Acad. Sci. U.S.A. 76, 4350–4354.
Tukey, R. H., Nebert, D. W., & Negishi, M. (1981) *J. Biol. Chem. 256*, 6969–6974.
Tweit, R. C., & Kagawa, C. M. (1964) *J. Med. Chem. 7*, 524–528.
Wood, A. W. (1979) *J. Biol. Chem. 254*, 5641–5646.
Wood, A. W., & Taylor, B. J. (1979) *J. Biol. Chem. 256*, 5647–5651.

Characterization of Native and Reconstituted Hydrogen Ion Pumping Adenosinetriphosphatase of Chromaffin Granules†

Gary E. Dean,‡ Pamlea J. Nelson, and Gary Rudnick*
Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510
Received November 22, 1985; Revised Manuscript Received April 3, 1986

ABSTRACT: The ATP-dependent H^+ pump from adrenal chromaffin granules is, like the platelet-dense granule H^+ pump, essentially insensitive to the mitochondrial ATPase inhibitors sodium azide, efrapeptin, and oligomycin and also insensitive to vanadate and ouabain, agents that inhibit the Na^+,K^+-ATPase. The chromaffin granule H^+ pump is, however, sensitive to low concentrations of NEM (*N*-ethylmaleimide) and Nbd-Cl (7-chloro-4-nitro-2,1,3-benzoxadiazole). These transport ATPases may thus belong to a new class of ATP-dependent ion pumps distinct from F_1F_0- and phosphoenzyme-type ATPases. Comparisons of ATP hydrolysis with ATP-dependent serotonin transport suggest that approximately 80% of the ATPase activity in purified chromaffin granule membranes is coupled to H^+ pumping. Most of the remaining ATPase activity is due to contaminating mitochondrial ATPase and Na^+,K^+-ATPase. When extracted with cholate and octyl glucoside, the H^+ pump is solubilized in a monodisperse form that retains NEM-sensitive ATPase activity. When reconstituted into proteoliposomes with crude brain phospholipid, the extracted enzyme recovers ATP-dependent H^+ pumping, which shows the same inhibitor sensitivity and nucleotide dependence as the native pump. These data demonstrate that the predominant ATP hydrolase of chromaffin granule membrane is also responsible for ATP-driven amine transport and granule acidification in both native and reconstituted membranes.

Until recently, all ion-translocating ATPases were thought to fall into one of two classes: the F_1F_0 class, represented by mitochondrial ATPase, and the phosphoenzyme class of eukaryotic ATP-dependent ion pumps, represented by the Na^+,K^+-ATPase. It has now become apparent that a third class of transport ATPase exists in acidic intracellular vesicles such as lysosomes, endosomes, coated vesicles, and secretory granules (Rudnick, 1986a,b). All of these organelles are thought to participate in the movement of their content to or from the extracellular medium. In all cases examined, ATP drives H^+ influx into the organelle, and this pumping is insensitive to many inhibitors of previously characterized transport ATPases.

Two approaches have been used to demonstrate the uniqueness of secretory granule ATPase. Cidon and Nelson (1983) demonstrated that chromaffin granule membranes from which all immunoreactive F_1 β-subunit had been removed by sodium bromide extraction still retained 70% of their ATPase activity and that this remaining ATPase would reconstitute ATP-dependent H^+ influx (Cidon et al., 1983). Since the β-subunit is highly conserved in F_1F_0 ATPase from all known sources, it is extremely unlikely that this major chromaffin granule ATPase represents a typical F_1F_0 enzyme. It has never been conclusively demonstrated, however, that this major ATPase activity represents the H^+ pump that normally drives amine transport. Our laboratory has used a second approach in studying the H^+-pumping ATPase of platelet-dense granule membrane. Dean et al. (1984) demonstrated that the platelet granule H^+ pump is insensitive to concentrations of inhibitors, which maximally inhibit F_1F_0 ATPase or Na^+,K^+-ATPase.

In this work, we have turned our attention to the ATP-dependent H^+ pump of adrenal chromaffin granules. Chromaffin granule membranes may be prepared in higher yield and purity than those from other secretory granules. These properties make possible the comparison of ATPase and H^+-pumping properties of the membrane. To avoid complications from other ATP-dependent H^+ pumps that might contaminate the preparation, we employed a coupled assay for H^+ pumping. In this assay, the electrochemical H^+ potential ($\Delta\bar{\mu}_{H^+}$)[1] (interior acidic and positive) generated by H^+ pumping is measured by serotonin accumulation catalyzed by the granule amine transporter. The amine transported catalyzes the reserpine-sensitive exchange of intragranular H^+ with cytoplasmic biogenic amines. The assay, therefore, is sensitive only to those ATPases in the preparation that pump H^+ across the granule membrane.

To ultimately determine if the secretory granule ATPase belongs to a new class of H^+ pump, structural information on

† This work was supported by a grant (HL-21217) from the National Institutes of Health (to G.R.).
‡ Present address: Department of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, OH 45267.

[1] Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; Nbd-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulfonyl fluoride; $\Delta\bar{\mu}_{H^+}$, transmembrane electrochemical potential for H^+; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; AMP-PNP, 5′-adenylyl imidodiphosphate.

0006-2960/86/0425-4918$01.50/0

the purified enzyme must be obtained. In solubilizing and purifying the enzyme, however, the primary function of the ATPase as an H^+ pump is lost unless reconstitution of pump activity can be demonstrated. In this work, we reconstitituted the solubilized H^+ pump in proteoliposomes and compared its catalytic properties to those of the native ATPase.

Experimental Procedures

Methods

Membrane Preparation. Chromaffin granule and mitochondrial membranes were isolated from bovine adrenal medullae essentially as described by Cidon and Nelson (1983) with the following modifications. Initial homogenates were centrifuged at 200*g* for 10 min to remove derbis instead of being filtered through cheesecloth. The Percoll gradient step was eliminated. Finally, chromaffin granules that remained intact after hypotonic lysis were resuspended in 18 mL (per 40 glands) of 10 mM MOPS, pH 7.5, containing 1 mM ATP and 0.1 mM PMSF. The suspension was stirred on ice for 15 min after addition of 3 mL of 30% glycerol, the suspension recentrifuged for 20 min at 39,000*g*, and the pellet rapidly resuspended in 100 mL of the same medium initially used to lyse the granules. The material was centrifuged at 200*g* for 10 min to remove debris and the resulting supernatant combined with the membranes formed in the initial lysis step. A final yield of 120–150 mg of membrane protein was routinely obtained from 120 g (wet wt) of dissected medullae.

Enzyme Assays. Mitochondrial and Na^+,K^+-ATP hydrolytic activity was assayed exactly as described previously (Dean et al., 1984). Chromaffin granule membrane ATPase activity was assayed by measuring release of inorganic ^{32}P from [γ-^{32}P]ATP, which was prepared by the method of Glynn and Chapel (1964). Membrane suspensions containing 5 mM labeled ATP (approximately 2×10^5 cpm per assay) in 10 mM HEPPS, pH 8.5, containing 0.12 M sucrose, 60 mM NaCl, 15 mM KCl, 0.2 mM EDTA, and 5 mM $MgSO_4$ were incubated for 10–30 min in 250-μL reaction mixtures at 37 °C. Reactions were stopped by addition of 1 mL of 10% activated charcoal (Sigma) in 0.1 M potassium phosphate, pH 2.0. The mixtures were allowed to sit at room temperature for at least 10 min, with periodic mixing, and the centrifuged for 5 min at 500*g*. Samples of 0.5 mL were then removed and counted by liquid scintillation spectrometry.

Serotonin transport driven by ATP hydrolysis or by an artifically imposed $\Delta\bar{\mu}_{H^+}$ was assayed exactly as described previously for platelet dense granule membrane (Dean et al., 1984).

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Solubilization and Reconstitution of ATP-Dependent H^+ Pump. A suspension of chromaffin granule membrane vesicles (24 mg mL^{-1} in 10 mM MOPS, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 0.5 mM ATP, 0.1 mM PMSF, and 0.1 mM *p*-aminobenzamidine) was solubilized by adding 0.1 volume of a mixture consisting of 10% octyl glucoside and 5% sodium cholate, pH 8.5, mixing, and incubating 5 min on ice. This detergent mixture was then centrifigued 1 h at 200000*g* at 4 °C and the supernatant carefully removed. In later experiments, insoluble material was removed by centrifugation for 5 min in an Eppendorf microfuge Model 5414 since all of the NEM-sensitive ATPase activity was in the soluble phase after high-speed centrifugation (see Results). The soluble extract was reconstituted by addition of lipid and removal of detergent by rapid gel filtration. Crude bovine brain phospholipid (0.74 mg/mg of membrane protein, added as a sonicated solution 23 mg/mL in 0.27% cholate) was added to the solubilized membrane solution, mixed, and a maximum of 80 μL was layered onto a 1-mL column (formed in a tuberculin syringe) of Sephadex G-50 equilibrated with 10 mM MOPS, pH 7.5, 0.3 M sucrose, 5 mM EDTA, and 0.5 mM ATP. The column was then centrifuged for 2 min at top speed in an IEC clinical centrifuge. Reconstituted proteoliposomes were recovered in the column effluent, approximately 500 μL.

Acidification Assays. A total of 50–100 μg of membrane protein was diluted into 0.7 mL of 10 mM Tris–HEPPS buffer, pH 8.5, containing 0.15 M KCl, 0.2 mM EDTA, 3–10 μM acridine orange, and 5 mM ATP. Acidification was initiated by the addition of $MgSO_4$ to 5 mM final concentration and was monitored by absorbance at 486 nm.

HPLC Gel Permeation Chromatography. Chromaffin granule membranes at a concentration of 10 mg mL^{-1} were solubilized in 1% octyl glucoside and 0.5% cholate for 10 min on ice, and the insoluble fraction was removed by centrifugation for 30 min at 250000*g*. A portion of the resulting supernatant (2.33 mg of protein) was then applied to an LKB TSK4000 column equilibrated with 10 mM MOPS, pH 7.5, containing 0.5 mM ATP, 0.3 M sucrose, 5 mM EDTA, 0.02% bovine brain phospholipid, 1% octyl glucoside, and 0.5% cholate at a rate of 1 mL min^{-1} and eluted with the same buffer at the same flow rate. Fractions (1 mL) were collected and 200-μl aliquots assayed for ATPase activity in the presence of 0.5 μM efrapeptin. NEM-sensitive activity was determined as the difference in ATP hydrolytic activity between samples treated with 0.5 mM NEM for 1 min and untreated samples.

Materials

Na^+,K^+-ATPase, from dog kidney outer medulla, was the generous gift of Dr. B. Forbush, Department of Physiology, Yale University (Forbush, 1983). Untreated specific activity was 1.7–1.8 μmol of phosphate released per minute per milligram. Efrapeptin was a gift from Dr. Robert L. Hamill of Lilly Research Laboratories. Nucleotide triphosphates were from Sigma and were used as the di- or trisodium salts. All other materials were of reagent grade or better obtained from commercial sources.

Results

Inhibitor Studies on Intact Membrane Vesicles. To examine the catalytic properties ot the chromaffin granule ATPase, we utilized an assay developed previously (Dean et al., 1984) for the H^+-pumping ATPase of platelet storage organelles. Both of these secretory granules store biogenic amines and contain, in their membrane, an amine transporter whose activity is driven by the transmembrane $\Delta\bar{\mu}_{H^+}$ generated by the ATPase. Measurements of ATP-dependent amine (serotonin) transport are not influenced by contaminating ATPases in the preparation, even by other H^+-pumping ATPases that are located in contaminating membranes such as mitochondria. In the series of experiments described below, various inhibitors were tested for their ability to inhibit the chromaffin granule ATPase as measured by ATP-dependent serotonin transport and, for comparison, the ATP-hydrolyzing activity of bovine adrenal mitochondrial membranes and purified canine Na^+,K^+-ATPase.

(*A*) *Inhibitors of "Phosphoenzyme" ATPases.* Most of the ATP-dependent ion pumps, such as Na^+,K^+-ATPase, found in the plasma membrane of eukaryotic cells are phosphorylated and dephosphorylated during each catalytic cycle. All of these enzymes have been shown to be sensitive to vanadium in the 5+ oxidation state. In addition, Na^+,K^+-ATPase is sensitive to the specific inhibitor ouabain. Figure 1 compares the in-

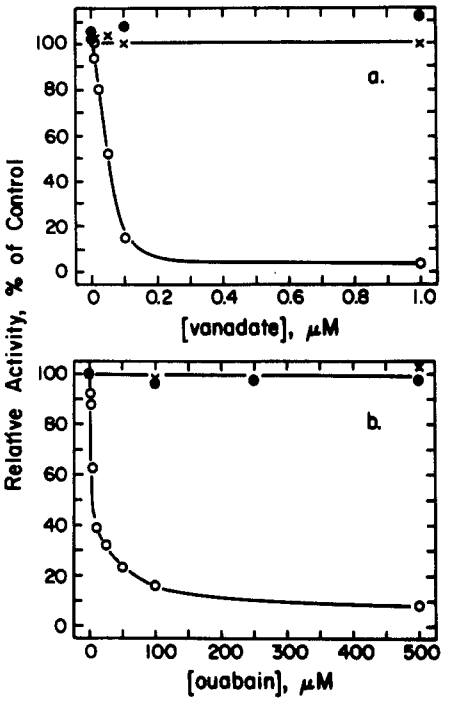

FIGURE 1: Inhibition of Na^+,K^+-ATPase, F_1F_0 ATPase, and chromaffin granule ATP-driven serotonin transport by vanadate (a) and ouabain (b). ATPase and transport assays were performed as described previously (Dean et al., 1984). Where indicated, membranes were preincubated with vanadate for 10 min in the presence of 25 mM $MgSO_4$. In all cases, the membrane protein concentration was 0.15 mg mL^{-1}. (O) Na^+,K^+-ATPase; (×) F_1F_0 ATPase; (●) chromafin granule serotonin transport.

hibitory effects of vanadate inhibition on the three test enzymes. It is clear that at 100 nM vanadate only slightly over 10% of the Na^+,K^+-ATPase activity remained, decreasing to 0% at 1 μM. Neither mitochondrial ATPase activity nor the chromaffin granule pump was affected at any of those vanadate concentrations. Ouabain (Figure 1b) also had no effect upon ATP hydrolysis by the mitochondrial ATPase or upon the chromaffin granule ATPase at concentrations that dramatically inhibited ATP hydrolysis by Na^+,K^+-ATPase.

(B) Inhibitors of F_1F_0 ATPases. Azide ion is known to inhibit F_1F_0 ATPases from a variety of sources including mitochondria, bacteria, and chloroplasts (Linnet & Beechey, 1979). The ability of azide to inhibit ATP hydrolysis by mitochondrial and Na^+,K^+-ATPase is compared, in Figure 2a, with its effect on the chromaffin granule H^+ pump. While azide inhibited the mitochondrial ATPase activity 91% at 2 mM, this concentration of azide had minimal effects upon both the Na^+,K^+-ATPase and the granule pump, inhibiting the granule pump approximately 15% and the Na^+,K^+-ATPase not at all. Figure 2 b,c also shows the inhibition of mitochondrial ATPase by two more specific inhibitors, oligomycin and efrapeptin, and the relative lack of inhibition of the Na^+,K^+-ATPase and the granule pump by these same inhibitors. ATP hydrolysis by F_1F_0 is inhibited 90% at 0.5 μM oligomycin (Figure 2b) or 1 μM efrapeptin (Figure 2c) while none of the concentrations tested inhibited Na^+,K^+-ATPase or the chromaffin granule ATPase.

DCCD (dicyclohexylcarbodiimide) inhibits the F_1F_0 complex by reacting with both the catalytic and membraneous portions of the enzyme (Racker, 1972; Cattell et al., 1971; Linnett & Beechey, 1979). This reagent, which covalently modifies carboxyl groups, inhibits all three types of H^+-pumping ATPases (Serrano, 1983). As shown by the data in Figure 3a, DCCD inhibits both mitochondria ATPase and

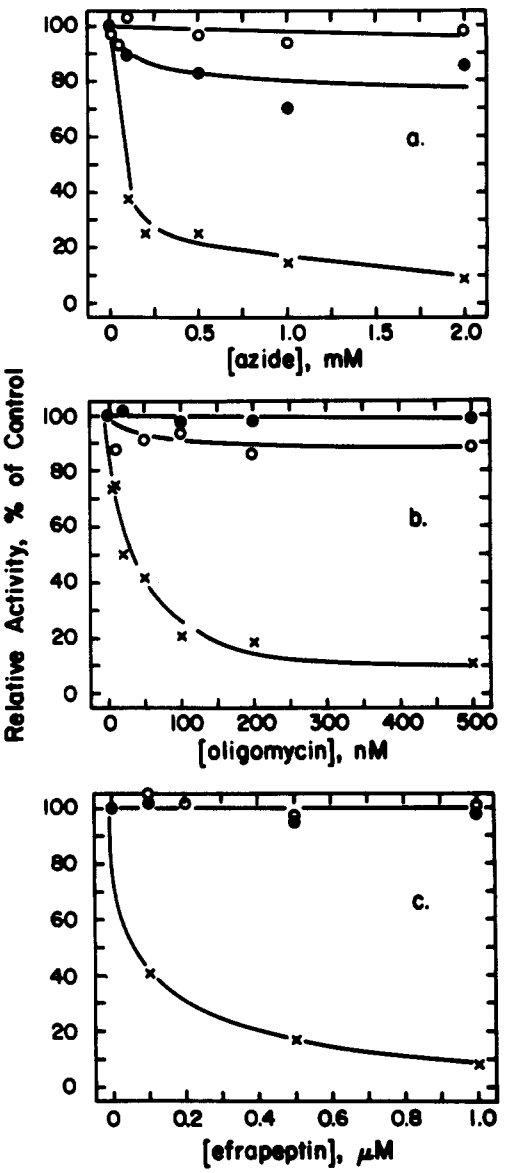

FIGURE 2: Inhibition by mitochondrial ATPase inhibitors. Conditions and symbols are as described in the legend to Figure 1.

ATP-driven serotonin transport in chromaffin granule membranes, although the mitochondrial enzyme is more sensitive (50% inhibition at 1.5 μM in mitochondria vs. 8.5 μM for the granule membrane). Even at the highest concentrations tested, however, Na^+,K^+-ATPase was completely resistant to DCCD. Inhibition of serotonin transport by DCCD could result from several other causes in addition to ATPase inactivation. Increasing the membrane's H^+ permeability or directly inhibiting the amine transporter will also block the coupled reaction. The data shown in Figure 3b demonstrate that although ATP-driven serotonin transport is more sensitive than transport energized by an artifically imposed $\Delta\bar{\mu}_{H^+}$ (interior positive and acidic), DCCD inhibits under both conditions. Thus, the membrane's H^+ permeability or amine transport or both are compromised in the presence of DCCD.

(C) Inhibitors of Granule ATPase. Both NEM (*N*-ethylmaleimide) and Nbd-Cl (7-chloro-4-nitro-2,1,3-benzoxadiazole) preferentially inhibit the granule ATPase, although Nbd-Cl also inhibits mitochondrial ATPase (Deters et al., 1975). The data shown in Figure 4 demonstrate the Nbd-Cl sensitivity of ATP-driven serotonin transport, which is totally inhibited at concentrations below 1 μM, while ATP hydrolysis by mitochondrial membranes or Na^+,K^+-ATPase is over 80%

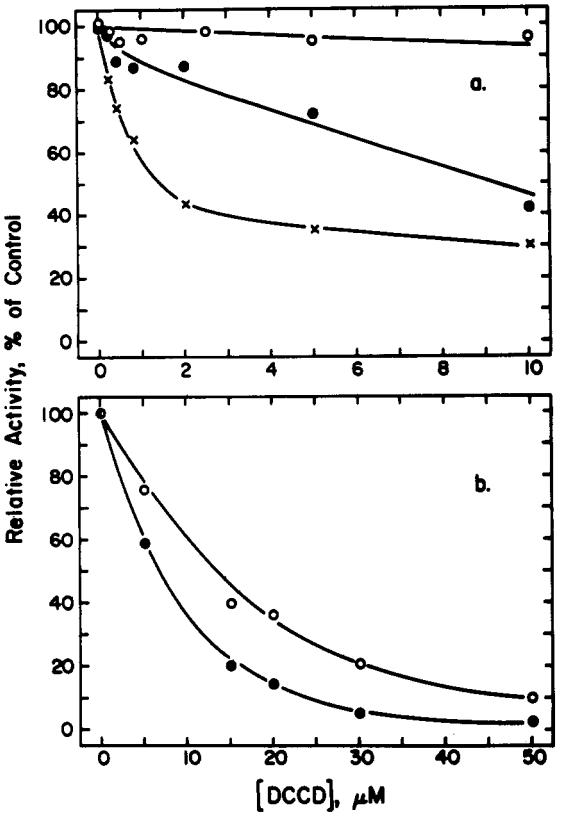

FIGURE 3: Inhibition by dicyclohexylcarbodiimide of Na^+,K^+-ATPase, F_1F_0 ATPase, and chromaffin granule serotonin transport. (Panel a) Conditions and symbols are as described in the legend to figure 1. (Panel b) Serotonin transport driven by ATP (●) and an artificially imposed $\Delta\bar{\mu}_{H^+}$ (○) was measured as described previously (Dean et al. 1984).

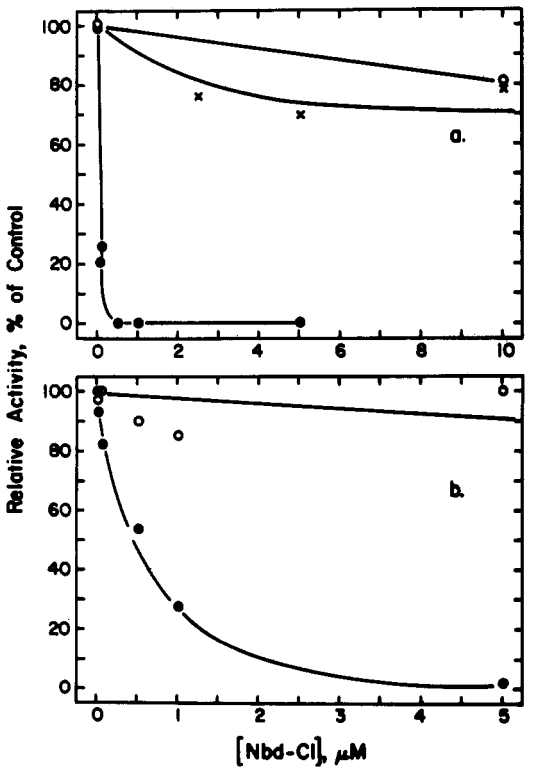

FIGURE 4: Inhibition by Nbd-Cl of Na^+,K^+-ATPase, F_1F_0 ATPase, and chromaffin granule serotonin transport. Conditions and symbols are as described in the legend to Figure 3.

resistant at 10 μM. Nbd-Cl failed to inhibit serotonin transport driven by an artifically imposed $\Delta\bar{\mu}_{H^+}$, indicating that

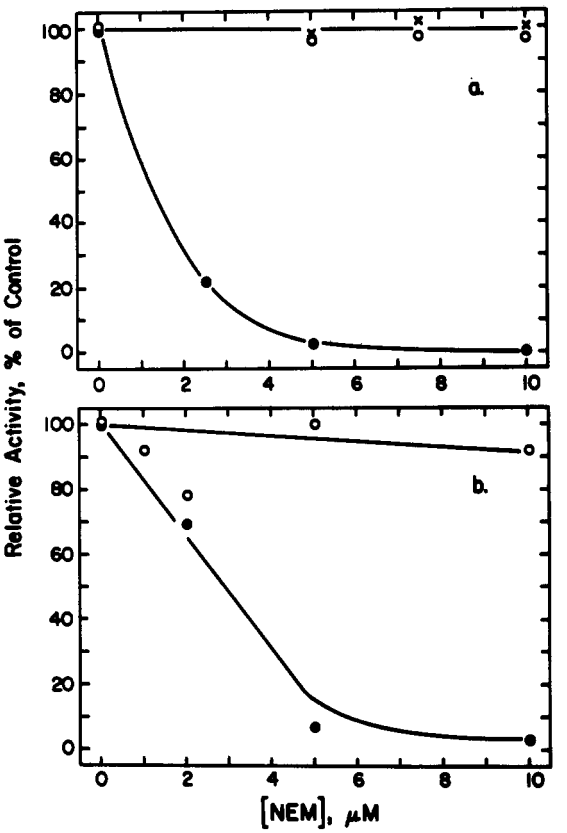

FIGURE 5: Inhibition by *N*-ethylmaleimide of Na^+,K^+-ATPase, F_1F_0 ATPase, and chromaffin granule serotonin transport. Conditions and symbols are as described in the legend to Figure 3.

Table I: Nucleotide Dependence of Na^+,K^+-ATPase, F_1F_0 ATPase, and Chromaffin Granule ATP-Dependent Serotonin Transport[a]

nucleotide	rate (relative to ATP)		transport (relative to ATP)
	F_1F_0	Na^+,K^+-ATPase	
dATP	95	79	70
GTP	37	0	17
dGTP	37	23	9
ITP	35	6	16
UTP	16	0	16
CTP	3	36	9
AMP-PNP	0	0	0

[a] ATPase and transport assays were performed as described previously (Dean et al., 1984) with the indicated nucleotide instead of ATP.

inhibition occurs at the level of the ATPase (Figure 4b). The slightly higher concentrations of Nbd-Cl necessary to inhibit ATP-driven transport in Figure 4b relative to Figure 4a reflects the different media used in the two experiments. The medium used in the experiment shown in Figure 4a was optimized for measuring ATPase activity in all three systems while the experiment shown in Figure 4b used a medium optimized for demonstrating $\Delta\bar{\mu}_{H^+}$-driven transport.

Figure 5 shows similar data for NEM. Complete inhibition of ATP-driven transport occurs at approximately 5 μM, while Na^+,K^+-ATPase and mitochondrial ATPase are completely resistant to 10 μM (Figure 5a). As with Nbd-Cl, all of the transport inhibition can be attributed to blockade of ATP-dependent H^+ pumping since transport driven by an artifically imposed $\Delta\bar{\mu}_{H^+}$ is resistant to NEM (Figure 5b). Again, the ATPase is somewhat more sensitive in the high-salt medium used for comparison with Na^+,K^+-ATPase and mitochondrial ATPase.

(*D*) *Nucleoside Triphosphate and Cation Dependencies.* Table I illustrates the nucleoside triphosphate dependence,

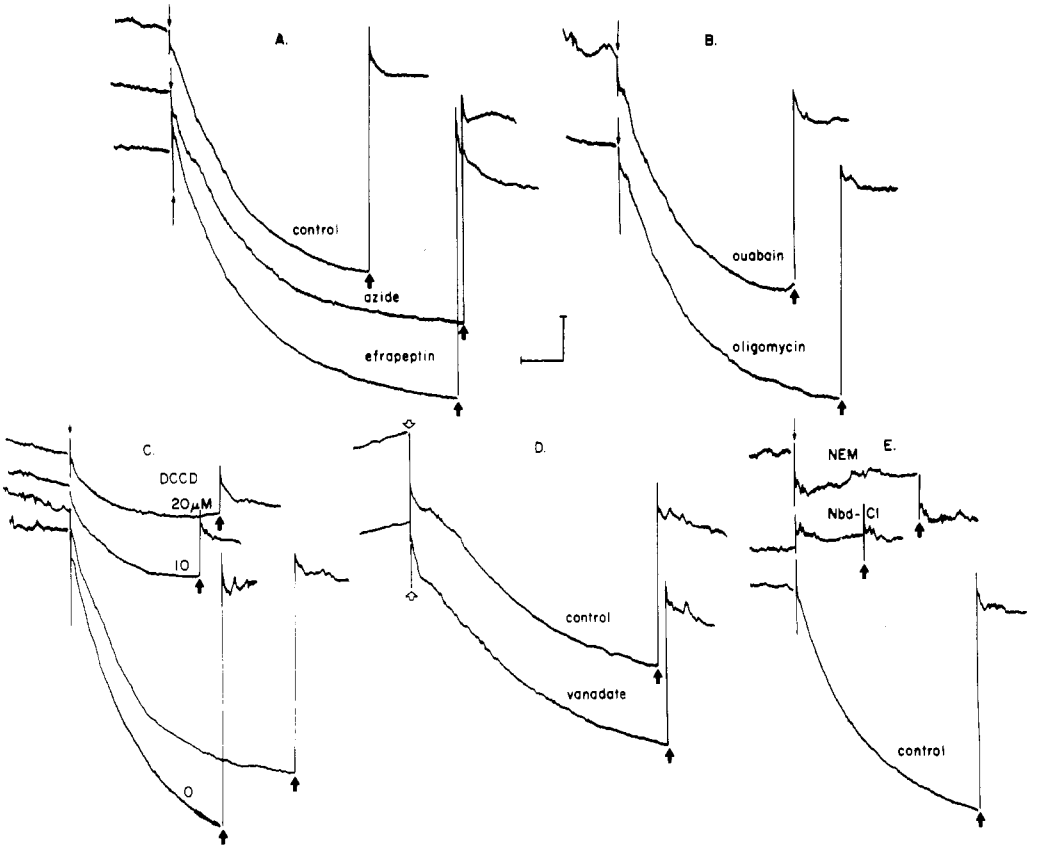

FIGURE 6: ATP-driven acidification of native chromaffin granule membrane vesicles. Acidification was measured by acridine orange absorbance quenching as described under Experimental Procedures. Reaction were initiated by addition of either 5 mM $MgSO_4$ (thin arrow) or 5 mM ATP (open arrow) and terminated by addition of 12.5 mM NH_4Cl (filled arrow). Where indicated, membranes were preincubated with 1 μM sodium vanadate for 10 min in the presence of 25 mM $MgSO_4$. Other inhibitors were present at the following concentrations: NaN_3, 1 mM; efrapeptin, 1 μM; ouabain, 0.5 mM; oligomycin, 0.5 μM; Nbd-Cl, 10 μM; NEM, 10 μM. The scale is 0.002 absorbance unit and 2 min.

Table II: Cation Dependence of Na^+,K^+-ATPase, F_1F_0 ATPase, and Chromaffin Granule ATP-Dependent Serotonin Transport[a]

cation	rate (relative to Mg^{2+})		transport (relative to Mg^{2+})
	F_1F_0	Na^+,K^+-ATPase	
Mn^{2+}	100	74	40
Ca^{2+}	14	20	2
Ba^{2+}	0	0	0
Ni^{2+}	5	19	3

[a] ATPase and transport assays were performed as described previously (Dean et al., 1984) with the indicated cation instead of Mg^{2+}.

relative to ATP, of Na^+,K^+-ATPase, mitochondrial ATPase, and ATP-driven serotonin transport into chromaffin granule membrane vesicles. The granule ATPase appears to be specific for ATP, showing little ability to use any of the other nucleotides, except for dATP. All of the ATPases appeared to be rather similar in their ability to use divalent cations other than magnesium (Table II). All three could utilize manganese to some degree and were relatively inactive in the presence of calcium, nickel, or barium.

Inhibition of H^+ Pumping. By measuring the absorbance quenching of acridine orange, we have determined the ability of each of the inhibitors mentioned above to inhibit acidification of chromaffin granule membrane vesicles. A weak base, acridine orange is concentrated in vesicles whose interior is more acidic than the medium. The optical properties of absorbance and fluorescence are quenched at higher concentrations and, thus, reflect internal acidification. The traces shown in Figure 6 demonstrates the influence of various inhibitors on ATP-driven acridine orange absorbance quenching in chromaffin granule membranes. In this experiment, membrane vesicles suspended in a medium containing 1 mM ATP are monitored at 486 nm, and the reaction is started by addition of 1 mM Mg $MgSO_4$. In the control, a rapid absorbance decrease indicates acidification of the vesicle interior (upper trace, panels A and D; lower trace, panels C and E). Addition of ouabain, oligomycin, azide, or efrapeptin has little effect on this acidification (panels A and B), but DCCD (panel C) and NEM and Nbd-Cl (panel E) all prevent the absorbance decrease. In the case of vanadate, the membranes were preincubated with VO_3^- in the presence of Mg^{2+}, a treatment that is required for maximal Na^+,K^+-ATPase inhibition, and the reaction was started with ATP, but regardless of the method of preincubation, vanadate fails to inhibit chromaffin granule membrane vesicle acidification (panel D).

Chromaffin Granule Membrane ATP Hydrolysis. Knowing the inhibitor sensitivity of the chromaffin granule ATPase, we can estimate what fraction of the ATP hydrolysis in the membrane preparation represents the H^+ pump. The data shown in Table III demonstrate the effect of various inhibitors on the rate of ATP hydrolysis by chromaffin granule membranes. At concentrations of oligomycin, azide, and efrapeptin previously shown to maximally inhibit mitochondrial ATPase, hydrolysis by chromaffin granule membranes decreases only 8–13%, probably due to contaminating mitochondrial membranes. Inhibition by 1 μM vanadate under conditions known to completely abolish hydrolysis by Na^+,K^+-ATPase resulted in a loss of only 7% of the hydrolysis; the fact that 500 μM ouabain decreased the rate by 6% of the total indicates that essentially all of the contamination by ATPases of the phos-

Table III: Inhibitor Sensitivity of ATP Hydrolysis by Chromaffin Granule Membrane Vesicles[a]

treatment	% activity remaining	treatment	% activity remaining
none	100	NEM	
efrapeptin, 2 μM	86	10 μM (15 min)	44
NaN_3, 1 mM	92	10 μM (30 min)	37
oligomycin, 1 μM	87	10 μM (60 min)	30
ouabain, 0.5 mM	94	FCCP, 2 μM	147
$NaVO_3$, 1 μM	93	nigericin, 1 μM	156
DCCD, 20 μM	57		
Nbd-Cl			
5 μM	43		
10 μM	37		
100 μM	13		

[a] ATPase activity was measured as described under Experimental Procedures. Unless otherwise indicated, inhibitors were added to the reaction mixture at the same time as membrane vesicles. Where indicated, membranes were preincubated with sodium vanadate for 10 min in the presence of 25 mM $MgSO_4$. NEM was preincubated with the membranes for the times shown. The absolute value of the control rate was 45 nmol mg^{-1} min^{-1}.

Table IV: Inhibition of ATP Hydrolysis by Nucleotides[a]

nucleotide	activity (% of control) 2 mM	5 mM	15 mM
dATP	75.8	50.8	20.8
GTP	94.1	81.3	46.7
dGTP	83.8	79	86
CTP	100	92	62
UTP	98	93	71
ITP	100	93	62
ADP	47	32	15
AMP-PNP	22	14	1

[a] ATPase activity was measured as described under Experimental Procedures in the presence of the indicated concentrations of nucleotide.

phorylated intermediate class was probably due to contamination by Na^+,K^+-ATPase.

DCCD, Nbd-Cl, and NEM all inhibited ATPase activity (Table III). Although none of these compounds completely inhibited ATP hydrolysis, Nbd-Cl inhibited 87% at 100 μM and approximately 63% at 10 μM, where mitochondrial AT-Pase Na^+,K^+-ATPase are relatively unaffected (Figure 4a). NEM also inhibited most of the ATP hydrolysis by the granule membranes. This inactivation required a prolonged incubation in NEM (60 min), while Nbd-Cl inactivation was maximal within 1 min. The inactivation by DCCD, NEM, and Nbd-Cl suggest that most of the ATPase activity in this preparation represents the granule H^+ pump. Another indication that most of the ATPase represents the H^+ pump is the observation that H^+ ionophores stimulate hydrolysis. Both nigericin and FCCP dissipate the $\Delta\bar{\mu}_{H^+}$ against which the ATPase must pump H^+ and consequently stimulate hydrolytic activity (Table III).

Nucleotides that fail to support H^+ pumping vary in their ability to inhibit ATP hydrolysis. As shown in Table IV, GTP, dGTP, CTP, UTP, and ITP inhibit ATPase activity only at high concentrations. Deoxy-ATP (a substrate) and ADP (the normal product) are slightly more potent, but the non-hydrolyzable analogue AMP-PNP is the most potent inhibitor tested.

To more closely correlate NEM-sensitive ATPase activity with H^+ pumping, we have measured the time course with which NEM inhibits both ATP-driven serotonin transport and ATP hydrolysis. In Figure 7 the extent of transport activity remaining at each time point is plotted against the amount of remaining ATPase. The roughly linear relationship between inactivation of the two activities indicates that inhibition of

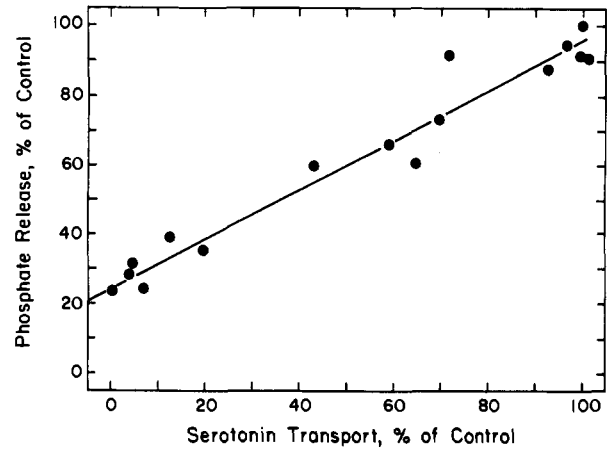

FIGURE 7: *N*-Ethylmaleimide inactivation of ATPase and ATP-driven serotonin transport. Chromaffin granule membrane vesicles were incubated in the presence of 10 μM NEM in 10 mM potassium HEPPS buffer, pH 8.5, containing 0.12 M sucrose, 60 mM NaCl, 15 mM KCl, and 0.2 mM EDTA at 25 °C, and samples were taken at times from 1 to 90 min. To each sample, 10 mM β-mercaptoethanol was added, and ATP-driven serotonin transport and ATPase activity were measured as described under Experimental Procedures.

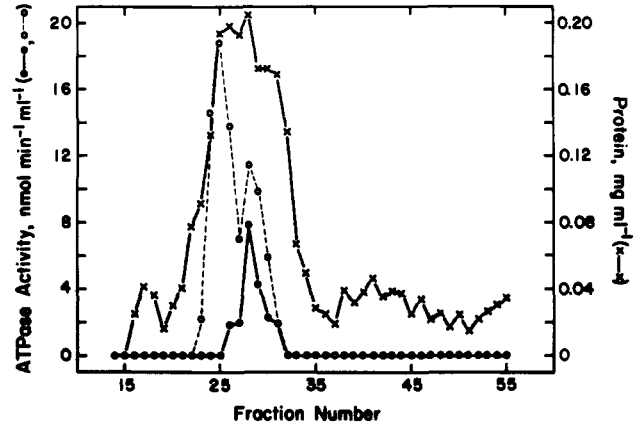

FIGURE 8: Gel permeation chromatography of solubilized chromaffin granule ATPase. Chromaffin granule membranes solubilized in cholate and octyl glucoside were applied, eluted, and assayed as described under Experimental Procedures. (×) Protein; (○) total ATPase; (●) NEM-sensitive ATPase.

hydrolysis follows the same time course as inhibition of serotonin transport and suggests that they result from the same process, namely, the nucleophilic attack of an essential sulfhydryl group on NEM. The limiting value of about 20% remaining hydrolysis when serotonin transport is completely inactivated suggests that roughly 80% of the ATP hydrolytic activity in our chromaffin granule membrane preparation was due to the H^+ pump.

Solubilization of ATPase. As described by Cidon and Nelson (1983), a mixture of cholate and octyl glucoside extracts NEM-sensitive ATPase activity from chromaffin granule membranes. This activity, which represented approximately half of the starting activity in the solubilized extract, did not sediment during centrifugation for 1 h at 200000*g*. To determine that NEM-sensitive ATPase activity was truly solubilized, we analyzed the detergent extract by gel permeation chromatography. The results shown in Figure 8 indicate that two peaks of ATPase activity eluted from the column. The second of these, which was NEM-sensitive, eluted well after the void volume of the column, with an apparent relative molecular weight of less than 250 000.

Reconstitution of H^+ Pump into Proteoliposomes. Solubilized NEM-sensitive ATPase activity was capable of cata-

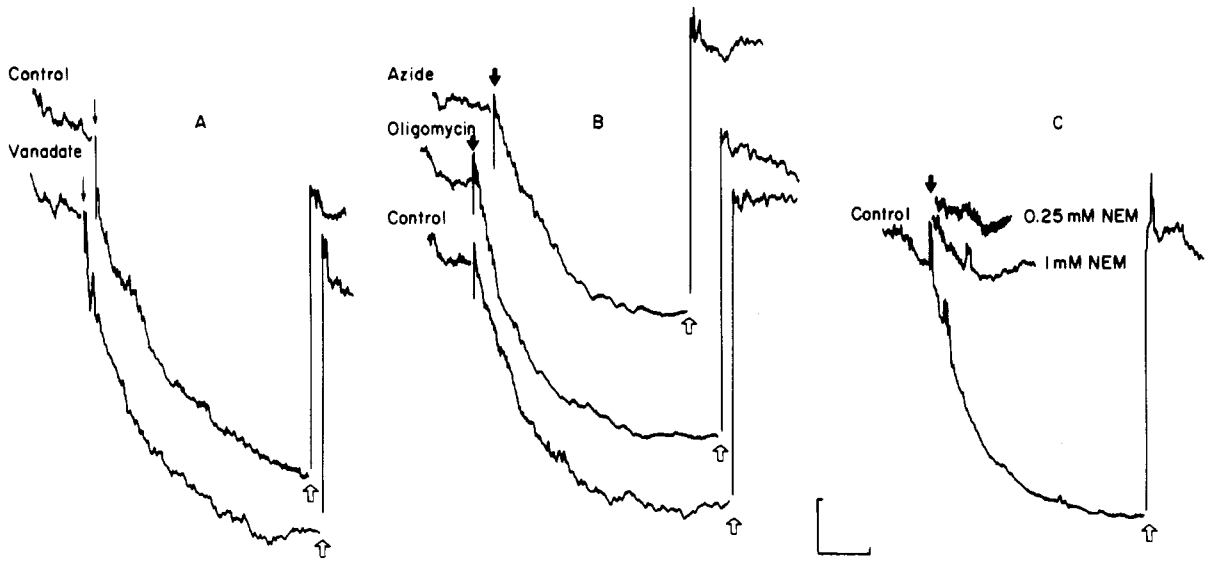

FIGURE 9: ATP-driven acidification of reconstituted chromaffin granule membrane vesicles. Proteoliposomes reconstituted with solubilized chromaffin granule ATPase were assayed for Mg-ATP-dependent acidification with acridine orange absorbance quenching as described under Experimental Procedures. Reactions were initiated by addition of either 5 mM $MgSO_4$ (thin arrow) or 5 mM ATP (filled arrow) and terminated by addition of 2 μM FCCP (open arrow). Where indicated, membranes were preincubated with sodium vanadate for 10 min in the presence of 25 mM $MgSO_4$. Other inhibitors were present at the following concentrations: NaN_3, 1 mM; oligomycin, 0.5 μM. The scale is 0.002 absorbance unit and 1 min.

lyzing H^+ pumping in reconstituted proteoliposomes, as measured by acridine orange absorbance quenching. Moreover, the reconstituted ATPase retains all of the properties that characterize the native enzyme. Solubilized ATPase spontaneously reconstituted into vesicles composed of crude bovine brain phospholipids upon detergent removal by rapid gel filtration. The traces shown in Figure 9 demonstrate the sensitivity of reconstituted H^+ pumping to NEM and FCCP and its resistance to vanadate, oligomycin, and azide. Efrapeptin and oligomycin (not shown) also failed to inhibit the reconstituted pump. In panel A, proteoliposomes preincubated with or without vanadate at high Mg^{2+} were diluted into a reaction mixture containing valinomycin and acridine orange, and the reaction was started by ATP addition. In panels B and C, proteoliposomes in the presence of ATP, valinomycin, acridine orange, and, where indicated, the appropriate inhibitor were added to the spectrophotometer cuvette, and reactions were initiated by addition of Mg^{2+}. In similar experiments (not shown), ATP was replaced with a variety of nucleotides. Of the compounds tested, only ATP and dATP served as substrates for the reconstituted ATP-dependent acidification, while CTP, GTP, dGTP, ITP, UTP, and ADP were ineffective.

Discussion

We have previously proposed Dean et al., 1984; Rudnick, 1986a,b) that acidic intracellular organelles, such as lysosomes, endosomes, coated vesicles, and secretory vesicles, contain an H^+-pumping ATPase distinct from previously characterized ATP-driven ion pumps. The results presented here demonstrate that the bovine adrenal chromaffin granule contains, in its membrane, an H^+-pumping ATPase similar to (if not identical with) the enzyme in porcine platelet-dense granule membranes (Dean et al., 1984). Moreover, the similarity between the ATPase in these two secretory vesicles and H^+ pumps in coated vesicles, endosomes, lysosomes, and plant vacuoles reinforces the suggestion that they represent a novel class of transport ATPase.

Characterization of the secretory vesicle ATPase rests on the ability to measure only those ATPases driving H^+ into the vesicle interior without also measuring other ATPases and ion pumps that may contaminate the preparation. We have addressed this problem by measuring ATP-driven serotonin transport. The reserpine-sensitive biogenic amine transporter of chromaffin granules couples H^+ efflux to serotonin influx. Contaminants in the preparation that are not components of the granule membrane will not contribute to ATP-driven serotonin transport even if they hydrolyze ATP or pump H^+ ions.

As with the previously described platelet granule ATPase Dean et al., 1984), the chromaffin granule H^+ pump displays an inhibitor sensitivity spectrum distinct from that of mitochondrial F_1F_0 ATPase or Na^+,K^+-ATPase. Insensitivity to azide and vanadate in particular render unlikely the possibility that the secretory granule ATPase belongs to the F_1F_0 or phosphoenzyme classes of transport ATPase. This pattern, plus sensitivity to NEM and Nbd-Cl, is characteristic of ATP-driven H^+ pumping in endosomes, lysosomes, and coated vesicles (Bowman, 1983; Forgac & Cantley, 1984; Galloway et al., 1983; Ohkuma et al., 1982; Stone et al., 1983).

The relatively high purity of the chromaffin granule membranes used here allows a comparison between hydrolytic and chemiosmotic properties of the ATPase. Approximately 80% of the ATPase activity of the membrane preparation represents the H^+ pump. ATP-driven amine transport, ATP hydrolysis, and vesicle acidification all share sensitivity to NEM and Nbd-Cl and resistance to azide, vanadate, oligomycin, ouabain, and efrapeptin. Furthermore, the observation that NEM inactivates ATP-driven serotonin transport and ATP hydrolysis with the same time course is consistent with the two activities being properties of the same enzyme. These results contradict earlier findings of Flatmark et al. (1982), which suggested that only H^+ pumping and not ATP hydrolysis is inactivated by NEM. The most likely explanation for the discrepancy between the two findings is that the earlier experiments may have used a preparation heavily contaminated with other ATPases, so that the decrease in ATP hydrolysis due to NEM inactivation of the granule H^+ pump was not measurable. Aside from this difference, the inhibitor sensitivity measured here agrees qualitatively with previous measurements of chromaffin granule ATPase inhibitor sensitivity [see Njus et al. (1981)

and Beers et al. (1982) for reviews].

Although the results discussed above indicate that the major ATP hydrolase of chromaffin granule membrane is also responsible for H^+ pumping and ATP-driven biogenic amine transport, the ultimate identification of this pump depends on purifying and characterizing the enzyme. The demonstration that the H^+ pump can be solubilized and reconstituted into proteoliposomes is an essential step in any attempt at purification. It is, therefore, especially significant that ATP-driven acidification in reconstituted vesicles displays the same inhibitor sensitivity and substrate specificity as the native enzyme. With these properties as a guide, it should be possible to identify the H^+-pumping ATPase in soluble subfractions and to purify it from other ATPases and ion pumps.

Registry No. ATPase, 9000-83-3; NEM, 128-53-0; Nbd-Cl, 10199-89-0; H^+, 12408-02-5.

References

Beers, M. F., Carty, S. E., Johnson, R. G., & Scarpa, A. (1982) *Ann. N.Y. Acad. Sci. 402*, 116–133.

Bowman, E. J. (1983) *J. Biol. Chem. 258*, 15238–15244.

Cattell, K. J., Lindop, C. R., Knight, I. G., & Beechey, R. B. (1971) *Biochem. J. 125*, 169–175.

Cidon, S., & Nelson, N. (1983) *J. Biol. Chem. 258*, 2892–2898.

Cidon, S., Ben-David, H., & Nelson, N. (1983) *J. Biol. Chem. 258*, 11684–11688.

Dean, G. E., Fishkes, H., Nelson, P. J., & Rudnick, G. (1984) *J. Biol. Chem. 259*, 9569–9574.

Deters, D. W., Racker, E., Nelson, N., & Nelson, H. (1975) *J. Biol. Chem. 250*, 1041–1047.

Flatmark, T., Gronberg, M., Husebye, E, Jr., & Berg, S. V. (1982) *FEBS Lett 149*, 71–74.

Forbush, B., III (1983) *Anal. Biochem. 128*, 159–163.

Forgac, M., & Cantley, L. (1984) *J. Biol. Chem. 259*, 8101–8105.

Galloway, C., Dean, G. E., Marsh, M., Rudnick, G., & Mellman, I. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3334–3338.

Glynn, I. M., & Chappell, J. B. (1964) *Biochem. J. 90*, 147–149.

Linnett, P. E., & Beechey, R. B. (1979) *Methods Enzymol. 55*, 472–518.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Njus, D., Knoth, J., & Zallakian, M. (1981) *Curr. Top. Bioenerg. 11*, 107–147.

Ohkuma, S., Moriyama, Y., & Takano, T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2758–2762.

Racker, E. (1972) in *Membrane Research* (Fox, C. F., Ed.) pp 97–114, Academic, New York.

Rudnick, G. (1986a) in *Physiology of Membrane Disorders* (Andreoli, T. E., Fanestil, D. D., Hoffman, J. F., & Schultz, S. G., Eds.) 2nd ed., pp 409–422, Plenum, New York.

Rudnick, G. (1986b) *Annu. Rev. Physiol. 48*, 403–413.

Serrano, R. (1983) *Arch. Biochem. Biophys. 227*, 1–8.

Stone, D., Xie, X.-S., & Racker, E. (1983) *J. Biol. Chem. 258*, 4058–4062.

Ligand-Induced Asymmetry As Observed through Fluorophore Rotations and Free Energy Couplings: Application to Neurophysin†

Suzanne F. Scarlata* and Catherine A. Royer‡

Department of Biochemistry, School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801

Received October 15, 1985; Revised Manuscript Received February 26, 1986

ABSTRACT: Changes that occur in subunit neurophysin structure upon ligand binding were explored by two methods. First, the thermal coefficient of the viscosity around the subunit tyrosine was monitored, which yields information on the environmental flexibility and free rotational space of the fluorophore. Initially, it was determined that the environmental flexibility and the free space around each subunit tyrosine are unperturbed upon dimerization. Binding of the tripeptide analogue of ocytocin causes the once homologous environments of the subunit tyrosines to become drastically different such that one moves onto a closely packed environment whereas the other moves into a region of larger free space. Even though the subunits as seen by each tyrosine are very different, the specific binding sites as seen by the ligands are similar. It was also found that ligand binding is stabilized by ring stacking and that energy transfer occurs between the tyrosine of the ligand and the neurophysin subunit tyrosine. Second, changes in subunit structure upon ligation were also followed by the determination of the order of free energy coupling between ligand binding and oligomerization, which tells how each ligand affects the subunit affinity. Since the binding of ligand is cooperative and induces dimer formation, there is second-order coupling between ligand binding and dimerization and the binding of the second ligand is responsible for the increase in subunit affinity.

Bovine neurophysin II is a small neurohypophyseal protein that serves as a carrier for the pituitary hormones ocytocin and vasopressin [for a general review, see Breslow (1979)]. Since this protein contains one centrally located tyrosine (49) and no tryptophans, it offers a fluorometrically simple system for protein studies. At high concentrations, neurophysin forms a dimer (Breslow et al., 1971; Nicolas et al., 1976), and hormone binding is cooperative and promotes dimer formation (Hope et al., 1975; Nicolas et al., 1978; Pearlmutter et al., 1977, 1980; Tellman et al., 1980). The dimer has two strong

† This work was supported by U.S. Public Health Service Grant GM 11223 to Gregorio Weber.

* Address correspondence to this author at AT&T, Princeton, NJ 08540.

‡ Present address: Laboratorie de Biologie Physico-Chimique, Universite de Paris VII, Paris 75005, France.

0006-2960/86/0425-4925$01.50/0
