

Isolation and Antigenic Characterization of Corn Mitochondrial F₁-ATPase¹

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ABSTRACT

Corn mitochondrial F₁-ATPase was purified from submitochondrial particles by chloroform extraction. Enzyme stored in ammonium sulfate at 4°C was substantially activated by ATP, while enzyme stored at -70°C in 25% glycerol was not. Enzyme in glycerol remained fully active (8-9 micromoles P_i released per minute per milligram), while the ammonium sulfate preparations steadily lost activity over a 2-month storage period. The enzyme was cold labile, and inactivated by 4 minutes at 60°C. Treatment with octylglucoside resulted in complete loss of activity, while vanadate had no effect on activity. The apparent subunit molecular weights of corn mitochondrial F₁-ATPase were determined by SDS-polyacrylamide gel electrophoresis to be 58,000 (α), 55,000 (β), 35,000 (γ), 22,000 (δ), and 12,000 (ϵ). Monoclonal and polyclonal antibodies used in competitive binding assays demonstrated that corn mitochondrial F₁-ATPase was antigenically distinct from the chloroplastic CF₁-ATPases of corn and spinach. Monoclonal antibodies against antigenic sites on spinach CF₁-ATPase β and γ subunits were used to demonstrate that those sites were either changed substantially or totally absent from the mitochondrial F₁-ATPase.

acterized by chemical, enzymic, and immunochemical methods. However, the situation in regard to the phosphorylating enzyme of plant mitochondria is quite different. In comparison to the amount of information that has been accumulated in animal systems, very little is actually known about the characteristics of plant mitochondrial F₁-ATPase. This is in large part due to the difficulties involved in isolating large quantities of plant mitochondria. Plant mitochondrial F₁-ATPase has been partially purified from pea cotyledons (7) and castor bean endosperm (28). Two recent reports (3, 8) have appeared which utilized the chloroform extraction procedure introduced by Beechey *et al.* (2) to solubilize F₁-ATPase from submitochondrial particles of bean and corn mitochondria. The isolation of sweet potato mitochondrial F₁-ATPase was also recently reported (9). No detailed comparisons of mitochondrial F₁-ATPase and chloroplastic CF₁-ATPase from the same plant species has as yet been accomplished, and the similarities or differences between these enzymes can only be inferred by studies of animal mitochondrial F₁-ATPases.

The fact that plant cells contain two unique organelles capable of energy production by oxidative phosphorylation in one case, or photosynthetic phosphorylation in the other, makes them an attractive model for comparative studies. Detailed studies of plant mitochondrial F₁-ATPase will provide the basis for comparison to chloroplastic CF₁-ATPase. Comparisons of these two enzymes from the same cell may provide valuable information on aspects of structure, regulation, biosynthesis, and evolution of the enzyme within these two organelles. This paper reports the isolation, purification, physical and enzymic properties of the F₁-ATPase from corn mitochondria. In addition, the antigenic properties of this enzyme are compared to those from CF₁-ATPases and mammalian and *E. coli* F₁-ATPases using both polyclonal and monoclonal antibodies.

MATERIALS AND METHODS

Purification of Corn Mitochondrial F₁-ATPase. About 700 g of etiolated 3- to 4-d-old corn seedlings (NB611) were homogenized for 20 s at full speed in a Waring Blendor with 3 volumes of 50 mM KH₂PO₄ buffer (pH 7.4) containing 220 mM D-mannitol, 70 mM sucrose, 0.5 mM EDTA, and 0.05% BSA (Buffer A). Debris was removed by centrifugation at 1500g × 5 min. Mitochondria were pelleted from the supernatant fluid by centrifugation at 12,000g × 15 min. The mitochondria were washed by resuspension in 1.5 L of Buffer A and centrifuged as above. Mitochondria were then resuspended in 200 ml of Buffer A without BSA, and 40 ml aliquots were disrupted by a 1-min sonication at maximum output (Bronwill, Biosonic III). Unbroken mitochondria were removed by centrifugation at 12,000g ×

Analogous proton-translocating ATPases are essential components of both mitochondrial respiration (oxidative phosphorylation) and of chloroplastic light-driven ATP synthesis. Both systems are comprised of two structurally distinct portions. The easily solubilized hydrophilic, peripheral membrane component possessing ATPase activity *in vitro* is generally abbreviated F₁-ATPase in mitochondrial systems and CF₁-ATPase in plant chloroplasts. Similar polypeptides designated as α , β , γ , δ and ϵ have been characterized in both systems. The second component of the proton-translocating ATPases functions as a proton translocator *in vivo* and consists of a group of hydrophobic integral membrane proteins abbreviated F₀ in both mitochondria and chloroplasts (for reviews, see Nelson [14], and Senior and Wise [23]). The mitochondrial F₁-ATPases from animals and the CF₁-ATPases from higher plants have been extensively char-

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10 min, and SMP² were collected by ultracentrifugation at 100,000g × 60 min. The SMP were resuspended in 50 ml of 40 mM Tris-Cl buffer (pH 7.4) containing 1 mM EDTA, 5 mM ATP, and protease inhibitors (3 mM *p*-ABA and 1 mM PMSF) (Buffer B). All above procedures were carried out at 4°C.

Corn mitochondrial F₁-ATPase was dissociated from SMP at room temperature by slow passage through chloroform. From 5 to 10 ml of the SMP suspension was slowly expelled from a pipette into the bottom of a 50-ml cylinder containing 40 ml chloroform. The aqueous phase containing soluble mitochondrial F₁-ATPase slowly bubbled to the top of the cylinder and was collected. Any remaining chloroform was removed by centrifugation at 12,000g × 10 min, and then the F₁-ATPase solution was cleared of membrane debris by centrifugation at 100,000g × 30 min.

The F₁-ATPase was precipitated from solution by addition of an equal volume of saturated (NH₄)₂SO₄. After a 30-min incubation at 4°C, precipitated protein was collected by centrifugation at 12,000g × 15 min at 4°C. The protein pellet was dissolved in 3 to 5 ml of Buffer B containing 8 mM ATP and 2 mM DTT, and then insoluble material was removed by centrifugation at 12,000g × 20 min (20°C). Finally, the F₁-ATPase was reprecipitated by (NH₄)₂SO₄ fractionation between 30 and 50%. The enzyme was stored as a 50% (NH₄)₂SO₄ slurry at 4°C for use as antigen, or redissolved in 25% glycerol containing 4 mM ATP, 1 mM EDTA, and 1 mM DTT and stored at -70°C for kinetic studies.

Isolation of Other ATPases. Corn and spinach CF₁-ATPases were isolated from thylakoid membrane fractions (12) by a chloroform release technique previously described (24, 25, 29). Glycerol (15%) was added after extraction to prevent loss of δ subunits during subsequent DEAE-cellulose chromatography. The CF₁-ATPases were eluted from DEAE-cellulose by 0.3 M NaCl in 40 mM Tris-Cl buffer (pH 7.4), 3 mM ATP, 1 mM EDTA, 15% glycerol. Further purification was achieved by gel filtration on Sepharose 4B equilibrated with 0.04 M Tris-Cl buffer (pH 7.4), 3 mM ATP, 1 mM EDTA. Beef heart F₁-ATPase was isolated as previously described (24, 25). *E. coli* F₁-ATPase was kindly provided by Dr. L. Heppel, Cornell University, Ithaca, New York.

SDS-Polyacrylamide Gel Electrophoresis. The subunits of corn mitochondrial F₁-ATPase were separated by SDS-PAGE as described by Ryrie and Gallagher (20), except the separating gel was 12% acrylamide, 0.4% methylene-bisacrylamide. The subunits of *E. coli* F₁-ATPase, beef heart F₁-ATPase, corn CF₁-ATPase, and spinach CF₁-ATPase were also separated as above for comparison of subunit mol wt. Average apparent mol wt for the subunits were derived from a calibration curve of seven protein markers plus the subunits of *E. coli* F₁-ATPase. The protein standards were BSA (6.6 × 10⁴), ovalbumin (4.5 × 10⁴), pepsin (3.47 × 10⁴), bovine erythrocyte carbonic anhydrase (3.0 × 10⁴), trypsinogen (2.4 × 10⁴), lactoglobulin (1.84 × 10⁴), and lysozyme (1.43 × 10⁴) (Sigma). The subunit mol wt of *E. coli* F₁-ATPase determined from nucleotide sequence data (10) were also used to establish the mol wt standard curve.

Densitometric analyses of the Coomassie brilliant blue R-250 stained polyacrylamide slab gels or their photo negatives were done with a LKB model 2202 densitometer.

Enzymatic Assay of Corn F₁-ATPase. Enzyme was stored at -70°C in 25% glycerol, 4 mM ATP, 1 mM EDTA. Under these conditions the preparations were stable over a period of up to 2 months. Prior to assay, storage nucleotide and glycerol were

removed by centrifugal filtration (17) using Biogel P-6DG equilibrated with 4mM ATP, 0.1 mM EDTA in 50 mM Tricine-NaOH (pH 8.0), and the enzyme incubated at 25°C for 1 h with 5 mM ATP. ATPase activity was determined at 30°C by following absorbance changes at 340 nm in a coupled assay system consisting of 50 mM Tricine-NaOH (pH 8.0), 5 mM ATP, 6 mM MgCl₂, 1.0 mM KCl, 2 mM phosphoenolpyruvate, 0.3 mM NADH, 12 units of pyruvate kinase, and 17 units lactate dehydrogenase in a total volume of 1 ml. During purification the enzyme activity was determined by P_i-released assay (22). The assay mixture (final volume, 0.5 ml) contained 50 mM Tricine-NaOH, 5 mM MgCl₂, 5 mM ATP, 4 mM DTT (pH 8.0). The reaction was started by the addition of enzyme (10–50 μg of protein) and followed for 5 min at 30°C. The reaction was stopped by the addition of 12.5 μl of 90% TCA and P_i was determined by the procedure of Ames (1).

Determination of pH Optimum. For pH studies, an isoionic buffer system consisting of 50 mM 2[*N*-morpholino]ethanesulfonic acid, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 100 mM diethanolamine, and 33.3 mM Na₂SO₄ replaced Tricine as the assay buffer.

Production of Rabbit Immune Sera. Purified corn CF₁-ATPase was used to immunize rabbits. The first immunizations (150 μg of protein) were emulsified in complete Freund's adjuvant, followed by two injections (100 μg of protein per injection) in incomplete Freund's adjuvant at 2-week intervals. Rabbits were bled 1 week after the third injection.

Production of Monoclonal Antibodies. BALB/c mice were immunized by intraperitoneal injection with 20 μg of spinach CF₁ in complete Freund's adjuvant followed by two injections of 20 μg CF₁ in incomplete Freund's adjuvant at monthly intervals. A final intravenous injection of 20 μg CF₁ was administered 4 d before the fusion. Hybridomas were produced by fusion of X63-AG8.653, a nonsecreting BALB/c myeloma cell line (11), with spleen lymphocytes from the immunized mouse according to the fusion protocol outlined by Oi and Herzenberg (16).

Antibody Screening. An enzyme-linked immunosorbent assay (ELISA) (26) was used to select for hybridomas producing antibody against spinach CF₁-ATPase. Vinyl microtiter plates (Costar) were coated with spinach CF₁-ATPase at 5 μg/ml by incubation at room temperature for 4 h. Unreacted protein binding sites were blocked by addition of 2% BSA in PBS (pH 7.2) for at least 1 h. The plates were then flicked dry, and washed three times with PBS containing 0.5% NP-40 (washing buffer). Antigen-coated plates could be used immediately or stored in a moist chamber at 4°C for up to several months. Hybridoma culture fluids were incubated in the antigen-coated wells for 2 h at 4°C, then rinsed off with washing buffer. Goat antimouse Ig (Meloy) at an appropriate dilution (usually 1:3200) was then incubated in the wells for 2 h at 4°C, followed by affinity-purified rabbit antigoat IgG conjugated to alkaline phosphatase (1:1000) (Sigma). The enzyme conjugate was incubated in the wells overnight at 4°C. All incubation volumes were 50 μl per well. Unbound phosphatase conjugate was removed by rinsing the plates three times in washing buffer followed by vigorous washing under running tap water. Dinitrophenyl phosphate (4 mM in 840 mM diethanolamine [pH 9.8], 0.25 mM MgCl₂) was added to each well (200 μl) and incubated for 1 h at 25°C. Absorbance was read on a Titertek MC interfaced to an IBM personal computer.³ Culture fluids were considered to contain antibody against CF₁-ATPase if the absorbance reading was greater than 2 SD above the absorbances of the negative controls. All culture fluids were also tested for nonspecific binding on plates coated with 2% BSA in the absence of spinach CF₁-ATPase.

² Abbreviations: SMP, submitochondrial particles; *p*-ABA, *p*-aminobenzoamidine; PMSF, phenylmethyl sulfonylfluoride; ELISA, enzyme-linked immunosorbent assay; NP-40, actylphenoxypolyethoxyethanol; Ig, immunoglobulin; IgG, immunoglobulin G; IgM, immunoglobulin M.

³ IBM-PC software was developed by G. H. Pfeiffer, Department of Agricultural Economics, University of Nebraska, Lincoln, NE 68583.

Positive clones were selected after from 2 to 6 weeks of growth following the initial fusion procedure and subcloned by limiting dilution (16) in media conditioned by Buffalo Rat liver (BRL-3A) cells (6). Four stable antibody-producing hybridoma lines were established from the fusion. The class specificities of these lines were determined by ELISA utilizing goat antimouse IgM, IgG₁, IgG_{2A}, and IgG_{2B} antisera (Meloy).

Competitive Binding Assays. Monoclonal antibodies were tested for cross-reactivity to corn CF₁-ATPase, corn mitochondrial F₁-ATPase, beef heart F₁-ATPase, and *E. coli* F₁-ATPase. A limiting amount of each monoclonal antibody was incubated in solution with each of the competing enzymes at various protein concentrations for 20 h at 4°C in 1% BSA-PBS. The antibody-protein solution was then incubated with spinach CF₁-ATPase on a solid phase (*i.e.* adsorbed to vinyl microtiter plates) for 2 h at 4°C. After plates were rinsed thoroughly, solutions of goat antimouse Ig, rabbit antigoat IgG conjugated to alkaline phosphatase and dinitrophenyl phosphate were added in sequence using the conditions described above for the ELISA. Competitive binding assays utilizing rabbit antisera against corn CF₁-ATPase were accomplished in a similar fashion, except bound antibody was detected with goat antirabbit IgG conjugated to alkaline phosphatase (Sigma).

Transfer of Electrophoretically Separated Proteins to Nitrocellulose Paper. Electrophoretic separation of the chloroplast and mitochondrial ATPases was carried out as described above, and then electrophoretically transferred to nitrocellulose paper utilizing a Trans-Blot cell (Bio-Rad). Transfers were performed at 30 v for 18 h according to the directions supplied with the Trans-Blot cell. Nitrocellulose transfer blots were either stained for protein using 0.1% amido black or reacted with antibody (15). For reaction with antibody, the nitrocellulose transfers were first incubated for 1 h with 3% ovalbumin in 10 mM Tris, 0.15 M NaCl (pH 8.2) (ovalbumin-Tris-saline), to block unreacted protein binding sites. Blots were then incubated for 2 h with hybridoma culture fluids diluted 1:50 in ovalbumin-Tris-saline. Free antibody was washed from the transfer blot using 10 mM Tris, 0.15 M NaCl (pH 7.4) (Tris-saline), and then goat antimouse Ig (Meloy) diluted 1:3200 in ovalbumin-Tris-saline was added for 2 h. After rinsing three times in Tris-saline, rabbit antigoat Ig conjugated to alkaline phosphatase (Sigma) diluted 1:1000 in 0.3% BSA, 20 mM Tris, 1 mM MgCl₂, 2.5 μM ZnCl₂, 0.15 M NaCl, 0.05% NaN₃ (pH 7.5), was added and incubated overnight at 4°C. Transfer blots were then washed thoroughly with at least five Tris-saline rinses over a 30-min interval. As a further rinse the blots were held under running tap water to remove all traces of unreacted enzyme conjugate. Failure to adequately remove free enzyme conjugate resulted in unacceptably high backgrounds. Transfer blots were then soaked in a substrate solution containing freshly prepared and filtered naphthol AS-MX phosphate (0.2 mg/ml) and Fast Red TR salt (3 mg/ml) in 0.1 M Tris-Cl (pH 8.2). After antibody-specific polypeptides were visible as red bands, the reaction was stopped by rinsing the blots in water (15). All incubations except as noted above were done at 23 ± 2°C. When rabbit-immune serum was used in place of hybridoma culture fluids, the blots were developed as above, except the sequence of reagents was rabbit immune serum followed directly by goat antirabbit IgG conjugated to alkaline phosphatase (Sigma).

Statistical Analysis. For competitive binding assays, seven replicates of each treatment were used, and results reported as the mean and standard deviations of those replicates. The Student's *t* test was used to compare treatment means at the 99% level of significance. Binding to a competing antigen was considered to be significant if the mean absorbance values with competing antigen were significantly lower than in the control treatment without soluble antigen.

Protein Analysis. The Coomassie dye-binding method of Bradford (4) (BioRad dye reagent concentrate) was used to determine the protein concentrations of the purified ATPases. Fraction V BSA (Sigma) was used for generation of a standard curve.

RESULTS

Isolation and Purification of Corn F₁-ATPase. The chloroform release method introduced by Beechey *et al.* (2) was used to release F₁-ATPase from SMP. However, in order to obtain reproducible results from this procedure, the special conditions of slow passage through chloroform described in "Materials and Methods" were extremely important. From 2 to 4 mg F₁-ATPase were purified from about 700 g of corn seedlings. The freshly purified enzyme had a specific activity in the range of 8 to 9 μmol P_i-released/min mg of protein. The enzyme preparation appeared to consist of five subunits as shown by SDS-PAGE (Fig. 1A), which is consistent with the subunit composition of F₁-ATPase as shown by other investigators (3, 8). Densitometric analysis of the Coomassie blue-stained gel demonstrated that these five bands comprised over 95% of the total protein present when 40 μg of protein were loaded onto the gel. Although the electrophoretic pattern suggests a high degree of enzyme purity, it is still possible, although unlikely, that one of these bands might represent a contaminating polypeptide.

Catalytic Properties of Corn F₁-ATPase. When the purified corn F₁-ATPase was stored as an (NH₄)₂SO₄ precipitate at 4°C in 4 mM ATP, 1 mM EDTA, low catalytic activities (<0.7 μmol/min mg at 5 mM ATP, 6 mM MgCl₂ [pH 8.0], 30°C) were observed unless the enzyme was incubated with ATP after removal of the (NH₄)₂SO₄. Following a 1-h incubation at 25°C in 4 mM ATP, 0.1 mM EDTA, 50 mM Tricine-NaOH (pH 8.0), the (NH₄)₂SO₄ precipitated enzyme typically had a specific activity of 2 μmol/min mg. However, enzyme preparations stored at -70°C in 25% glycerol, 4 mM ATP, 1 mM EDTA typically had ATPase activities of 8 to 9 μmol/min mg under the above conditions, and were not markedly activated by incubation with ATP. Unlike the chloroplast CF₁-ATPase (15), a 4-min incubation at 60°C in 35 mM ATP and 5 mM DTT inactivated the mitochondrial F₁-ATPase. The isolated corn F₁-ATPase was cold labile. After 1 h at 0 to 2°C, the enzyme lost practically all activity in the absence of (NH₄)₂SO₄.

Contamination of the preparations with chloroplast CF₁-ATPase was directly addressed by examining the effect of octylglucoside on activity. Conditions which activate the Mg-dependent CF₁-ATPase (18), (10 mM octylglucoside) completely inhibited the activity of the purified mitochondrial enzyme. Sodium vanadate, which inhibits the plasma membrane ATPases, and soluble phosphatases of corn root cells (5) had no effect on activity at concentrations of up to 2 mM. In addition, NaN₃, which inhibits F₁-ATPases, but not the plasma membrane or cytoplasmic enzymes (5), caused 60% inhibition of the preparations at a concentration of 2.5 μM.

Physical Properties of Corn F₁-ATPase. The apparent mol wt of corn mitochondrial F₁-ATPase subunits were determined by SDS-PAGE using *E. coli* F₁-ATPase subunits in addition to protein standards as the calibration curve (10). These mol wt are presented in Table I, and are compared to the subunits of beef heart F₁-ATPase, and spinach and corn CF₁-ATPases determined simultaneously in the same gel. Simultaneous determination of mol wt of the subunits of F₁-ATPase from a number of sources allowed us to avoid the discrepancies in apparent mol wt which can arise between electrophoretic gels. The mol wt of the α, β, and δ subunits of corn mitochondrial F₁-ATPase appeared to be similar to the corresponding subunits of the mitochondrial enzyme from beef heart. In contrast, the mol wt of the δ and ε subunits of corn mitochondrial F₁-ATPase appeared to be larger than the corresponding subunits from other mitochondrial

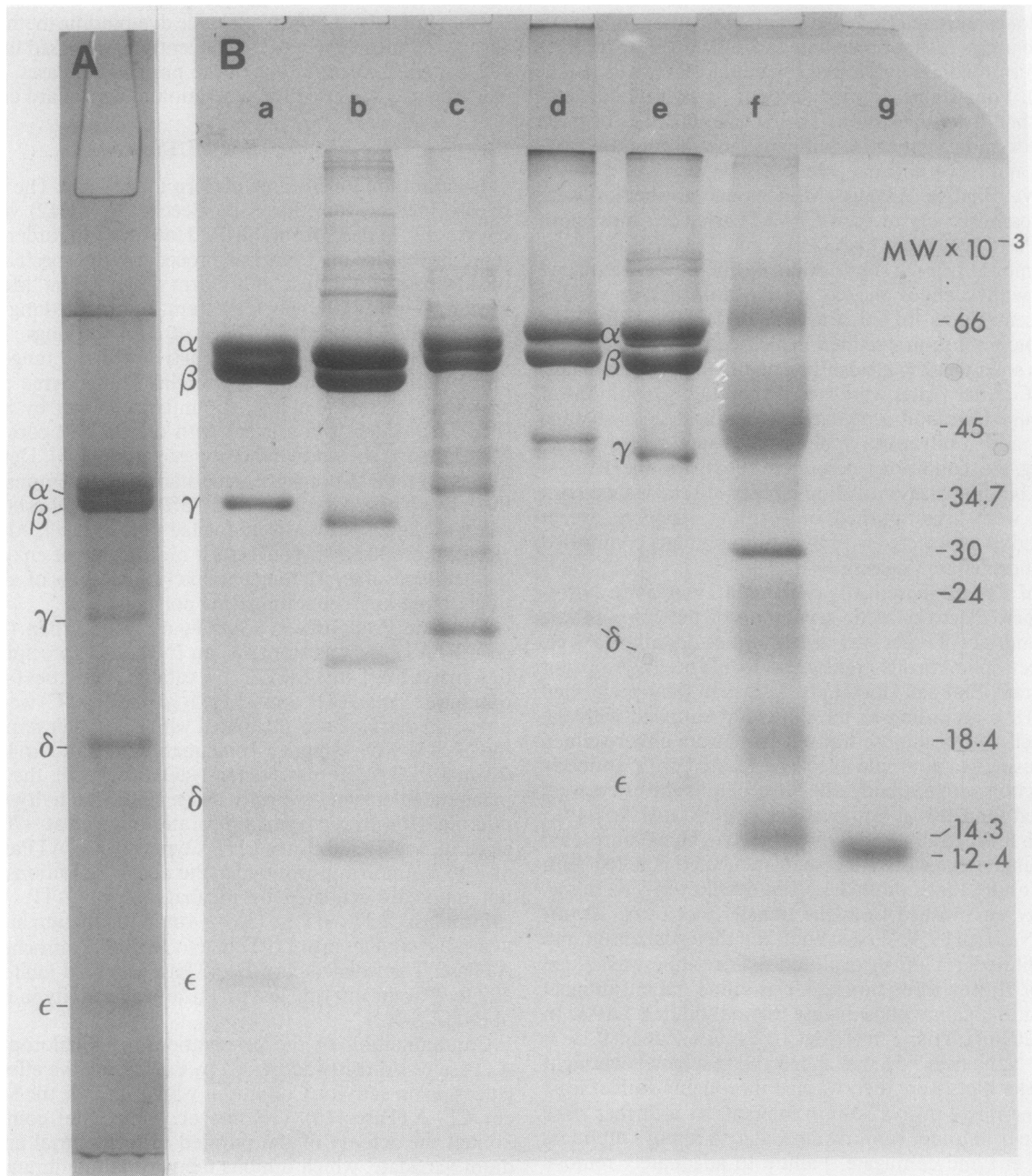


FIG. 1. SDS-polyacrylamide slab gel electrophoresis of (A) corn mitochondrial F_1 -ATPase (40 μ g) and (B) a comparison of the subunit structure of various ATPases. (a), Beef heart F_1 -ATPase; (b), *E. coli* F_1 -ATPase; (c), corn mitochondrial F_1 -ATPase; (d), corn CF_1 -ATPase; (e), spinach CF_1 -ATPase; (f), marker proteins (BSA, ovalbumin, pepsin, carbonic anhydrase, trypsinogen, β -lactoglobulin, lysozyme); (g), Cyt *c*.

Table I. Comparison of Subunit Mol Wt ($n \times 10^{-3}$) of Corn Mitochondrial F_1 -ATPase to F_1 -ATPase Subunits from Other Sources

All mol wt were determined simultaneously by SDS-polyacrylamide slab gel electrophoresis (modified Laemmli system as described in "Materials and Methods").

Subunit Type	Corn Mitochondria	<i>E. coli</i>	Beef Heart	Corn Chloroplast	Spinach Chloroplast
α	58.0	57.2	58.0	60.0	62.0
β	55.0	52.5	54.0	56.0	55.0
γ	35.0	31.5	34.0	40.0	39.5
δ	22.0	20.5	15.5	22.5	21.0
ϵ	12.0	14.5	10.0	15.5	16.0

sources.

Densitometric analysis of the SDS-polyacrylamide slab gel stained with Coomassie blue suggested a stoichiometry of 3:3:1 for α , β , and γ subunits for all investigated F₁-ATPases. The mol wt of corn mitochondrial F₁-ATPase was calculated to be 430,000 based on a subunit stoichiometry of ($\alpha_3\beta_3\gamma\delta_2\epsilon$) and on the apparent subunit mol wt estimated by SDS-PAGE (see Table I).

A subunit stoichiometry of ($\alpha_3\beta_3\gamma\delta_2\epsilon$) for the corn mitochondrial F₁-ATPase subunits was suggested on the basis of densitometric analysis of Coomassie blue-stained bands of F₁-ATPase on SDS-PAGE (see Fig. 1). The generally accepted stoichiometry of $\alpha_3\beta_3\gamma$ for beef heart mitochondrial F₁-ATPase was first proposed by Senior and Brooks (21) on the basis of similar densitometric measurements. The stoichiometry $\alpha_3\beta_3\gamma\delta_2\epsilon$ for corn mitochondrial F₁-ATPase gives a mol wt of 430,000 which is close to the mol wt of the spinach CF₁ recently estimated by sedimentation equilibrium and by light scattering (13). Additional experiments with gel filtration using Sepharose 4B \times 200 cm showed that the R_f for the corn mitochondrial enzyme and spinach CF₁ were very close (data not shown). This indicates that the corn mitochondrial F₁-ATPase isolated by us was in a high mol wt form similar to other isolated ATPases.

Polyclonal Antibody Characterization. Antibodies produced in rabbit against corn CF₁-ATPase were tested for specificity by the nitrocellulose transfer technique (15). When the subunits of corn CF₁-ATPase were separated by SDS-PAGE followed by transfer to nitrocellulose, antibody reacted with the α , β , γ , δ , and ϵ subunits of the enzyme. Thus, specific polyclonal antibodies appeared to be present for all subunits.

Production and Characterization of Monoclonal Antibodies. Four stable hybridoma cell lines producing antibody against spinach CF₁-ATPase have been established. Two hybridoma lines were found to produce antibody of the IgM class, and thus proved unsuitable for competitive binding assays due to the large number of antigen combining sites on each antibody molecule. In addition, attempts to determine the subunit specificity of these IgM antibodies were unsuccessful because they did not bind to nitrocellulose transfer blots. Of the two remaining cell lines, one produced IgG₁ (7D10) and the other produced IgG_{2A} (8C4). Antibodies produced by these two cell lines were suitable for nitrocellulose blotting and competitive inhibition studies. Subclones of the monoclonal antibody designated 7D10 were found to be specific for an antigenic site on the β subunit of spinach CF₁-ATPase by nitrocellulose blotting, and subclones of the monoclonal antibody designated 8C4 reacted only with the γ subunit.

Comparison of Antigenic Determinants. Specific polyclonal antibodies against corn CF₁-ATPase, and monoclonal antibodies against spinach CF₁-ATPase were utilized in solid phase competitive binding assays to generate information about the antigenic sites of corn mitochondrial F₁-ATPase in relation to other mitochondrial and chloroplastic enzymes. The binding specificity of polyclonal antisera raised against corn CF₁-ATPase appeared to be identical for both corn and spinach CF₁-ATPase (Fig. 2). Prior incubation of this antibody with either corn or spinach CF₁-ATPase in solution was effective in inhibiting binding of the antibody to solid-phase enzyme. Fifty per cent inhibition occurred at 0.6 μ g/ml of either corn or spinach CF₁-ATPase. Antigenic similarity was also shown between the chloroplastic and mitochondrial forms of the enzyme, as evidenced by the inhibition curves for the beef heart, corn mitochondrial and *E. coli* enzymes. However, neither the mitochondrial nor bacterial enzymes were as effective as the chloroplastic enzymes in preventing antibody binding to the solid phase, which indicated substantial differences must exist between the antigenic determinants of the chloroplastic and mitochondrial enzymes.

Competitive binding curves using the monoclonal antibody

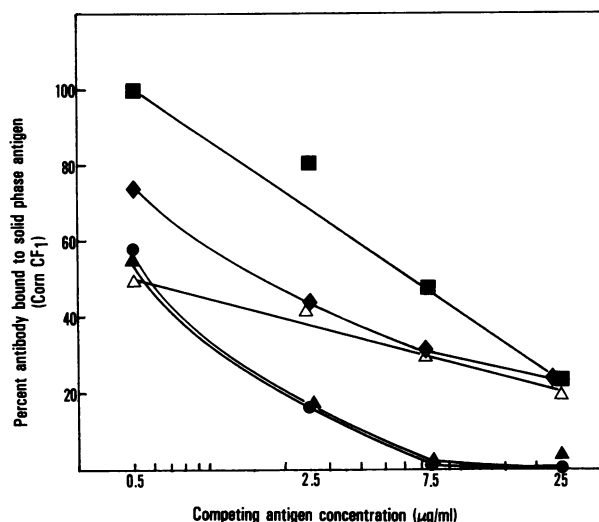


FIG. 2. Binding specificities of rabbit antibodies to corn chloroplastic CF₁-ATPase. Limiting concentrations of antibody were incubated in solution with competing antigens (▲), corn CF₁; (●), spinach CF₁; (■), corn mitochondrial F₁; (◆), beef heart mitochondrial F₁; (Δ), *E. coli* F₁ at the concentrations shown for 20 h at 4°C. Inhibition of antibody binding by the competing ATPases was then determined by incubation of the ATPase-antibody complexes with solid phase corn CF₁ at 5 μ g/ml (attached to vinyl microtiter plates) for 2 h. After rinsing to remove unbound protein, affinity purified goat antirabbit Ig conjugated to alkaline phosphatase was incubated overnight, followed by 4 mM dinitrophenyl phosphate for 1 h at 25°C. The absorbance of competing antigen (in PBS) was used to determine maximum antibody binding.

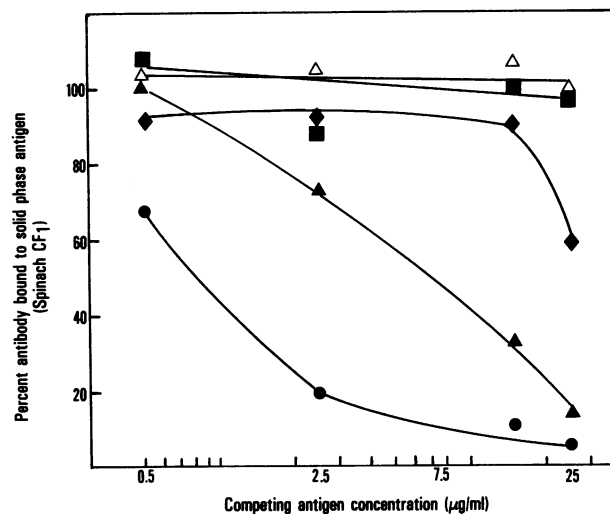


FIG. 3. Binding specificities of monoclonal antibody 8C4 (γ subunit specific). Limiting concentrations of antibody were incubated in solution with competing antigens (●), spinach CF₁; (▲), corn CF₁; (■), corn mitochondrial F₁; (◆), beef heart mitochondrial F₁; (Δ), *E. coli* F₁ at the concentrations shown for 20 h at 4°C. Binding inhibition was then determined by incubation of the antigen-antibody solutions with solid phase spinach CF₁ at 5 μ g/ml (attached to vinyl microtiter plates) for 2 h. Plates were then developed as stated in "Materials and Methods", and absorbance readings corrected with negative controls. The absorbance readings of antibody bound to the solid phase in the absence of competing antigen (in PBS) was used to determine maximum antibody binding.

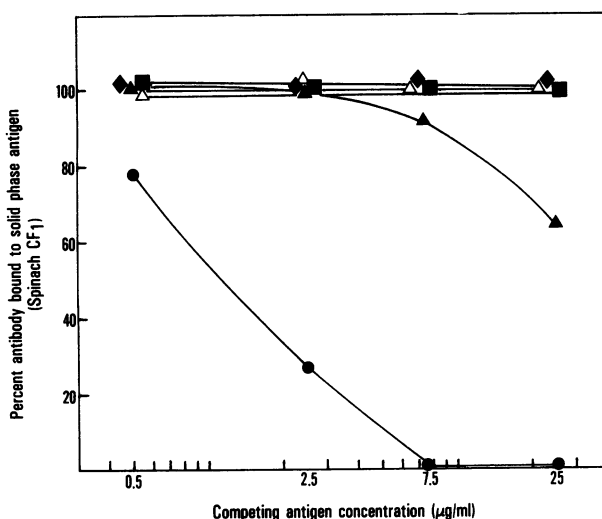


FIG. 4. Binding specificities of monoclonal antibody 7D10 (specific for β subunit). Limiting concentrations of antibody (culture fluid diluted in BSA-PBS) were incubated in solution with competing antigens (●), spinach CF₁; (▲), corn CF₁; (■), corn mitochondrial F₁; (◆), beef heart mitochondrial F₁; (△), *E. coli* F₁ at the concentrations shown for 20 h at 4°C. Inhibition of antibody binding to solid-phase spinach CF₁ was determined as described in Figure 3.

8C4, which is specific for a single antigenic determinant on the γ subunit of CF₁-ATPase, are shown in Figure 3. When the homologous enzyme (spinach CF₁-ATPase) was used as the competing antigen in solution, an inhibition binding curve was obtained that closely resembled those observed with homologous enzyme and polyclonal antibody (Fig. 2). Fifty per cent inhibition occurred at 0.8 $\mu\text{g/ml}$ competing antigen. Thus, competition for the monoclonal antibody between homologous soluble enzyme and enzyme bound to the solid phase was similar to that observed with the polyclonal antibodies. However, when the other ATPases were used as competitive antigens, they were much less effective. Corn CF₁-ATPase was able to compete for antibody binding with the solid phase spinach enzyme, but a high concentration (7 $\mu\text{g/ml}$) of competing antigen was necessary to obtain 50% binding inhibition. The mitochondrial enzymes, on the other hand, including corn F₁-ATPase, exhibited almost no binding to the antibody (Fig 3).

Competitive binding curves for the monoclonal antibody designated 7D10, which was specific for an antigenic determinant on the β subunit of spinach CF₁-ATPase, are shown in Figure 4. This antibody appears to be extremely specific. Even the chloroplastic enzyme from corn was not able to compete with the solid-phase spinach enzyme for antibody binding, and 50% binding inhibition was not achieved at even the highest concentration of competing antigen (25 $\mu\text{g/ml}$). The mitochondrial enzymes were totally unable to bind to this antibody under the conditions of the assay.

DISCUSSION

The F₁-ATPase from corn mitochondria has been isolated and purified by a chloroform-release technique and (NH₄)₂SO₄ precipitation. The chloroform-release method first reported by Beechey *et al.* (2) is currently being used for isolation of F₁-ATPase from many different sources but we have found that the conditions of extraction of corn mitochondrial F₁-ATPase from SMP had to be closely controlled in order to obtain reproducibly good results. The modification of the chloroform treatment described in "Materials and Methods" provides those conditions. Our method of isolation of corn mitochondrial F₁-ATPase is

simpler than the method reported by Hack and Leaver (8); it does not require the sucrose gradient centrifugation or DEAE-Sephadex (or cellulose) chromatography steps. This modification can serve as a standard for isolation of F₁-ATPases from other plant sources. In addition, the possibility that contaminating ATPases of chloroplastic, plasma membrane origin, or soluble nonspecific phosphatases were extracted by this procedure has been eliminated by kinetic analyses using heat, octylglucoside, vanadate, and NaN₃.

A careful comparison of apparent mol wt of the subunits of corn mitochondrial F₁-ATPase with ATPases from bacterial, mitochondrial, and chloroplastic sources has revealed some significant differences in the mitochondrial enzyme from corn (Fig. 1; Table I). While the establishment of proper mol wt for the α , β and γ subunits of corn mitochondrial F₁-ATPase from SDS-PAGE was straightforward, the proper identification of the δ and ϵ subunits of the corn enzyme was more difficult. The mol wt of the δ subunit (22×10^3) of corn F₁-ATPase appears to be close to that of the δ subunit of the *E. coli* and chloroplastic enzymes, rather than to the mitochondrial enzyme. The ϵ subunit (12×10^3) may be analogous to the ϵ subunit of beef F₁-ATPase (10×10^3) or corn chloroplastic F₁-ATPase (15.5×10^3). The recent work of Walker *et al.* (27) revealed that bovine heart oligomycin-sensitivity conferring protein (OSCP) is related to δ subunits of *E. coli* and bacterial ϵ is homologous to δ subunits of mammalian F₁-ATPase. The questions thus raised regarding the exact determination of the counterparts for δ and ϵ subunits of corn mitochondrial F₁-ATPase as well as the stoichiometry of the small subunits are currently under investigation by us. The subunit mol wt of corn F₁-ATPase determined in the present study agree quite closely with those determined recently by Hack and Leaver (8), except that they identified an 8,000 mol wt polypeptide as the ϵ subunit, whereas our work suggests that the ϵ subunit has a mol wt of 12,000, which is much less than 22,900 proposed for the ϵ subunit of *Vicia faba* (3).

Competitive binding assays were used in conjunction with both polyclonal and monoclonal antibodies to compare the antigenic sites of corn mitochondrial F₁-ATPase with those from other sources. Competitive binding assays utilizing specific polyclonal antisera (Fig. 2) yield information generated from a large number of antibody molecules differing in affinities and specificities. The advantage of using a polyclonal antiserum is that it is possible to obtain an overall antigenic picture of complex enzymes such as the mitochondrial and chloroplastic ATPases, each having many different antigenic sites distributed among the five subunits. Thus, an overall picture of the similarities and differences between the ATPases from diverse sources was obtained. The binding data shown in Figure 2 demonstrate a very high degree of antigenic similarity between the chloroplastic CF₁-ATPases from corn and spinach, as might be expected. Cross-reaction between the chloroplastic and mitochondrial enzymes was also apparent, although the degree of binding inhibition was significantly less. This large amount of cross-reaction using polyclonal antisera was expected since a high degree of sequence homology (at least for the β subunit) between ATPases from *E. coli* and both beef heart (19) and spinach chloroplast (30) has been determined.

In contrast to polyclonal antisera, monoclonal antibodies are highly specific for a single antigenic determinant. Thus information may be obtained about a very specific part of the polypeptide chain (*i.e.*, in the vicinity of a single antigenic determinant), and comparisons between enzymes from diverse species will reflect differences in that part of the molecule only. The disadvantage of monoclonal antibodies used in this context, of course, is that a complete antigenic map of a complex enzyme containing five subunits would require the generation of a large number of monoclonal antibodies. Hopefully, this will be accom-

plished in the future. The monoclonal antibodies used in this study, however, have demonstrated their potential for antigenic mapping. The extreme specificity of the two monoclonal antibodies to sites on the β and γ subunits of spinach CF₁-ATPase have demonstrated that although the chloroplastic enzymes from corn and spinach are antigenically very similar, they are by no means identical, at least at these two sites (Figs. 3 and 4). In addition, these monoclonal antibodies have demonstrated that these two antigenic regions are either changed substantially or totally absent from the mitochondrial F₁-ATPases. The extreme specificity of the monoclonal antibody which recognizes an antigenic site on the β subunit (7D10) was somewhat surprising, considering the highly conserved nature of the β subunit (19, 30). The fact that the monoclonal antibody which is directed against an antigenic site on the γ subunit of spinach CF₁-ATPase (8C4) did not react with the γ subunit of *E. coli*, beef heart, or corn mitochondrial F₁-ATPases may be a reflection of the smaller size of the later polypeptides (Table I). This antigenic determinant may be totally absent from the smaller subunits of *E. coli* and mitochondrial enzymes.

In summary, the investigation of corn mitochondrial F₁-ATPase isolated in this study has shown it to be similar in many respects to the more intensively studied mammalian mitochondrial F₁-ATPases with regard to kinetic parameters and subunit mol wt, although it is by no means identical. In addition, reactions with polyclonal and monoclonal antibodies have suggested that the corn mitochondrial F₁-ATPase isolated in this study is antigenically more similar to mitochondrial enzymes from diverse sources than to the chloroplastic enzyme isolated from the same species of plant.

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LITERATURE CITED

- AMES BN 1966 Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol* 8: 115–118
- BEECHY RB, SA HUBBARD, PE LINNETT, AD MITCHELL, EA MUNN 1975 A simple and rapid method for the preparation of adenosine triphosphatase from submitochondrial particles. *Biochem J* 148: 533–537
- BOUTRY M, M BRIQUET, A GOFFEAU 1983 The α subunit of a plant mitochondrial F₁-ATPase is translated in mitochondria. *J Biol Chem* 258: 8524–8526
- BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254
- GALLAGHER SR, RT LEONARD 1982 Effect of vanadate, molybdate and azide on membrane associated ATPases and soluble phosphatase activities of corn roots. *Plant Physiol* 70: 1335–1340
- GISS B, J ANTONIOU, G SMITH, J BRUMBAUGH 1982 A method for culturing chick melanocytes: the effect of BRL-3A cell conditioning and related additives. *In Vitro* 18: 817–826
- GRUBMEYER C, M SPENCER 1979 Effects of anions on a soluble ATPase from mitochondria of pea cotyledons. *Plant Cell Physiol* 20: 83–91
- HACK E, CJ LEAVER 1983 The α -subunit of the maize F₁-ATPase is synthesized in the mitochondrion. *EMBO J* 2: 1783–1789
- IWASAKI Y, T ASAHI 1983 Purification and characterization of the soluble form of mitochondrial adenosine triphosphatase from sweet potato. *Arch Biochem Biophys* 227: 164–173
- KANAZAWA H, M FUTAI 1982 Structure and function of H⁺-ATPase: What we have learned from *E. coli* H⁺-ATPase. *In* E Carafoli, A Scarpa, eds, *Transport ATPases*, Annals of the New York Academy of Sciences, New York, pp 45–64
- KEARNEY JF, A RADBRUCH, B LIESEGANG, K RAJEWSKY 1979 A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cells. *J Immunol* 123: 1548–1550
- LIEN S, E RACKER 1971 Preparation and assay of chloroplast coupling factor CF₁. *Methods Enzymol* 23: 547–555
- MORONEY JV, L LOPRESTI, BF MCEWEN, RE MCCARTY, GG HAMMES 1983 Mr-Value of chloroplast coupling factor 1. *FEBS Lett* 158: 58–62
- NELSON N 1981 Proton ATPase of chloroplasts. *In* D Sanadi, ed, *Current Topics in Bioenergetics*, Vol 11. Academic Press, New York, pp 1–33
- O'CONNOR CG, LK ASHMAN 1982 Application of the nitrocellulose transfer technique and alkaline phosphatase conjugated anti-immunoglobulin for determination of the specificity of monoclonal antibodies to protein mixtures. *J Immunol Methods* 54: 267–271
- OI VT, LA HERZENBERG 1980 Immunoglobulin-producing hybrid cell lines. *In* B Mishell, S Shiigi, eds, *Selected Methods in Cellular Immunology*, WH Freeman and Co, San Francisco, pp 351–367
- PENEFSKY HS 1977 Reversible binding of P_i by beef heart mitochondrial adenosine triphosphate. *J Biol Chem* 252: 2891–2899
- PICK U, S BASSILIAN 1983 The effect of octylglucoside on the interactions of chloroplast coupling factor 1 (CF₁) with adenine nucleotides. *Eur J Biochem* 133: 289–298
- RUNSWICK MJ, JE WALKER 1983 The amino acid sequence of the β -subunit of ATP synthetase from bovine heart mitochondria. *J Biol Chem* 258: 3081–3089
- RYRIE IJ, A GALLAGHER 1979 The yeast mitochondrial ATPase complex. Subunit composition and evidence for a latent protease contaminant. *Biochim Biophys Acta* 545: 1–14
- SENIOR AE, JC BROOKS 1971 The subunit composition of the mitochondrial oligomycin-insensitive ATPase. *FEBS Lett* 17: 327–329
- SENIOR AE, JC BROOKS 1970 Studies on the mitochondrial oligomycin-insensitive ATPase I. An improved method of purification and the behavior of the enzyme in solutions of various depolymerizing agents. *Arch Biochem Biophys* 140: 257–266
- SENIOR AE, JG WISE 1983 The proton-ATPase of bacteria and mitochondria. *J Membr Biol* 73: 105–124
- SPITSBERG VL, JE BLAIR 1977 Evidence supporting the identity of beef heart mitochondrial chloroform-released adenosine triphosphatase (ATPase) with coupling factor 1. *Biochim Biophys Acta* 460: 136–141
- SPITSBERG VL, HP MORRIS, SHP CHAN 1979 Isolation and comparative studies of mitochondrial F₁-ATPase from morris hepatoma and rat liver. *Arch Biochem Biophys* 195: 136–144
- STOCKER JW, F MALAVASI, M TRUCCO 1981 Enzyme immunoassay for the detection of hybridoma products. *In* I Lefkowitz, B Pernis, eds, *Immunological Methods*, Vol 2. Academic Press, New York, pp 299–308
- Walker JE, MJ Runswick, M Saraste 1981 Subunit equivalence in *E. coli* and bovine heart mitochondrial F₁F₀ ATPases. *FEBS Lett* 146: 393–396
- YOSHIDA K, Y TAKEUCHI 1970 Properties of a soluble ATPase from castor bean endosperm mitochondria. *Plant Cell Physiol* 11: 403–409
- YOUNIS HM, GD WINGET, E RACKER 1977 Requirement of the δ subunit of chloroplast coupling factor 1 for photophosphorylation. *J Biol Chem* 252: 1814–1818
- ZURAWSKI G, W BOTTOMLEY, PR WHITFIELD 1982 Structures of the genes for the β and ϵ subunits of spinach chloroplast ATPase indicate a dicistronic mRNA and an overlapping translation stop/start signal. *Proc Natl Acad Sci USA* 79: 6260–6264