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On the Subunit Composition of the *Neurospora* Plasma Membrane H^+-ATPase*

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The resolution-reconstitution approach has been employed in order to gain information as to the subunit composition of the *Neurospora* plasma membrane H^+-ATPase. Proteoliposomes prepared from sonicated asolectin and a highly purified, radiolabeled preparation of the 105,000-dalton hydrolytic moiety of the H^+-ATPase by a freeze-thaw procedure catalyze ATP hydrolysis-dependent proton translocation as indicated by the extensive 9-amino-6-chloro-2-methoxyacridine fluorescence quenching that occurs upon the addition of MgATP to the proteoliposomes, and the reversal of this quenching induced by the H^+-ATPase inhibitor, vanadate, and the proton conductors, carbonyl cyanide *m*-chlorophenylhydrazone and nigericin plus K^+. ATP hydrolysis is tightly coupled to proton translocation into the liposomes as indicated by the marked stimulation of ATP hydrolysis by carbonyl cyanide *m*-chlorophenylhydrazone and nigericin plus K^+. The maximum stimulation of ATPase activity by proton conductors is about 3-fold, which indicates that at least two-thirds of the hydrolytically active ATPase molecules present in the reconstituted preparation are capable of translocating protons into the liposomes. Furthermore, as estimated by the extent of protection of the reconstituted 105,000-dalton hydrolytic moiety against tryptic degradation by vanadate in the presence of Mg^{2+} and ATP, the fraction of the total population of ATPase molecules that are hydrolytically active is at least 91%. Taken together, these data indicate that at least 61% of the ATPase molecules present in the reconstituted preparation are able to catalyze proton translocation. This information allows an estimation of the amount of any polypeptide in the preparation that must be present in order for that polypeptide to qualify as a subunit that is required for proton translocation in addition to the 105,000-dalton hydrolytic moiety, and an analysis of the radiolabeled ATPase preparation by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and urea rules out the involvement of any such polypeptides larger than 2,500 daltons. This indicates that the *Neurospora* plasma membrane H^+-ATPase has no subunits even vaguely resembling any that have been found to be associated with other transport ATPases and that if this enzyme has any subunits at all other than the 105,000-dalton hydrolytic moiety, they must be very small.

It is now fairly clear that most of the membranous structures in cells contain ion-translocating ATPases that function either to synthesize ATP in response to a transmembrane electrochemical proton gradient or, conversely, to hydrolyze ATP and generate transmembrane ion gradients which are in turn used to energize solute transport and a variety of cellular signalling mechanisms as well. The ion-translocating ATPases that have been characterized thoroughly enough to permit classification can be subdivided into two major categories. The first of these comprises the proton-translocating $F_1F_0H^+$-ATPase/ATP synthases of mitochondria, bacteria, and chloroplasts, which are characterized by a relatively complex subunit structure, the absence of a covalent phosphoryl-enzyme intermediate in the catalytic cycle, and ready reversibility for ATP synthesis in the presence of a transmembrane electrochemical proton gradient of sufficient magnitude (1). The second category of ion-translocating ATPases is the aspartylphosphoryl-enzyme intermediate family, which is characterized by a more simple subunit structure, the existence of a catalytically competent covalent phosphoryl-enzyme intermediate, apparently less ready reversibility under physiological conditions, and a broader range of transported ions. Well characterized members of this class include the Na^+/K^+-translocating ATPase of most animal cell plasma membranes (2), the Ca^{2+}-translocating ATPase of sarcoplasmic reticulum (3), the H^+/K^+-translocating ATPase of gastric mucosa (4), and the electrogenic proton-translocating ATPase of the fungal plasma membrane (5, 6, 10, 11). The fungal enzyme has been the primary subject of experimental attention in this laboratory for several years (7–14).

An understanding of the molecular mechanisms by which the ion-translocating ATPases transduce the chemical energy of ATP hydrolysis into transmembrane electrical and/or chemical ionic potential differences is a major goal of contemporary biological science. An important prerequisite to the attainment of such a goal for any transport ATPase is knowledge of the subunit composition[1] of that enzyme, but unfortunately, such knowledge has not been easy to obtain. Although it is possible to state that the subunit composition of an ATPase is relatively complex or relatively simple, a precise description of the number of individual subunits that constitute a functional transport unit is available for virtually none of these enzymes. A possible exception is the bacterial $F_1F_0H^+$-ATPase/ATP synthase (1). The major impedance to progress along these lines in the case of F_1F_0-ATPases has been the great complexity of these enzymes; in the case of the aspartylphosphoryl-enzyme intermediate ATPase family, confusion has arisen primarily from the recognition that small (M_r

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[1] The term "subunit composition" refers only to the types of different subunits present in a given enzyme and not to the relative stoichiometries of such subunits.

~ 6,000–13,600) proteolipids are present in most of the standard ATPase preparations and attendant suggestions that such proteolipids may play a functional role in the transport process (15–17).

Theoretically, the resolution-reconstitution approach, pioneered for membrane proteins by Racker and his associates (18), is an ideal approach to determining the subunit composition of the transport ATPases. In principle, it should be possible to simply resolve a functional ATPase preparation into its individual components and then assay for necessary components on the basis of reconstitutivity of function. In practice, however, this approach has not been lucrative, probably because no conclusions can be drawn without an estimation of the reconstitution efficiency,[2] and such information is quite difficult to acquire. In this article, we report reconstitution of the *Neurospora* plasma membrane H⁺-ATPase, describe an experimental approach for estimating the efficiency of the reconstitution process, and provide quantitative information as to the composition of the ATPase preparation used for the reconstitution studies. In light of the results obtained, the probable subunit composition of this enzyme is then discussed.

EXPERIMENTAL PROCEDURES

Growth of Radiolabeled Cells—Cells of the cell wall-less *fz, sg, os*-1 strain of *Neurospora crassa* were maintained by daily transfer of 1 ml of an overnight culture into 50 ml of fresh medium followed by rotary shaking (150 rpm) at 30 °C. The maintenance growth medium was Vogel's N medium (19) supplemented with 2% (w/v) D-mannitol, 0.75% (w/v) yeast extract, and 0.75% (w/v) nutrient broth. The day before the ATPase isolation procedure was carried out, the cells contained in 20 ml of an overnight culture were pelleted by centrifugation (1100 × *g*, 20 min, room temperature) under sterile conditions and resuspended in 10 ml of radioactive growth medium (see below), and the resulting cell suspension was added to an additional 490 ml of radioactive growth medium. The culture was then grown overnight with rotary shaking (150 rpm) at 30 °C with a stream of sterile O_2 passing over the surface at a flow rate of 1.5 l/min. During this time, the optical density of the culture increased 6-fold. The radioactive growth medium was Vogel's N medium supplemented with 2% (w/v) D-mannitol, 0.1% (w/v) yeast extract, 0.1% (w/v) D-fructose, 0.004% (w/v) L-alanine, 0.0027% (w/v) L-arginine, 0.0034% (w/v) L-leucine, 0.0018% (w/v) L-phenylalanine, 0.0025% (w/v) L-proline, 0.002% (w/v) L-serine, 0.0014% (w/v) L-tyrosine, 0.0006% (w/v) L-valine, and 250 μCi of D-[U-^{14}C]fructose (specific activity of 326 Ci/mol), 125 μCi of L-U-^{14}C-amino-acid mixture (specific activity of approximately 55 Ci/g atom of carbon in each individual amino acid), 2.2 μCi of L-[3-^{14}C]tryptophan (specific activity of 54.4 Ci/mol), and 2 μCi of L-[^{35}S] methionine (specific activity of >400 Ci/mmol) per 500 ml. In this medium, all principal carbon sources (*i.e.* fructose and 17 different L-amino acids)[3] are present with an approximate specific activity of 375–500 μCi/g.

Purification of the Plasma Membrane H⁺-ATPase—The H⁺-ATPase was purified from the radiolabeled cells as described (20), but on a scale 30 times smaller. The only major deviation from strict proportionality was that a 32-ml glycerol density gradient was used. The two fractions containing the greatest amount of ATPase activity were pooled and stored at −20 °C. The specific activity of the ATPase in the pooled fractions was 51 μmol of P_i released/min/mg of protein, using the assay described previously (20) (10 μl of Folch fraction I suspension).

Preparation of Asolectin Liposomes—Asolectin was purified by the procedure of Kagawa and Racker (21) and stored under N_2 at −20 °C as a chloroform solution (116 mg/ml). At least one day before the liposomes were prepared, 0.86 ml of the asolectin solution (100 mg) was placed in a 15-ml Corex tube, dried under a stream of N_2, and stored *in vacuo* at room temperature. The liposomes were prepared by sonicating the 100 mg of dried asolectin with 2 ml of Buffer I (10 mM MES[4] containing 50 mM potassium acetate (pH 6.8 with KOH)) under N_2 for 30 min in a cylindrical bath type sonicator (Special Ultrasonic Cleaner, Laboratory Supplies Co., Inc., Hicksville, NY). The opalescent mixture was then centrifuged (25,000 × *g*, 30 min, 4 °C) and the supernatant fluid used as a source of liposomes.

Reconstitution of the H⁺-ATPase—The ATPase was reconstituted into asolectin liposomes by a procedure closely modeled after the procedure of Dufour *et al.* (22). Two hundred and forty μl of the liposome suspension was mixed with 340 μl of Buffer I and 20 μl of the purified ATPase solution (2.5 μg of protein) in a polypropylene tube on ice. The tube was then capped and placed in liquid N_2. Just prior to use in the various assays, the mixture was thawed in a 30 °C water bath for 2.5 min.

Measurement of ATP Hydrolysis Catalyzed by the Reconstituted H⁺-ATPase—Assay mixtures contained 100 μl of the reconstituted ATPase preparation, 850 μl of Buffer I, 50 μl of 0.2 M disodium ATP/$MgSO_4$ (pH 6.8 with Tris), and the indicated additions in a total volume of 1 ml. The reactions were started by the addition of the reconstituted ATPase preparation. After incubation for 10 min at 30 °C, the reactions were terminated by the addition of 3 ml of room temperature 7% (w/v) sodium dodecyl sulfate solution followed by the addition of 400 μl of 2.5% (w/v) $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 5 N H_2SO_4 and then the addition of 40 μl of a solution containing 1-amino-2-naphthol-4-sulfonic acid (1.92 mg/ml), $Na_2S_2O_5$ (11.54 mg/ml) and Na_2SO_3 (11.54 mg/ml). The samples were mixed by vortexing after each addition. After 20 min at room temperature, the $A_{660\ nm}$, 1 cm was determined, and after subtraction of the values obtained in appropriate zero time controls, the amount of P_i liberated during the incubations was estimated by comparison with a KH_2PO_4 standard treated in identical fashion.

ACMA Fluorescence Assay—Conditions of the ACMA fluorescence assay were virtually identical to those of the ATPase assay except that the assays were carried out at room temperature (~22 °C). 1.7 ml of Buffer I was added to a cuvette followed by 2 μl of ACMA solution (0.3 mg/ml in methanol), 200 μl of the reconstituted ATPase preparation, and, except for the experiment described by Fig. 1, *trace D*, 1 μl of valinomycin solution (0.5 mg/ml in dimethyl sulfoxide). It was found to be important to add valinomycin after the liposomes. The cuvette was then placed in the sample chamber of a Perkin-Elmer Hitachi MPF-2A recording spectrophotofluorometer, the chamber closed, and the recorder adjusted to 100% fluorescence by varying the emission slit width. Subsequent additions (see legend to Fig. 1) were made via a light-tight port at the top of the chamber. Mixing was accomplished magnetically. Fluorescence was measured at 90° with a Farrand interference filter (peak wavelength, 515 nm; half-band width, 14 nm; 40% transmission) in the path of the emission beam. The excitation wavelength was 412 nm and light emission was monitored at 520 nm.

Treatment of the Reconstituted H⁺-ATPase with Trypsin under Several Conditions and SDS-PAGE Analysis of the Tryptic Cleavage Patterns—Each of two 90-μl aliquots of the reconstituted H⁺-ATPase preparation was mixed on ice with 5 μl of 0.2 M disodium ATP/$MgSO_4$ (pH 6.8 with Tris), 1 μl of 10 mM Na_3VO_4, and 2 μl of 1 mM HCl (samples 1 and 2). Another 90-μl aliquot was mixed on ice with 5 μl of 0.2 M Na_2SO_4 in 0.2 M H_3PO_4 (pH 6.8 with Tris), 1 μl of 10

[2] The absolute requirement for an estimation of the reconstitution efficiency in considerations of subunit composition is best explained by an example. Consider a highly purified ATPase preparation that contains 99% by mass of a 100,000-dalton hydrolytic moiety and 1% by mass of a 10,000-dalton proteolipid. In this case, 1 mg of the preparation contains about 6×10^{15} molecules of the hydrolytic moiety and about 6×10^{14} molecules of the proteolipid. Reconstitution of the enzyme's transport function with an efficiency of 100% virtually rules out the participation of the proteolipid in the transport process because there are roughly 10 times more functional transport molecules than there are proteolipid molecules. On the other hand, if the reconstitution efficiency is only 10%, no conclusion as to the role of the proteolipid can be drawn because the number of functional transport molecules and the number of proteolipid molecules is roughly equivalent, leaving open the possibility that 10% of the molecules of the hydrolytic moiety reconstitute in 1:1 stoichiometry with all of the proteolipid molecules.

[3] The approximate amino acid composition of yeast extract was kindly provided by Difco.

[4] The abbreviations used are: MES, 2-(*N*-morpholino)ethanesulfonic acid; ACMA, 9-amino-6-chloro-2-methoxyacridine; SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; Δp, transmembrane electrochemical protonic potential difference; Δψ, transmembrane electrical potential difference, ΔpH, transmembrane pH difference; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

mM Na_3VO_4, and 2 μl of 1 mM HCl (sample 3). All three samples were then preincubated at 30 °C for 5 min with shaking in a water bath, after which samples 2 and 3 each received 2 μl of trypsin (L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) solution (1 mg/ml in 1 mM HCl). The samples were all shaken for an additional 3 min, and the reactions were then terminated by the addition of 0.5 ml of ice-cold 10% (w/v) trichloroacetic acid. Sample 1 then received 2 μl of the trypsin solution. Sample 1 is referred to elsewhere in the text as "control," sample 2 as "trypsin-treated, Mg^{2+}-vanadate-ATP-protected," and sample 3 as "trypsin-treated." The samples were then centrifuged (1100 × *g*, 30 min, 4 °C), the pellets resuspended in 0.6 ml of ice-cold 10% (w/v) trichloroacetic acid, and the resulting suspensions centrifuged again under the same conditions. The pellets were then dissolved in 50 μl of SDS-PAGE disaggregation buffer (0.125 M Tris (pH 6.8 with H_3PO_4) containing 5% (w/v) SDS, 25% (w/v) glycerol, 2 mM EDTA, 0.1% (w/v) dithiothreitol, and a small amount of pyronin Y as a tracking dye) and allowed to stand for 1 h at room temperature, and the entire samples were then subjected to slab SDS-PAGE as described (12) except that both the stacking and resolving gels contained 1% (w/v) SDS and 1 mM EDTA, the resolving gel was cast with 8.5% (w/v) acrylamide, the gel was 0.75 mm thick, 0.15% (w/v) ammonium persulfate and 0.025% (v/v) TEMED were used for polymerization of the resolving gel, and 0.13% (w/v) ammonium persulfate and 0.07% (v/v) TEMED were used for polymerization of the stacking gel. Electrophoresis was conducted at constant current (20 mA) for about 3 h in a Bio-Rad Model 220 gel electrophoresis apparatus. After electrophoresis, the gel was fixed overnight in methanol/acetic acid/water (5:1:5, v/v/v) and stained with silver according to the procedure of Oakley *et al.* (23). The densities of the ATPase bands were quantitated with the aid of an LKB 2202 Ultroscan Laser Densitometer interfaced with an Apple II computer programmed to integrate the densitometer signal.

SDS-PAGE of the Purified H⁺-ATPase Preparation—Ninety μl of the purified ATPase solution were mixed with an equal volume of disaggregation buffer containing 2.56% (w/v) SDS, 2.57% (v/v) β-mercaptoethanol, and 0.0257 M H_3PO_4 (pH 6.8 with Tris), and the resulting mixture was adjusted to 8 M urea by the addition of solid urea. The sample was then heated at 60 °C for 10 min followed by the addition of pyronin Y to 0.007% (w/v). Two hundred and twenty-two μl of the resulting sample (10.7 μg of protein) were then subjected to SDS-PAGE analysis in a cylindical tube gel (0.6 × 10 cm) according to the method of Merle and Kadenbach (24) (3.6 M urea and 13% (v/v) glycerol) except that the resolving and stacking gels were adjusted to their respective pH values with H_3PO_4. Electrophoresis was conducted at 70 V overnight, adjusted to 100 V the next morning, and allowed to run until the pyronin Y tracking dye was approximately 1 cm from the bottom of the gel. Polypeptide standards and radioactive phosphatidylinositol were prepared and electrophoresed in a similar fashion. The gels were water cooled (~15 °C) during the run. After electrophoresis, the gels were isolated by shattering the casting tubes, and the gels containing the polypeptide standards were stained in 0.25% (w/v) Kenacid blue R in methanol/acetic acid/H_2O (5:1:5, v/v/v) for 2 h at room temperature and then destained in 7.5% (v/v) acetic acid and 5% (v/v) methanol in water overnight at 37 °C. The gels containing the radiolabeled H⁺-ATPase and radioactive phosphatidylinositol were sliced into 1-mm segments with the aid of a Macrotome GTS gel slicer (Yeda Research and Development Co. Ltd., Rehovot, Israel), the slices transferred individually to scintillation vials containing 630 μl of NCS tissue solubilizer and 70 μl of H_2O, and the resulting mixtures incubated at 50 °C overnight (25) in capped vials. Ten ml of the Triton X-100/toluene (2:1, v/v) scintillation fluid described by Patterson and Greene (26) were then added to each vial, and the radioactivity was determined by liquid scintillation counting. Background radioactivity was determined by counting several slices of a gel that contained no sample.

Determination of Protein—Protein was estimated by the method of Lowry *et al.* (27) after precipitation of the protein by the deoxycholate/trichloroacetic acid method of Bensadoun and Weinstein (28). Bovine serum albumin was used as a standard.

Materials—The sources of most of the materials employed in these studies have been identified previously (9, 12, 13, 20). D-[U-^{14}C] Fructose, L-U-^{14}C-amino-acid mixture, L[3-^{14}C]tryptophan, and L-[^{35}S]methionine were from New England Nuclear. NCS tissue solubilizer and 1-stearoyl-2-[1-^{14}C]oleoylphosphatidylinositol (specific activity of 54 Ci/mol) were from Amersham Corp. Kenacid blue R and molecular weight range 2,512–16,949 polypeptide standards were from Gallard-Schlesinger Chemical Mfg. Corp. Somatostatin, L-tyrosylglycylglycyl-L-phenylalanyl-L-leucyl-L-arginyl-L-lysyl-L-arginine, valinomycin, and Folch fraction I lipids from bovine brain were from Sigma. Ultrapure urea was from Schwarz/Mann. ACMA was the generous gift of R. Kraayenhof, Biological Laboratory, Free University, Amsterdam, the Netherlands. All other reagents were of the highest commercially available grade.

RESULTS AND DISCUSSION

Fig. 1 shows the changes in fluorescence of the substituted aminoacridine, ACMA, that occur upon the addition of MgATP and several other agents to purified ATPase-bearing liposomes prepared as described under "Experimental Procedures." While the precise mechanism by which aminoacridine fluorescence changes are induced is the subject of some controversy (29–31), it is generally agreed that these molecules are useful qualitative indicators of an interior acid pH gradient (ΔpH) in vesicular systems (29–31). Fig. 1 *trace A* shows that the addition of MgATP to the proteoliposomes causes an instantaneous decrease in ACMA fluorescence, which is probably an artifact, followed by a marked, time-dependent additional quenching of much of the remaining fluorescence. Upon the addition of the ATPase inhibitor vanadate, the time-dependent fluorescence quenching is totally reversed. These results indicate that at least some of the reconstituted H⁺-ATPase molecules are capable of catalyzing ATP-dependent proton translocation into the interior of the proteoliposomes, thus generating an inside acid ΔpH. This conclusion is corroborated in *trace B* which shows that nigericin, which dissipates pH gradients via an electroneutral H^+/K^+ exchange (K^+ is present in the buffer), also reverses the fluorescence quenching, and in *trace C*, which shows that the quenching is also reversed by the protonophore, CCCP. The CCCP response is complicated by the fact that CCCP itself partially quenches the ACMA fluorescence (data not shown). However, reversal is complete as evidenced by the fact that nigericin does not bring about further reversal of the fluorescence quenching. *Trace D* shows an experiment similar to the others, but with valinomycin added after MgATP instead of before it as in *traces A–C*, and *E*. This trace demonstrates that the great majority of the ATP hydrolysis-dependent fluorescence quenching response is not seen in the absence of valinomycin.[5] *Trace E* is included to show that dimethyl sulfoxide, the ionophore vehicle used in these experiments, does not affect

[5] There are at least two feasible explanations for the valinomycin requirement. First, because proton translocation catalyzed by the ATPase is electrogenic (7), valinomycin may be required to prevent membrane potential ($\Delta\psi$) generation by catalyzing countermovement of K^+, thus allowing electrically unimpeded proton translocation and maximum ΔpH generation, which is reflected as maximum ACMA fluorescence quenching. Such an explanation was suggested by Dufour *et al.* (22) on the basis of similar results obtained with the reconstituted yeast plasma membrane ATPase. While this explanation may well be correct, it should be kept in mind that it assumes, without evidence, that $\Delta\psi$ predominates the transmembrane electrochemical protonic potential difference (Δp) in these proteoliposomes in the absence of valinomycin. If this assumption is not valid, as would be the case if the proteoliposomes are unexpectedly permeable to anions or cations or are extremely small, then the valinomycin requirement cannot be explained in this way. An alternative or additional explanation, which does not require assumptions as to the values of $\Delta\psi$ and ΔpH, centers upon the probe response. Because electrostatic binding of the ACMA molecules to the surface of the proteoliposomes appears to be necessary for the fluorescence quenching response (30), it may be that valinomycin is needed to dissipate an inside positive membrane potential (of unspecified magnitude relative to ΔpH) that would otherwise inhibit binding of the cationic ACMA molecules. Clearly, more information, beyond the scope of these experiments, will need to be obtained before firm conclusions as to the nature of the valinomycin effect can be drawn. Fortunately, for the purposes of the present argument, such information is unnecessary.

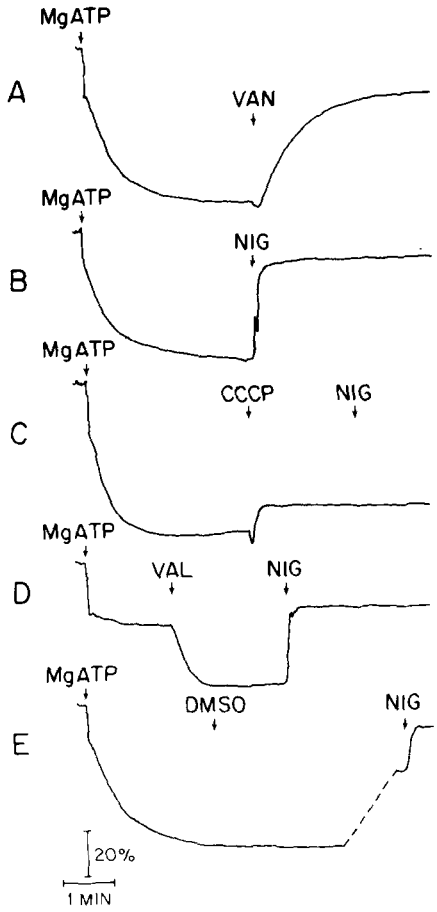

FIG. 1. **AMCA fluorescence quenching as an indicator of proton translocation catalyzed by the reconstituted H⁺-ATPase.** See "Experimental Procedures" for details. Where indicated, the following additions were made: MgATP, 100 μl of 0.2 M disodium ATP/$MgSO_4$ (pH 6.8 with Tris); vanadate (*VAN*), 20 μl of 0.1 M Na_3VO_4; nigericin (*NIG*), 2 μl of 5 mg/ml of nigericin in dimethyl sulfoxide; CCCP, 2 μl of 50 mM CCCP in dimethyl sulfoxide; valinomycin (*VAL*), 1 μl of 0.5 mg/ml valinomycin in dimethyl sulfoxide; dimethyl sulfoxide (*DMSO*), 2 μl of dimethyl sulfoxide. As described under "Experimental Procedures," in all of the experiments except the one described by *trace D*, valinomycin was added before the recordings began. The *dashed line* in *trace E* approximates the position of the recording pen over a period of 83 min, during which the chart drive was turned off.

the fluoresence quenching response and to show how stable the reconstituted proteoliposomes are. The fluorescence quenching signal decreases only slowly over the course of nearly 90 min. Although not shown, ATP hydrolytic activity measured under the same conditions is nearly linear for the same period of time. As a final point regarding this data, the reconstituted proteoliposomal membranes appear to be somewhat permeable to protons. This conclusion is drawn from the fact that after the addition of vanadate (*trace A*), the time-dependent fluorescence quenching is totally reversed in only a few minutes.

Given that some of the reconstituted H⁺-ATPase molecules do translocate protons into the proteoliposomes, if the proteoliposomal membrane is sufficiently impermeable to protons, those ATPase molecules might be expected to become inhibited when a sufficient Δp across the liposomal membrane is generated. If so, the activity of these molecules should be stimulated by agents that dissipate Δp. Table I shows that the reconstituted ATPase does indeed behave in a manner consistent with these notions. The specific ATP hydrolytic activity of the enzyme in the control samples is around 10.8

TABLE I

Effects of ionophores on ATP hydrolysis catalyzed by the reconstituted H⁺-ATPase

See "Experimental Procedures" for details of the ATPase assay. Entries are the averages of duplicate determinations. Where indicated, the following additions were included in the assay mixtures: dimethyl sulfoxide, 1 μl of dimethyl sulfoxide; valinomycin, 0.5 μl of 0.5 mg/ml of valinomycin in dimethyl sulfoxide; CCCP, 1 μl of 50 mM CCCP in dimethyl sulfoxide; nigericin, 1 μl of 5 mg/ml of nigericin in dimethyl sulfoxide.

Addition	Specific ATPase activity
	μmol of P_i released/ min/mg protein
None	10.8
Dimethyl sulfoxide	10.7
Valinomycin	15.2
CCCP	27.0
Nigericin	30.3
Valinomycin plus CCCP	30.8
Valinomycin plus nigericin	32.0
CCCP plus nigericin	24.4

μmol of P_i released per min/mg of protein, and this value is augmented to a small extent in the presence of valinomycin. The specific ATP hydrolytic activity is increased markedly to 27 in the presence of CCCP and even more markedly to 30.3 in the presence of nigericin. The effects of valinomycin plus CCCP or valinomycin plus nigericin are only roughly additive, and the combination of nigericin plus CCCP does no more than either ionophore alone. Although not shown, the ATPase can be activated to levels similar to those shown in Table I by substituting small amounts of Folch fraction I lipids from bovine brain for the asolectin liposomes in an otherwise similar assay. ATPase activated in this manner is not stimulated by CCCP or nigericin, which means that neither of these ionophores *per se* affect the ATPase activity.[6] Our interpretation of these results is as follows. The properly reconstituted ATPase molecules catalyze ATP hydrolysis-driven proton translocation into the liposomes, which leads to the generation of a Δp consisting of both Δψ and ΔpH of unspecified relative magnitudes. The Δp thus generated inhibits further proton translocation and hence ATP hydrolysis. The addition of valinomycin elicits K^+ efflux from the liposomes, which dissipates Δψ and allows additional proton translocation until a ΔpH roughly equivalent to the dissipated Δψ is generated. This results in a transient small increase in ATP hydrolysis. The low intraliposomal pH generated in the presence of valinomycin could also increase the proton permeability of the liposomes, which would also stimulate ATP hydrolysis. In any case, the effects of valinomycin are small. The addition of CCCP causes dissipation of most of the Δp, which allows proton translocation and ATP hydrolysis to proceed at a much higher rate. And finally, the addition of nigericin elicits an H^+/K^+ exchange that results in the dissipation of ΔpH, which again allows proton translocation and ATP hydrolysis to proceed much faster.[7]

[6] Presumably the ATPase activated by Folch fraction I lipids does not translocate protons into a sealed space, either because no ATPase-bearing proteoliposomes are formed with these lipids or because the proteoliposomes formed are leaky to protons.

[7] The marked stimulation by nigericin alone, and the lack of a large additional effect of valinomycin when added with nigericin may suggest that the ΔpH component predominates the protonic potential difference, for the reasons mentioned in Footnote 5. Alternatively, regardless of the relative magnitudes of Δψ and ΔpH, these results would also be expected if Δψ and ΔpH are not equipotent with respect to their effectiveness in reversing the ATPase reaction, with ΔpH being more effective than Δψ. That is to say, because intraliposomal protons are a product of the ATPase reaction, it stands to reason

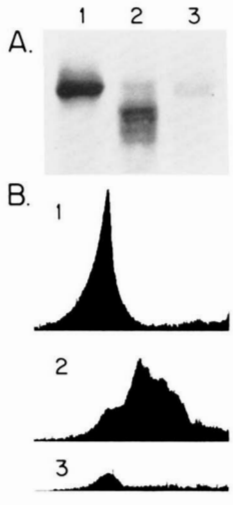

FIG. 2. **Differential trypsin sensitivity of the reconstituted H⁺-ATPase.** See "Experimental Procedures" for details regarding the preparation of the various samples. In *A* and *B*, 1 refers to the control sample, 2 refers to the trypsin treated, Mg^{2+}-vanadate-ATP-protected sample, and 3 refers to the trypsin-treated sample. *A*, photograph of the ATPase region of a silver-stained SDS-PAGE gel. *B*, densitometer tracings of the individual lanes of the same gel shown in *A*.

In addition to providing evidence that some of the ATPase molecules present in the reconstituted proteoliposome preparation are capable of generating a Δp of a magnitude sufficient to cause inhibition of ATP hydrolysis, the data presented in Table I provide an estimate of the fraction of the total population of hydrolytically active ATPase molecules that are involved in such activity. If the specific ATPase activity measured in the presence of valinomycin and nigericin is taken as 100%, then the maximally inhibited (control) specific activity is 33–34%. This means that at least two-thirds of the hydrolytically active ATPase molecules in the reconstituted preparation are inhibited by Δp, which indicates that they are capable of proton translocation. This is probably a minimum estimate because, as mentioned above, the reconstituted proteoliposomes are somewhat leaky to protons. The specific ATPase activity in the control samples is thus probably higher than it would be in less proton-permeable liposomes.

The data presented thus far provide a measure of the fraction of catalytically active ATPase molecules in the reconstituted proteoliposomes that are capable of proton translocation, but do not contribute any information as to the percentage of the total population of ATPase molecules that are catalytically active. Fig. 2 presents information bearing upon this point. We have previously reported evidence that the H⁺-ATPase undergoes several conformational changes during its catalytic cycle (13). This conclusion was based upon the characteristics of protection of the ATPase against tryptic degradation by a variety of ATPase ligands. Of the several ligands employed in those experiments, the most effective proved to be the ATPase inhibitor vanadate (in the presence of Mg^{2+}), and vanadate protection was further enhanced in the presence of ATP. Thus, in the absence of any ligand, the hydrolytic moiety of the ATPase ($M_r \sim 105{,}000$) in isolated plasma membranes is rapidly degraded by trypsin to small fragments, but in the presence of Mg^{2+}, vanadate, and ATP, the $M_r \sim 105{,}000$ ATPase is rapidly degraded to an enzymatically active $M_r \sim 95{,}000$ form, but further degradation (via an $M_r \sim 88{,}000$ form) occurs only slowly. As elaborated upon in that report, the evidence strongly suggested that vanadate, which is most likely a transition state analogue of the enzyme dephosphorylation reaction (36, 37), binds to the ATPase and "locks" it in the conformation that it normally assumes in the transition state that occurs during the enzyme dephosphorylation reaction. Fig. 2 shows that similar results are obtained with the purified, reconstituted H⁺-ATPase. In the absence of any ligand,[8] trypsin rapidly degrades the 105,000-dalton hydrolytic moiety to small fragments, but in the presence of Mg^{2+}, vanadate, and ATP, degradation is restricted to the generation of a $M_r \sim 95{,}000$ form and several slightly smaller forms (Fig. 2A). Fig. 2*B* shows densitometer tracings of the ATPase areas of the three lanes shown in Fig. 2*A*. The scans were performed on a track roughly ⅓ of the distance from the left-hand to the right-hand edge of each lane. Computer-assisted integration of these tracings, and correction for the fact that the control well is approximately 12% wider than the others, indicates that 91% of the ATPase is degraded by trypsin in the absence of ligands, and virtually 100% remains in the high molecular weight region when the trypsin treatment is carried out in the presence of Mg^{2+}, vanadate, and ATP. If the densities are corrected for mass loss in the clipped forms, the sum is greater than 100%, which strengthens the conclusion that the ATPase is completely protected except for minor nicking. After subtraction of 9% of the total, which represents unreacted ATPase, these numbers indicate that vanadate (in the presence of Mg^{2+} and ATP) is able to lock at least 91% of all of the ATPase molecules present in the reconstituted ATPase preparation in a conformation that presumably represents the conformation that the enzyme assumes in the transition state of the enzyme dephosphorylation reaction. Because the ability of any enzyme to firmly bind to the transition state configuration(s) of the reaction(s) that it catalyzes is the essence of enzymic catalysis (38–41), we conclude that at least 91% of the ATPase molecules in the reconstituted proteoliposomes are catalytically active. To the extent that this is true, and to the extent that the above conclusion that a minimum of two-thirds of the catalytically active ATPase molecules translocate protons is true, the data presented thus far demonstrate that the efficiency of the ATPase reconstitution procedure is 61% or greater.

The foregoing data and conclusions set specific limits on the quantity of any polypeptide other than the $M_r \sim 105{,}000$

that some finite intraliposomal concentration of protons may be necessary for ATPase reversal, and in the absence of that concentration of protons, even a relatively large $\Delta\psi$ might not reverse the ATP hydrolysis reaction, at least not at a rate sufficient to lead to significant ATPase inhibition. In this case, nigericin could markedly stimulate ATP hydrolysis while dissipating only a relatively small ΔpH. It should be remembered in this regard that in the case of the F_1F_0 H⁺-ATPase/ATP synthases, $\Delta\psi$ and ΔpH are thought to be equipotent because of the presence in such enzymes of a "proton well" or "proton trap" in the subunits that make up the F_0 sector (32–34). The evidence presented in this paper suggests that the *Neurospora* plasma membrane H⁺-ATPase probably has no such device. Finally, it should be mentioned that in view of the above mentioned possibilities, there is no compelling reason to invoke variable nigericin H^+/K^+ stoichiometry or ATPase-catalyzed K^+ movements to explain these data, as has been done with similar data obtained with the reconstituted yeast plasma membrane H⁺-ATPase (22, 35), although such explanations must still be considered plausible.

[8] Vanadate, a potential ATPase ligand, was present during the trypsin treatment of sample 3 (see "Experimental Procedures") but, as was shown previously (13), this compound does not protect well against tryptic degradation of the ATPase in the absence of Mg^{2+}. This presumably means that vanadate binds to the ATPase only weakly or not at all in the absence of Mg^{2+}.

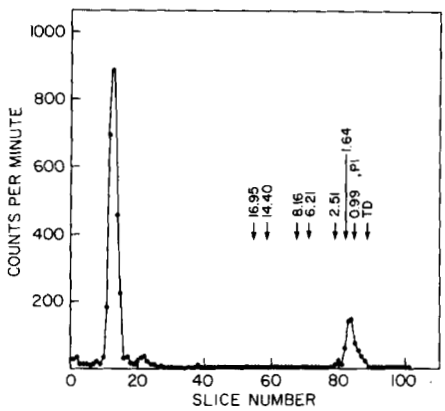

FIG. 3. **Composition of the purified H⁺-ATPase preparation used for the reconstitution experiments.** See "Experimental Procedures" for details regarding sample preparation, the SDS-PAGE system used, and the gel staining, slicing, and counting procedures. *Arrows* point to the position of migration of seven polypeptide standards, phosphatidylinositol (*PI*), and the tracking dye (*TD*). The molecular mass (in kilodaltons) of each polypeptide standard is indicated above the corresponding arrows. The standard polypeptides used with their individual M_r values in parentheses are as follows: horse myoglobin cyanogen bromide fragments (16,949, 14,404, 8,159, 6,214, and 2,512), somatostatin (1,639) and L-tyrosylglycylglycyl-L-phenylalanyl-L-leucyl-L-arginyl-L-lysyl-L-arginine (996). The major peak at the top of the gel (*left*) is the hydrolytic moiety of the H⁺-ATPase (M_r ~ 105,000).

hydrolytic moiety that must be present in the ATPase preparation in order to qualify as a subunit. With 61% reconstitution efficiency, for every mole of the hydrolytic moiety present in the ATPase preparation used for the reconstitution, there must be 0.61 mol of any subunit, assuming a subunit stoichiometry of 1:1. For a 10,000-dalton subunit, this amounts to a mass ratio (subunit/hydrolytic moiety) of about 0.058. By the same argument, any subunits of higher molecular weight require a higher mass ratio. Fig. 3 shows the composition of the H⁺-ATPase preparation used for all of the reconstitution experiments described in this communication, as indicated by SDS-PAGE. The ATPase preparation was made from cells grown in the presence of radioactive carbon sources so that any *Neurospora* proteins present in the ATPase preparation could be detected, regardless of whether or not they could be stained by protein stains. About 2,515 cpm are present in the amount of M_r ~ 105,000 hydrolytic moiety applied to the gel, and thus, at a mass ratio of 0.058, 146 cpm of an essential 10,000-dalton subunit would need be present. More than this amount would need be present for higher molecular weight subunits. Clearly, there are no components present in anywhere near sufficient amounts in the 10,000-dalton and higher molecular weight range. The argument holds down to about 2,500 daltons where about 37 cpm would be needed, but are not present. Below this value, the situation becomes less clear because very few counts/min are required for an essential subunit, and more than an adequate amount of radioactive material is present in the relatively broad band that migrates near the tracking dye. Because a phospholipid standard (phosphatidylinositol) also migrates as a similarly broad band in about the same position, it is quite possible that this radioactive material is lipid, in which case, the hydrolytic moiety is the only polypeptide component of the H⁺-ATPase. However, the involvement of a very small oligopeptide subunit that migrates in the phospholipid region cannot be excluded by the present analysis, and thus, further investigation will be required before it can be stated with certainty whether or not the hydrolytic moiety alone is capable of efficient proton translocation. In any case, two reasonably firm conclusions can be drawn from the data presented in this communication. First, it is clear that no conventional protein or proteolipid subunits of the type that have been reported to be associated with certain other ATPases in the aspartylphosphoryl-enzyme intermediate family of transport ATPases and no peptides similar to those that constitute the F_1 or F_0 sectors of the F_1F_0 ATPase/ATP synthases are involved in the mechanism of proton translocation catalyzed by the *Neurospora* plasma membrane H⁺-ATPase. And second, if this enzyme contains any subunits at all other than the hydrolytic moiety, they must be very small.

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