, identical proteins.

MATERIALS AND METHODS

Bovine heart mitochondria were prepared by the large scale method of Crane et al. [3]. Submitochondrial particles were prepared by the procedure of Senior and Brooks [4].

At the time of the isolation procedure, the activity of the enzyme was determined by the change in pH of the reaction medium with a Beckman Expandomatic SS2 pH meter and Soltec MARC-II recorder at 30 °C [5]. The assay mixture (final volume 5 ml) contained 50 mM Tris · HCl, 4 mM $MgCl_2$, 2 mM ATP, and 20–200 μg protein at pH 8.5. ATPase activity of the finally purified enzyme was assayed by measuring the rate of P_i production according to the procedure of Senior and Brooks [4]. The inorganic phosphate released was determined by the method of Lindberg and Ernster [6]. One unit of ATPase is defined as the amount of enzyme which catalyzes the release of 1 μmol of P_i or 1 μmol of H^+ per minute at 30 °C. Determinations of inhibitor action of DCCD were done according to Penefsky's procedure [7]. SDS gel electrophoresis was performed according to the procedure of Dunker and Rueckert [8]. Sedimentation velocity experiments were performed in a Spinco Model E ultracentrifuge equipped with Schlieren optics. Runs were made at 20 °C. When we studied the cold inactivation of ATPase, the run was made at 5 °C. The molecular weight was calculated from the data of the high-speed sedimentation equilibrium experiments [9]. The partial specific volume was taken to be 0.74 g/ml [10]. A particles were prepared by the method of Fessenden and Racker [11]. The rate of NAD^+ reduction by succinate was assayed in the system described by Fessenden and Racker [12]. P/O rates were measured using an oxygen electrode with an ATP-regenerating system as described by Tzagoloff et al. [13]. The substrate was either succinate or NADH. Phospholipid content of the preparations of ATPase was determined by the method of Chen et al. [14]. Protein was determined by the method of Lowry et al. [15]. For rapid estimation the biuret method was used [16]. The chemicals were analytical in grade or highest grade commercially available.

Release of ATPase from mitochondrial particles (about 10 g of protein) by treatment with chloroform was done according to the procedure of Beechey et al. [1] except that the concentration of submitochondrial particles in the aqueous phase was taken to be equal to 25–35 mg of protein/ml. The following purification of the enzyme was done by chromatography on a column of DEAE-Sephadex A-50 according to the procedure proposed by Senior and Brooks [4] with small changes. After chloroform treatment, the solution of enzyme was diluted in half with a solution of 80 mM in Tris sulfate, 2 mM EDTA, and 4 mM ATP at pH 7.6 and applied to column (8 × 4 cm) of DEAE-Sephadex A-50 (in a salt form) equilibrated with a buffer containing 40 mM Tris sulfate, 1 mM EDTA, 2 mM ATP at pH 7.6 (ATP-containing buffer). The rate of flow was 10 ml/min. The enzyme was eluted by 350 ml of ATP containing buffer which contained 0.3 M KCl and then the enzyme was precipitated by adding a saturated solution of ammonium sulfate (pH 7.5) to 60 % saturation ammonium sulfate in the presence of 4 mM ATP and 1 mM EDTA. Finally, the prepared enzyme was treated by fractionation of the saturated solution of ammonium sulfate (pH 7.5)

TABLE I

PURIFICATION OF ATPase

Purification stage		Yield (mg)	Specific activity (units/mg)	Day
Step 1	Submitochondrial electron transport particles	10 000	1.1*	
Step 2	Treatment with chloroform	554	12.7	1st
Step 3	DEAE-Sephadex A-50	140	36.5	
Step 4	$(NH_4)_2SO_4$ fraction (33–60 %)	130	70–90**	2nd

* Specific activity was measured by the change in pH of the medium [5].
** Specific activity was assayed by measuring the rate of P_i production [4].

in the presence of 4 mM ATP and 1 mM EDTA. The protein fraction which precipitated in the $(NH_4)_2SO_4$ of saturation range 33–60 % was kept at 4 °C a few weeks with hardly any loss of activity.

RESULTS AND DISCUSSION

The ATPase which was prepared by the method described above had a high degree of homogeneity in an analytical centrifugation as demonstrated by the linearity of the log fringe displacement vs. r^2 plot of the equilibrium data. The protein sedimented as a single symmetric peak and had a sedimentation coefficient ($S_{20,w}$) of 12.2 S, which is in good agreement with data reported by other researchers [4, 10].

The molecular weight for the ATPase was determined in a solvent in which the enzyme was quite stable (40 mM Tris sulfate, 4 mM ATP, 1 mM EDTA, at pH 7.5). The molecular weight, determined by high speed sedimentation equilibrium was determined to be 330 000 daltons. Specific activity of the prepared enzyme was 70–90 units/mg protein. Maximal inhibition (90 %) of isolated ATPase by DCCD was achieved at molar ratio of inhibitor to protein of 30 : 1 as was shown by Penefsky [6].

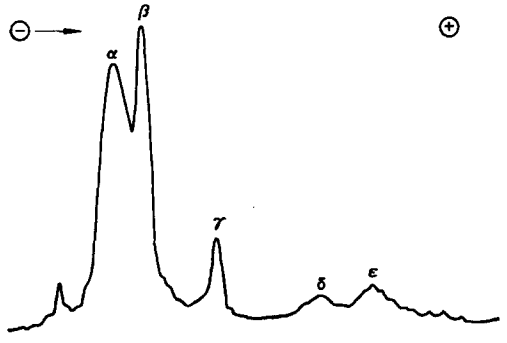

Fig. 1. Densitometric tracing of ATPase in 10 % acrylamide gel containing 0.1 % SDS. Electrophoresis was carried out for 3 h at 8 V/cm at room temperature.

TABLE II

EFFECT OF ATPase ON P/O RATIOS AND REVERSED ELECTRON TRANSFER

For the restoration of oxidative phosphorylation function of A particles the following procedure was used. To 0.5 ml of A particles (12.5 mg/ml) were added 0.05 ml of ATPase (6 mg/ml), which was dissolved in a solution containing 0.25 M sucrose and 1 mM EDTA at pH 7.5, and the mixture was dialyzed for one half hour against a 0. 25 M sucrose, 1 mM EDTA solution at pH 7.5 at room temperature, Then the protein mixture was put in an ice bath (0 °C) for 20 min to inactivate excess unbound ATPase.

	P/O ratio with		NAD^+ reduction rate (nmol/min/mg)
	NADH	Succinate	
A particles	0.13	0.17	10.5
A particles+ATPase	0.52	0.65	46.9

Analysis of this ATPase by SDS polyacrylamide gel electrophoresis revealed a typical pattern for a mitochondrial ATPase (Fig. 1); i.e. five bands corresponding to the five subunits of ATPase with molecular weights 53 000, 50 000, 25 000, 12 500, and 7500 [17, 18].

For a proof of the functional completeness of the isolated ATPase; i.e. identity with coupling factor I, we used experiments in which the restoration of different coupling functions of A particles (oxidative phosphorylation and reversed electron transfer energized by ATP hydrolysis) was studied after the addition of ATPase to them. Table II shows that ATPase, isolated by our method, enhanced the P/O ratio of A particles when the substrates were NADH and succinate. ATPase isolated by our method was able to increase the rate of reversed electron transfer of A particles from 10.5 to 46.9 nmol NAD^+ reduction per min per mg protein. Observed results are similar to those reported for bovine heart coupling factor I by MacLennan and Paulson [19].

In agreement with data reported by Beechey et al. [1], the solvent-released bovine heart ATPase possesses cold stability and becomes cold-labile following precipitation with $(NH_4)_2SO_4$ and redissolving.

It was well known that mitochondrial ATPase has a cold lability; i.e. enzyme incubated at 0 °C loses activity and dissociates into subunits with sedimentation coefficients of 3.55 and 9.15 S [10]. Also, it was shown that cold inactivation of the ATPase could be prevented with various solvents; e.g. ethylene glycol, methanol, ethanol, and glycerol [10].

We proposed that cold stability which was observed by Beechey et al. [1] could be explained by the protective effect of the chloroform which dissolved in the aqueous phase when the suspension of submitochondrial particles was shaken with chloroform. In order to check out this possibility, the following experiments were done. Solutions of mitochondrial bovine heart ATPase, isolated by the method of Senior and Brooks [4] were prepared in ATP-containing buffer and in an ATP-containing buffer saturated with chloroform by shaking with a half volume of chloroform at room temperature. Both solutions of protein were dialyzed at 4 °C for 16 hours against ATP-containing buffer and ATP-containing buffer saturated with chloroform, respectively. Solution were then analyzed in an analytical centrifuge at 5 °C. In the

absence of chloroform, this ATPase dissociated into the subunits with sedimentation coefficients of 3.15 and 7.70 S and a small quantity of the intact enzyme with 11.6 S. In this case, ATPase activity was practically absent. In the presence of chloroform, the ATPase also had peaks of 3.10 S and 7.7 S, but the quantity of intact 11.6 S enzyme was 40–50 %. Activity of ATPase in this case was approximately 40 % of the initial activity.

From the results presented in this paper, it is concluded that the described method may be used to isolate mitochondrial ATPase which resembles other coupling factor I preparations in its sedimentation coefficient, SDS-polyacrylamide gel electrophoresis properties, molecular weight, specific activity, inhibition by DCCD, and in its ability to restore the oxidative phosphorylation function of A particles [4, 18, 19, 20]. This method requires only two days (Table I). The enzyme isolated by the described method was practically free of phospholipids, as was the ATPase prepared by other authors [17].

The investigation of cold inactivation of the mitochondrial ATPase in the presence of chloroform in an aqueous medium confirmed once more the complex nature of the forces stabilizing the ATPase molecule. It is interesting to note that methanol protects membrane-associated Mg^{2+}-stimulated ATPase of *Escherichia coli* against cold inactivation [21]. The cold lability of various ATPases (coupling factor I) and its protection by solvents such as methanol, glycerol, and chloroform could reflect identical macromolecular structure of these enzymes which are involved in the process of oxidative phosphorylation.

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REFERENCES

1 Beechey, B. R., Hubbard, S. A., Linnett, P. F., Mitchell, D. A. and Munn, E. A. (1975) Biochem. J. 148, 533–537
2 Barrett, R. H. and Selwyn, M. J. (1976) Biochem. J. 156, 315–322
3 Crane, F. L, Glenn, J. L. and Green, D. E. (1956) Biochim Biophys. Acta 22, 475–487
4 Senior, A. E. and Brooks, J. C. (1970) Arch. Biochem. Biophys. 140, 257–266
5 van de Stadt, R. J., de Boer, B. L. and van Dam, K. (1973) Biochim. Biophys. Acta 292, 338–349
6 Lindberg, O. and Ernster, L. (1956) Methods Biochem. Anal. 3, 1–22
7 Penefsky, H. S. (1967) J. Biol. Chem. 242, 5789–5795
8 Dunker, A. K. and Rueckert, R. R. (1969) J. Biol. Chem. 244, 5074–5080
9 Yphantis, D. A. (1964) Biochemistry 3, 297–317
10 Penefsky, H. S. and Warner, R. C. (1965) J. Biol. Chem. 240, 4694–4702
11 Fessenden, J. M. and Racker, E. (1966) J. Biol. Chem. 241, 2483–2489
12 Fessenden, J. M. and Racker, E. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.). Vol. 10, pp. 530–532, Academic Press, New York
13 Tzagoloff, A., Byington, K. and MacLennan, D. (1968) J. Biol. Chem. 243, 2405–2412
14 Chen, P. S., Toribara, T. Y. and Warner, H. (1958) Anal. Chem. 28, 1756–1758
15 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–295
16 Gornall, A. G., Bardawill, C. T. and David, M. M. (1949) J. Biol. Chem. 177, 751–766

17 Pedersen, P. L. (1975) Bioenergetics 6, 243–275
18 Brooks, J. C. and Senior, A. E. (1971) Arch. Biochem. Biophys. 147, 467–470
19 MacLennan, D. M. and Paulson, C. W. (1970) Can. J. Biochem. 48, 1079–1081
20 Pullman, M. E., Penefsky, H. S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329
21 Vogel, G. and Steinhart, R. (1976) Biochemistry 15, 208–216