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Methods in the Preparation of F-Type ATP Synthase

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Abstract: As the energy currency of the cell, ATP is mainly produced by the catalysis of ATP synthase in the mitochondria. In the past 50 years, scientists have done much on ATP synthase's structure and its function already, but people have known more about function but fewer about the mechanism, so there is much more work to do. High purified ATPase which should come first is the key factor in the study of enzyme's structure and catalytic mechanism. So isolation of the ATP synthase has become the primary work for the researchers who want to study it. This article has summarized and introduced some valuable methods in the isolation of mitochondria and submitochondria, and purification of ATP synthase, and the assay of the enzyme's activities in details respectively.

Key words: mitochondria; submitochondrial particles; ATP synthase; extraction; purification; activity
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1 Introductions

The enzyme ATP synthase (EC 3.6.1.34), also known as H^+-ATPase, F_1F_0-ATPase or proton-translocating ATPase, has a very complex and intricate structure and is more complicated than any other ion-translocating ATPase[1]. The enzyme is composed of a water-soluble F_1 moiety and a transmembrane, detergent-soluble F_0 moiety, in which F_1-ATPase is the complex that catalyses the terminal step of oxidative phosphorylation. The phosphorylation of ADP by this enzyme is driven by a proton electrochemical gradient, generated by electron transport in the membrane. And this process takes place in an extra-membrane assembly F_1, which is attached to an intrinsic membrane assembly F_0. In which, F_0 is buried in the inner membrane of the mitochondrion and contains a proton channel through which the proton electrochemical gradient is coupled to the synthesis of ATP and F_1 is exposed to the mitochondrial matrix[1,3-4]. It can be detached from F_0 and is an ATPase. In the past 50 years[1-18], hundreds of experiments and researches[5] have been done in this field, which have involved many different types of materials such as bovine heart, rat liver, yeast and so on and many new methods of purification and assay of enzyme's activities. Here we just give the bovine heart as the example.

2 Mitochondria Preparation

Fresh bovine hearts[3] without fat connective tissue are cut into small pieces (about 2.5 cm^3) and passed through a precooled electric meat grinder (plate with hole of 4mm diameter). The material is then homogenized in a large capacity, high-speed blender in lots of 400 grams. To every 400 grams are added 1 200 mL of 8.5% sucrose (0.25 $mol \cdot L^{-1}$) con-

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taining 1.85 grams of K_2HPO_4 per liter(about 0.01 mol•L^{-1}). The blender is operated for 30 s at full speed. The pH should be around 7.2 to 7.4 by adding 1 to 2 mL of 6 mol•L^{-1} KOH at the outset of blending and 2 to 3 mL at the end. The homogenate is immediately centrifuged for 13 min at 1 900 r. p. m. in a refrigerated International Serum centrifuge. The red supernatant is decanted through a single layer of cheesecloth and the sediment and a partially sedimented buff layer are discarded. There are two different but quite common solutions of mitochondria as follows:

I. Sucrose-Mitochondria　8.5% sucrose solution is added in the proportion of one volume to four volumes of supernatant. And the mixture is now passed through a refrigerated supercentrifuge at 50 000 r. p. m. Anozzle is used which perimite the passage of 10 liters for 7 min[3]. The mitochondria which have been sedimented in the bowl of the centrifuge by this procedure, are scraped out in a room They are blended with 400 mL of 8.5% sucrose solution for 10 to 15 s at low speed until they form a sooth paste. The mixture is finally brought to a volume of 2 liters per contents of one bowl, with the same sucrose solution. The resulting suspension is again passed through the sharples centrifuge at a rate of 10 L per 20 min. The residue is removed as before and blended with the desired volume of 8.5% sucrose solution[3]. This is the so-called sucrose-mitochondria.

II. KCl-mitochondria　With 0.9% KCl solution, we can get the KCl-mitochondria through the similar procedure as the sucrose-mitochondria do.

3　Sub-mitochondrial Preparation

Two types of procedures have been developed for the preparation of submitochondrial particles (SMP)[3-5]. Both of them are remarkably similar in general composition and catalytic properties but they differ in their content of non-heme iron and cytochrome c. At 0~ 4 ℃, bovine heart mitochondria are suspended in a solution of 0.25 mol•L^{-1} sucrose, 0.01 mol•L^{-1} tris-Cl, and pH 7.8 at 60 mg protein per 1 mL and frozen at - 20 ℃. The mitochondria suspension is then thawed and diluted to the concentration of 40 g•L^{-1} with the same buffer. The pH is adjusted to 7.5 with HCl. The mitochondrial suspension is sonicated for 1 min in 100 mL beakers at room temperature. The beakers should be cooled on ice for 1 min. This sonication is repeated twice with each batch. And the sonicator is a Branson Model - S125 which should be equipped with 1/2-in probe and set at number 8 and full power. The suspension is adjusted to pH 7.5 and centrifuged (35 000 g for 10 min). The supernatant would collect and recentrifuged (78 000 g for 90 min). The pellets are suspended in a solution of 0.25 mol•L^{-1} sucrose, 0.01 mol•L^{-1} Tri-Cl pH 7.8 at 20 mg protein per 1 mL. These submitochondrial particles could be stored frozen for several months.[1]

4　Extraction of F_1-ATPase

In the experiments[1,3], bovine hearts mitochondria have been used as the source material and prepared extracts containing soluble ATPase by sonic oscillation of mitochondrial suspensions. Then, they applied kinds of purification techniques such as polyacrylamide gel electrophoresis in phenol-urea-acetic acid, polyacrylamide gel electrophoresis in sodium dodecylsulfate, sedimentation equilibrium ultra-centrifugation in 8 mol•L^{-1}-guanidine hydrochloride and protein estimation. However, the yields and activities obtained are very low, and the major problem is at the stage of protamine sulfate precipitation. Conversely, heat treatment always gives a good purification of the enzyme and an activation of the ATPase activity[7]. By checking on the effect of pH, ionic strength, time of sonication, temperature, etc, at each step, the method described below consistently yields large amounts of enzyme of high activity.

Step 1　Release ATPase by Sonication at High pH.

The submitochondrial particles proteins are routinely used as the starting materials; 0.3 mL of 0.5 mol•L^{-1} EDTA, pH 7.5 and 0.2 mL of 0.5 mol•L^{-1} ATP are added to each 100 mL suspension at 0~ 4 ℃(described previously). The pH should be adjusted to 7.4 with KOH or HCl. Batches of 42 mL suspension are sonicated at full power at room temperature in 50 mL beakers until the temperature reaches 38~ 40 ℃. Then, the suspension that has been pre-

warmed to 25 ℃ would be centrifuged (150 000 g for 45 min) and the supernatants should be discarded. The pellet is homogenized with buffer again and the suspension can stand at least 18 hours at room temperature. The pH should be adjusted to 9.2 with 1 mol·L^{-1} NH_4OH and then the suspension can be sonicated in a tap water-cooled chamber until the temperature reaches 50 ℃. Then the suspension would be centrifuged in rotors warmed to 30 ℃ (100 000 g for 90 min) and at last the supernatant should be collected.[7]

Step 2 Ammonium Sulfate Fractionation.

At 25 ℃, saturated $(NH_4)_2SO_4$ solution could be added slowly with stirring to 37.5% saturation and the pH can be maintained at 7.5 with KOH[8]. The suspension is stirred for 15 min at 25 ℃ to remove the precipitate. The supernatant can be adjusted to 52.5% $(NH_4)_2SO_4$ saturation with saturated $(NH_4)_2SO_4$ solution, and again stirred for 15 min. The precipitate would be collected by centrifugation at 0 ℃ and then the supernatant will be discarded.

Step 3 Ion-exchange Chromatography.

The operations' temperature should be kept at 25 ℃ during this step and the next one (step 4). A column of DEAE-Sephadex A-50 should be equilibrated with the a buffer containing 40 mmol·L^{-1} Tris-SO_4, 1 mol·L^{-1} EDTA, 2 mmol·L^{-1} ATP, pH 7.5 (called TEA). The final column dimensions are 6 cm high and 4 cm in diameter. The protein from the latest step would be dissolved in the minimum volume of TEA buffer, then desalted in Sephadex G-25 in the same buffer and applied to the DETA-Sephadex column. The columns are washed with 150 mL TEA buffer that contains 0.125 mol·L^{-1} KCl, pH 7.5 and the effluent should be discarded. Then the column can be washed with 200 mL of TEA buffer containing 0.2 mol·L^{-1} KCl at pH 7.5. The effluent would be collected, and saturated $(NH_4)_2SO_4$ solution would be added to give 55.0% saturation.

Step 4 Gel Filtration.

The ammonium sulfate suspension is centrifuged and the protein will be dissolved in TEA buffer at 30 g·L^{-1}. Insoluble material would be removed by brief centrifugation. The solution would be then applied to a column of Sephadex G-200. Upward flow will be maintained at 20 mL/hour with a pump; the column buffer is TEA[7-8]. The ATPase eluted in a single symmetrical peak, V_e = 240 mL, V_o = 180 mL. Some inactive, low molecular weight material eluted in later fractions. An approximate molecular weight of 260 000 is obtained from the V/V ratio. The protein should be collected by precipitation in 55% saturation $(NH_4)_2SO_4$.[7]

Step 5 Heat.

The protein should be dissolved in TEA buffer to a final protein concentration of 10 g·L^{-1}. ATP would be added to give a final concentration of 4 mmol·L^{-1}. The solution should be heated for 2 min at 65 ℃ in a water bath. The precipitate will be removed by brief centrifugation. The soluble protein remaining in the supernatant should be collected by precipitation in 55% saturation $(NH_4)_2SO_4$. And that is the right thing we need.

5 Extraction of F_0-ATPase

As one moiety of the ATP synthase, the detergent-soluble F_0 is buried in the inner membrane of the mitochondria. Due to the fact[1,4,6], we can perform the solvent extraction from the submitochondria particles (SPM), and those are experimental methods, i.e., plans of the experiments will be improved with the development of the mechanisms of the ATP synthase.

Step1 Solvent Extraction.

The final SMP fraction from above would be used for solvent extraction and for every 100 μL of this fraction, 2.5 mL of 2∶1 chloroform: methanol mixture will be added and mixed end over end for 1 hour. Then this mixture will be centrifuged for 5 min in a Beckman GPR bench centrifuge at 2 800 g and supernatant should be kept[1]. The pellet is resuspended in 100 μL of buffer (0.4 mol·L^{-1} Tris-HCl, pH 7.4) and re-extracted with 2∶1 chloroform: methanol mixture as above and supernatants should be pooled. To this pooled supernatant, 0.2 volume of water is added, vortexed

and mixed end over end for 10 min, and centrifuged as previously. The upper phase is removed and discarded and the lower phase is divided into 1.5 mL aliquots and placed in Eppendorf tubes. The volume in each Eppendorf tube is reduced to about 0.1 mL by evaporation at 30 ℃, then ice-cold ether is added to 1.4 mL and vortexed to mix and tubes are stored at - 7 ℃[6] until needed for future experiments. The chloroform: methanol solubilized material precipitated out in the presence of the added ether when stored at - 70 ℃, forming a loose pellet that is referred to as "pellet" throughout the text. This loose pellet is then used for solubilization experiments. A typical preparation, using 50~ 60 g of liver will result in enough material to give 24~ 25 Eppendorf tubes containing these pellets.[6]

Step 2　Solubilization of Pellets Using Various Detergents.

Ether-developed pellets are removed from - 70 ℃ storage and centrifuged at 19 000 g for 30 min at - 20 ℃ and supernatants, which are essentially the added ether, would be discarded. The pellets are then mixed with 100 mL of 50 mmol• L^{-1} Tris-HCl, pH 7.4, at room temperature, containing any one of the following detergents: Triton X-100, C12E8, n-octyl b-D-glucopyranoside, Zwittergent series, sodium cholate, sodium deoxycholate, NP-40, and LDS[1]. All detergents should be used at their CMC values and at several concentrations above and below their CMC[1]. Once the buffer containing a specific detergent, at a specific concentration, is added to the pellet, the sample would be gently vortexed and then sonicated for 20 s at an amplitude of 6~ 8 mm and allowed to stand for 5 min. Sonication is repeated as earlier for three to four more times. If any of the pellets were solubilized completely, the resulting samples would be then used directly or stored at - 20 ℃ for future use. For pellets that could not be solubilized completely by the added buffer that contained a given detergent at any concentration and after repetitive sonication, they should be centrifuged at 16 000 g in a bench centrifuge for 30 min to sediment the undissolved pellets[6]. The supernatants would be removed and their protein concentrations are determined, then the supernatant is the right thing we need.[1]

4 Discussion

Our experiments would be the processes to continue probing on the relations of the buffers and regents conditions to the characters of the ATPase[4, 16- 17], that is to say, the techniques of the extractions and purifications on the ATPase are the challenged things in the way of studying on the structures and functions of the ATPase. High purified ATPase led our work to be true, at least can be used for studying their structure with the assistantance of AFM, TEM, SEM, X-ray, optic tweezers. With other biophysical and biochemistry techniques, the experiment would try a comprehensive study in the catalytic mechanism of ATPase. High purified ATPase will give us much more valuable work to do.

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线粒体 F 型 ATP 合成酶的制备方法研究与探讨

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摘 要: 科研工作者们在过去的 50 年前赴后继的工作中深入研究了 ATP 合成酶的功能, 并努力尝试解析该酶的空间结构以便能够从结构基础上对 ATP 合成酶的催化机理进行阐明; 但蛋白的纯化工作却一直是困扰研究顺利进展的最大障碍. 蛋白的纯化技术是与特定阶段科技发展及科研工作者思维模式的直接反应, 因而是一门不断发展的艺术. 文章主要介绍了 ATP 合成酶的提取与纯化工作, 在详细对比了现有方法的基础上, 给出了纯化此酶复合体的详细方案和线粒体、亚线粒体、ATP 合成酶的提取与纯化方法; 并在方法讨论的基础上对线粒体 F 型 ATP 合成酶的研究提出了前瞻性的方案.

关键词: 线粒体; 亚线粒体; ATP 合成酶; 亚基; 提取与纯化; 活性
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