Journal of Chromatography, 157 (1978) 396–400

CHROM. 11,097

Note

Preparation and properties of beef heart mitochondrial ATPase covalently bound to Sepharose 4B*

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(Received April 10th, 1978)

The most significant recent advancements in the understanding of the process of ATP synthesis have occurred in the realm of the molecular mechanism of the mitochondrial ATPase. Recent reviews[1–3] emphasize the importance of these results in obtaining a complete understanding of the process of ATP synthesis. The mitochondrial ATPase has been shown to contain an essential arginine[4] and tyrosine[5] and a sulfhydryl group is necessary for anion activation[6]. A number of studies concerning the kinetic and regulatory properties of the enzyme have appeared[7–9] as well as work on the nucleotide[10,11] and phosphate[12] binding properties.

Recently, the role of the loosely associated proteins that make up the "ATP synthase complex" has come under serious scrutiny. The role of these proteins in regulation or membrane attachment is currently being rigorously studied[1–3], but the lack of rapid isolation techniques for these complex proteins has been a severely limiting step.

The preparation of an insolubilized mitochondrial ATPase preparation that maintains all of the kinetic properties of the soluble enzyme is described here. In addition, this preparation is stable under conditions which will enable it to be used as an "affinity probe" for the proteins that can normally associate with the ATPase molecule.

MATERIALS AND METHODS

Sepharose 4B (200 ml packed gel) was thoroughly washed with distilled water and allowed to react with 60 g CNBr (300 mg/ml packed gel) at 20° according to the method of Cuatracasas[13]. After the Sepharose had been activated, it was washed with water and 100 ml of the activated product was immediately mixed with an ice cold

* Published as paper No. 5517, Journal Series, Nebraska Agricultural Experiment Station. The work reported was conducted, in part, under Project No. 15-019.

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solution (100 ml) of 1,7-diamino heptane (0.1 *M*) adjusted to pH 10.0 with 6 *N* HCl. The remainder of the activated Sepharose 4B was mixed with an ice cold solution (100 ml) of 0.1 *M* ethylenediamine adjusted to pH 10.0 with 6 *N* HCl. These solutions were incubated at 4° with gentle stirring for 24 h. The resins were washed with water, incubated in 0.1 *N* NaOH for 15 min at room temperature, then washed to neutrality with water. The presence of primary amino groups bound to the resin was tested by allowing a small portion of resin to react with an alkaline solution of trinitrobenzene sulfonate[13]. The appearance of an orange color, associated with the resin bead, confirmed the synthesis of amino heptyl and amino ethyl Sepharose 4B.

The succinyl derivative of each resin was prepared by allowing the resin to react with a solution of succinic anhydride at pH 6.0 (ref. 13). The N-hydroxysuccinimide mixed anhydride of the succinylated resin was prepared exactly according to Cuatracasas and Parikh[14]. F_1-ATPase was attached to the activated resin[14] by incubating 20 ml solution containing 6.0 mg/ml in 0.1 *M* phosphate pH 7.5, with 20 ml of packed activated resin at room temperature for 2 h. The resulting resin was washed with a large volume of 0.1 *M* Tris–glycine buffer pH 7.5 at room temperature and, subsequently, with a large volume of a buffer containing 0.1 *M* sucrose and 50 m*M* Tris–Cl pH 8.0. The resin was used without further treatment.

ATPase activity was assayed by modified measurement of phosphate release. The washed resin was mixed with about 4 ml of buffer (0.1 *M* sucrose, 50 m*M* Tris–Cl, pH 8.0) for each ml of resin, and suspended using gentle stirring. A small volume of the suspension was rapidly pipetted to a reaction tube equilibrated at 30°. After 15 min of incubation at 30°, the reaction was terminated by addition of cold perchloric acid to a final concentration of 3%. The resultant slurry was filtered and washed with a small volume of water, and the combined filtrate and wash were assayed for inorganic phosphate by the procedure of Sumner[15]. In all cases, equivalent blanks were prepared by adding the perchloric acid prior to addition of resin-bound ATPase.

The ATPase used in these studies was prepared as described by Knowles and Penefsky[16] and protein was determined by a biuret procedure[17]. All chemicals were from common commercial suppliers and were of the highest purity available.

RESULTS

Kinetic properties of enzymes are sensitive probes of structure and stability. Consequently, we used the known kinetic properties of the mitochondrial ATPase to monitor the effect of immobilization on the ATPase molecule. When ATP is used as the substrate, a double reciprocal plot shows that in the absence of activating anions, negative cooperativity is observed, a property of the soluble enzyme under similar assay conditions[18]. Likewise when a saturating amount of an activating anion, such as bicarbonate, is added to the assay medium, the double reciprocal plot becomes linear, and the initial velocity of ATP hydrolysis is more rapid at any given substrate concentration (Fig. 1).

A distinctive feature of the mitochondrial ATPase is that with substrates other than ATP, the observed kinetics yield linear double reciprocal plots[18]. Fig. 2 clearly shows that insolubilized ATPase maintains this property. The double reciprocal plot of ITP hydrolysis rates by the insolubilized ATPase shows a K_m value of approximately 4.5 m*M* and a $V_{max.}$ of 200 nmoles/min/ml gel.

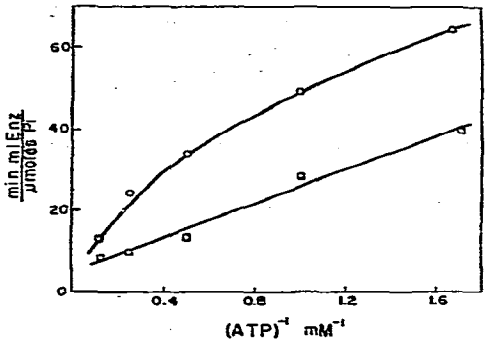

Fig. 1. A double reciprocal plot of the hydrolysis of ATP by immobilized ATPase in the absence (circles) and presence (squares) of 20 m*M* bicarbonate.

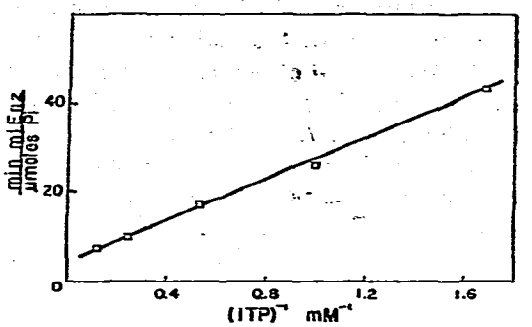

Fig. 2. A double reciprocal plot of the hydrolysis of ITP by immobilized ATPase.

It has been recently shown[19] that the substrate specificity of the mitochondrial ATPase is dependent upon the compositions of the assay medium. In purely aqueous medium, the ATPase shows negative cooperativity with respect of ATP concentration, but ITP hydrolysis exhibits no apparent cooperativity. If the medium becomes less polar (*i.e.*, 20% methanol), ATP hydrolysis becomes non-cooperative (and therefore shows enhanced hydrolytic rates) while ITP hydrolysis is inhibited. The data in Table I show that the same phenomenon occurs with the insolubilized mitochondrial ATPase. The insolubilized ATPase shows enhanced ATP hydrolyzing activity in 20% methanol as compared to the aqueous control, while ITP hydrolysis activity is inhibited in the methanolic medium.

TABLE I

EFFECT OF 20% METHANOL ON THE RATES OF IMMOBILIZED ATPASE CATALYZED ATP OR ITP HYDROLYSIS

Substrate	*Concentration of methanol (%)*	*V (nmoles/min/ml)*
ATP (8 m*M*)	0	81
ATP (8 m*M*)	20	126
ATP (1 m*M*)	0	20
ATP (1 m*M*)	20	47
ITP (8 m*M*)	0	130
ITP (8 m*M*)	20	68
ITP (1 m*M*)	0	37
ITP (1 m*M*)	20	20

A feature that distinguishes the soluble and immobilized forms of ATPase is its stability to cold inactivation. In an aqueous buffer system, the immobilized ATPase is more stable at 0° than the soluble enzyme (Fig. 3). In a medium containing 70% glycerol, the enhanced stability of the immobilized ATPase is more dramatic (Fig. 3). Immobilized ATPase was therefore stored routinely in 70% glycerol at 0–4° for 2 to 3 weeks without noticeable loss in activity.

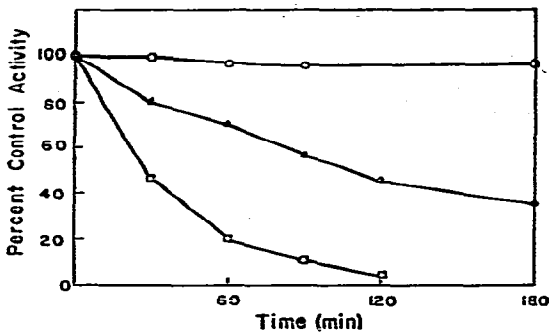

Fig. 3. The effect of storage at 4° on the activity of soluble and immobilized ATPase. The squares represent the activity of soluble ATPase in a completely aqueous medium, the triangles represent the immobilized ATPase in a completely aqueous medium and the circles represent the immobilized ATPase in 70% glycerol.

DISCUSSION

Recently Swanljung and Frigeri[20] demonstrated the feasibility of using the immobilized ATPase inhibitor protein of Pullman and Munroy[21] as an affinity chromatography tool for ATPase isolation. This technique has potential use in problems requiring rapid ATPase isolation, especially from small tissue sources. Conversely, the preparation of a fully functional immobilized ATPase may play a key role in the isolation of the other proteins of the mitochondrial ATP synthetase complex, and in the subsequent elucidation of the nature of the complex itself.

The data presented in this communication clearly demonstrate that under the proper conditions, the mitochondrial ATPase can be covalently linked to Sepharose 4B via a spacer. This preparation is shown to possess all the kinetic properties of the soluble ATPase except that it is more stable in glycerol at low temperatures. Studies are underway to employ the immobilized ATPase as an affinity column for the associated mitochondrial proteins of the ATP synthetase complex.

REFERENCES

1 R. B. Beechey, *Biochem. Soc. Spec. Publ.*, 4 (1974) 41.
2 H. S. Penefsky, in P. D. Boyer (Editor), *The Enzymes*, Academic Press, New York, 1974, p. 375.
3 P. L. Pederson, *Bioenergetics*, 6 (1975) 243.
4 F. Marcus, S. M. Schuster and H. A. Lardy, *J. Biol. Chem.*, 251 (1976) 1775.
5 S. J. Ferguson, W. J. Lloyd, M. H. Lyons and G. K. Radda, *Eur. J. Biochem.*, 54 (1975) 117.
6 P. L. Pederson, *Biochem. Biophys. Res. Commun.*, 64 (1975) 610.
7 S. M. Schuster, R. E. Ebel and H. A. Lardy, *Arch. Biochem. Biophys.*, 171 (1975) 656.
8 S. M. Schuster, R. E. Ebel and H. A. Lardy, *J. Biol. Chem.*, 250 (1975) 7848.
9 P. L. Pederson, *J. Biol. Chem.*, 251 (1976) 934.
10 W. A. Catterall and P. L. Pederson, *J. Biol. Chem.*, 247 (1972) 7969.
11 D. A. Hilborn and G. G. Hammes, *Biochemistry*, 12 (1973) 983.
12 H. S. Penefsky, *J. Biol. Chem.*, 252 (1977) 2891.
13 P. Cuatracasas, *J. Biol. Chem.*, 245 (1970) 3059.
14 P. Cuatracasas and I. Parikh, *Biochemistry*, 11 (1972) 2291.
15 J. B. Sumner, *Science*, 100 (1944) 413.

16 A. F. Knowles and H. S. Penefsky, *J. Biol. Chem.*, 247 (1972) 6617.
17 E. Layne, *Methods Enzymol.*, 3 (1957) 447.
18 R. E. Ebel and H. A. Lardy, *J. Biol. Chem.*, 250 (1975) 191.
19 S. M. Schuster, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 36 (1977) 3329.
20 P. Swanljung and L. Frigeri, *Biochem. Biophys Acta*, 283 (1972) 391.
21 M. E. Pullman and G. C. Munroy, *J. Biol. Chem.*, 238 (1963) 3762.