H* ATPase of Chromaffin Granules

KINETICS, REGULATION, AND STOICHIOMETRY*

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The chromaffin granule ATPase mediates an inwardly directed transport of H* against concentration gradients, thereby forming and maintaining an electrochemical transmembrane H* gradient. The kinetics of this ATPase, its activity modulation by changes in electrochemical H* gradients, and the stoichiometry between H* transport and ATP hydrolysis were studied in intact bovine chromaffin granules, resealed chromaffin granule ghosts, and highly purified fragmented chromaffin granule membranes. In fragmented membranes the H* ATPase has a Km for ATP of 60 μM, a maximum of activity at pH 7.5, and a Vmax of 111 nmol/min/mg of protein at 20 °C. Trimethyl tin inhibits the ATPase at much lower concentrations than dicyclohexylcarbodiimide, whereas oligomycin, reserpine, and other inhibitors were without effect. In intact chromaffin granules, the ATPase activity was stimulated up to 300% by collapsing the H* transmembrane gradients. H*/ATP stoichiometry was measured in resealed chromaffin ghosts devoid of ATP and catecholamines under conditions where no net pH changes occur upon ATP hydrolysis. After addition of ATP, the rates of H* accumulation in the ghosts and ATP hydrolysis were both linear for about 60-100 s, and the ratio of H* to ATP was 1.71. These data indicate that the H* ATPase of chromaffin granules has both kinetic similarities and dissimilarities with other known H* ATPases. The regulation by changes in H* gradients and the fixed H*/ATP ratio of this ATPase is further evidence of its primary role in establishing electrogenic H* translocation and H* gradients in chromaffin granules.

The chromaffin granule is a highly specialized subcellular organelle which sequesters most of the catecholamines in the chromaffin cells of the adrenal medulla (1-6). A large body of recent evidence obtained with isolated chromaffin granules and ghosts has unequivocally established that the electrochemical proton gradient across the chromaffin granule membrane is the driving force for biogenic amine accumulation (7-19). In keeping with the tenets of the chemiosmotic hypothesis, transport of the catecholamines requires an obligatorily electrogenic H* pump, genuine insight into its basic properties, functioning, and regulation has lagged behind. In addition, review of the literature reveals rather wide disagreement concerning many of the fundamental properties of the ATPase so far investigated, such as values for Vmax and pH optimum (3, 18, 23-24).

The lack of knowledge in some important aspects of the chromaffin granule ATPase properties and regulation and the large variability in the activities measured so far can be attributed to several factors: 1) impurity of some biological preparations which contain other ATPases of mitochondria and lysosomes; 2) nonstandardization of protein assays and lack of appreciation of the large interfere from catecholamines in several of these assays; 3) use of nonoptimal techniques for measurement of ATP hydrolysis; 4) utilization of intact chromaffin granules with high endogenous ATP content and transmembrane proton gradients; 5) failure to consider that ATPase activity is regulated by existing H* gradients; and 6) technical limitations in the ability to simultaneously measure ATPase activity, H* fluxes, and the electrochemical H* gradients.

In this study, some of these drawbacks and limitations in experimental approach have been overcome by using: 1) highly purified chromaffin granule membranes, 2) formation of chromaffin ghosts devoid of ATP and transmembrane gradients, 3) multiple methodologies for the measurement of ATPase activity, and 4) sensitive potentiometric and spectrophotometric measurement of electrochemical proton gradients. With these approaches, it was possible to investigate for the first time important kinetic parameters of the chromaffin granule H* ATPase as well as its regulation by transmembrane H* gradients and its stoichiometry with H* transport. In addition, the comparison of the data reported here with previous measurements of other properties of the ATPase permits clarification of many important ambiguities. Several of the experiments were previously reported in abstract form (35).

1 The abbreviations used were: ΔpH, transmembrane proton gradient; ΔΨ, transmembrane potential gradient; Mes, 3-(N-morpholino)ethanesulfonic acid; FCCP, carbonyl cyanide p-trifluoromethoxy phenylhydrazone; DCCD, dicyclohexylcarbodiimide; Di-S-C3(5), 3,3'-dipropyl-2,2'-thiodiacyrbocyanine.
H+ ATPases of Chromaffin Granules

EXPERIMENTAL PROCEDURES

Preparation of Chromaffin Granules, Ghosts, and Fragmented Membranes—Chromaffin granules were isolated in isotonic media and in isotonic Percoll gradients as described previously (36). Chromaffin ghosts were prepared according to the method previously described (37) with the following modification. Intact purified chromaffin granules (150 mg), suspended in 5 ml of isolation medium, were subjected to lysis by incubation in 200 ml of 6 mM Tris-maleate, 0.06% bovine serum albumin, and 5 mM EDTA at pH 7.0 for 15 min at 4 °C. The membranes were pelleted at 23,000 X g for 20 min and the supernatant discarded. After resuspension and gentle homogenization of the membranes to free the lysing medium, the above lysing procedure was repeated. Ghosts were formed by suspension of the doubly lysed membranes in 10 ml of 185 mM KCl, 1 mM Mes at pH 6.10, placed in a dialysis tubing, and dialyzed overnight at 4 °C against 3 liters of the same medium. After 16-18 h, the contents of the bag were removed, washed by dialysis in 100 ml of fresh dialysis medium, and pelleted by high speed centrifugation. The ghosts were resuspended in fresh medium and used immediately in experiments.

Chromaffin granule fragmented membranes were prepared from doubly lysed membranes through resuspension in 20 mM Tris-maleate at pH 7.0 and stored at 4 °C until use. In these fragmented membranes the space accessible to [3H]polydextran is virtually identical with that of H2O, the amount of endogenous ATP and catecholamines is negligible, and no detectable H+ gradients are present in either the presence or absence of ATP.

Protein was measured by the method of Bradford (38), using bovine serum albumin as the standard.

Measurement of ATPase Activity—Three different methods were used to measure enzymatic ATP hydrolysis.

1) Colorimetric determination of inorganic phosphate (P) by the method of Fiske and Subbarow (39). After addition of MgATP to a 4 ml reaction mixture, 300-μl aliquots were withdrawn at various times, added to 600 of 1.5% sodium dodecyl sulfate or 600 μl of 15% perchloric acid, and centrifuged in an Eppendorf desk centrifuge for 5 min to precipitate protein. Six hundred μl of each supernatant was then added in 1.4 ml of distilled H2O, 400 μl of 1.25% ammonium molybdate in 2.5 x H2SO4, and 100 μl of Fiske and Subbarow reducing reagent. The samples were incubated for 10 min at 23 °C and absorbance at 660 nm was determined using a Varian 210 C spectrophotometer and compared to a linear standard curve prepared under identical conditions using H3PO4.

2) Appearance of 32P from [γ-32P]ATP by the method of Seals et al. (40). The γ-32P[ATP used in these experiments contained a specific activity of 0.5-1.0 μCi/μmol.

3) Production of ADP measured spectrophotometrically as the rate of NADH oxidation in an enzymatically coupled reaction (41). In a reaction cuvette were placed 1.9 ml of reaction medium (including 10 mM K+ and 10 mM Mg2+), chromaffin protein (0.2 mg), 1.4 mM F-enolpyruvate, 40 μg of pyruvate kinase-lactate dehydrogenase suspension, and 0.11 m NADH; a reference cuvette containing all reagents but NADH was also prepared. The reaction was started by the addition of ATP and the rates of absorbance decrease at 340 nm were recorded in a Varian 210 split-beam spectrophotometer. Standards used to quantify absorbance changes were prepared by adding various concentrations of ADP to the reaction cuvette.

Measurement of ATP-driven H+ Translocation—ATP driven H+ translocation was measured by the ATP pulse technique described by Mitchell and Moyle (42) and by Thayer and Hinkle (43), in which H+ fluxes across the vesicles are measured as changes in the pH of the medium. Measurements were carried out in a magnetically stirred 4-ml glass reaction vessel fitted with a plastic water jacket held constant at 25 °C. The pH was measured using a Thomas model 2480-T1 pH electrode connected to an Orion model 701-A pH meter and strip chart recorder with built-in scale expander (1-mV full scale). The pH electrode, reaction vessel, and magnetic stirrer were enclosed within a Faraday cage constructed of copper screen and grounded to the recorder to minimize electrical interference.

Chromaffin ghosts (1-3 mg), prepared as described above, were placed in the reaction vessel containing 185 mM KCl, 500 μM Mes (pH 6.10), and 1.7 mM MgSO4 (conditions shown by Alberty (44) to inhibit any H+ production due to ATP hydrolysis. Valinomycin (7.5 μg/mg of protein) was added to prevent the generation of transmembrane potentials (positive inside) which would retard proton translocation. Reserpine (0.37 μg/mg of protein) was added to prevent any proton movements caused by carrier-mediated catecholamine fluxes across the vesicular membranes. Final volume was 3.0 ml. The pH was adjusted to 6.1 with HCl and the suspension was allowed to equilibrate 10-15 min until a stable pH baseline was observed. Proton translocation was initiated by the addition of 10 μl of 20 mM ATP 40 mM MgSO4 (pH 6.1). Measurements were quantitated by adding known amounts of a HCl standard.

Measurement of Intravesicular pH and Transmembrane Potential—Intravesicular pH and ΔpH across the membrane was measured by [14C]methylamine distribution as described previously (8). The transmembrane electrical potential across the chromaffin granule membrane was measured spectrophotometrically using the membrane potential dye Di-S-C3(5) by the method of Salama et al. (45). Potential dependent changes in transmitted light of Di-S-C3(5) were monitored using a Johnson Foundation dual wavelength spectrophotometer at 665-655 nm. The dye signal was calibrated by quantitatively varying the Nerst potential for protons using FCCP and HCl, as previously described (45).

Materials—All standard reagents were purchased from Sigma. Pyruvate kinase-lactate dehydrogenase suspension and F-enolpyruvate were obtained from Boehringer Mannheim. FCCP was purchased from Pierce Chemical Co. Tris[methyl)lin, DCCD, and other substituted carbodiimides were obtained from K & K Rare Chemicals, Inc., Plainview, NJ. Di-S-C3(5) was a generous gift from Dr. A. Waggoner of Amherst College, Amherst, MA.

RESULTS

Kinetic Properties of the ATPase—Incubation of the fragmented chromaffin granule membranes with increasing concentrations of ATP produced a dose-dependent increase in the ATPase activity until a plateau was reached. Analysis of the data in terms of a double reciprocal plot (Fig. 1) permits calculation of a Km of 69 μM and a Vmax of 111 nmol/min/mg of protein at 20 °C.

The enzyme exhibited a pH dependence with a maximum of activity at pH 7.30 (Fig. 2). The relationship of the pH to
the rate of hydrolysis was relatively broad with the specific activity varying only by approximately a factor of 2 between pH 6.0 and pH 9.0.

The relationship between the ambient temperature and the rate of the ATPase was examined between 5 and 40 °C (not shown). Increasing the temperature from 5 to 25 °C produced nearly a 10-fold increase in the ATPase activity (16 to 148 nmol/min/mg of protein), while the increase from 25 to 40 °C was by a factor of 3 (148 to 525 nmol/min/mg of protein). An Arrhenius plot of these data revealed two distinct slopes with an intersection at 15 °C.

Inhibitors—Several agents which selectively inhibit H* ATPase in mitochondria, chloroplasts, chromatophores, and bacteria (46-49) were tested for their effects upon the ATPase activity of fragmented chromaffin granule membranes (Table I). In these representative experiments, the baseline ATPase activity was 110 nmol/min/mg of protein. While ethanol had little effect upon the ATPase rate, it was of interest that dimethyl sulfoxide, a commonly used solvent for solubilization of these inhibitors, increased the ATPase rate 50% over that of the control. Rotenone and antimycin A (two electron transport inhibitors), oligomycin (a mitochondrial ATPase inhibitor), and reserpine (a biogenic amine transport inhibitor) all failed to significantly influence the rates of ATPase. DCCD has been shown to be an effective inhibitor of chromaffin granule ATPase (11). The effects of DCCD could be reproducibly titrated in terms of inhibition of the ATPase activity (Fig. 3), and 50% inhibition was observed at 310 nmol/mg of protein. Analogues of DCCD were studied to determine whether a more sensitive inhibitor could be identified. The results showed that among the various carbodiimides tested, DCCD was the most effective inhibitor in its class on a molar basis, closely followed by N,N'-disopropylcarbodiimide (NIPCD) and N-ethyl-N'(3-dimethylaminopropyl)carbodiimide (EPICD). Antimycin A (30 nmol/mg) had a little effect upon the ATPase rate, it was of interest that dimethyl sulfoxide, a commonly used solvent for solubilization of these inhibitors, increased the ATPase rate 50% over that of the control. Rotenone and antimycin A (two electron transport inhibitors), oligomycin (a mitochondrial ATPase inhibitor), and reserpine (a biogenic amine transport inhibitor) all failed to significantly influence the rates of ATPase. DCCD has been shown to be an effective inhibitor of chromaffin granule ATPase (11). The effects of DCCD could be reproducibly titrated in terms of inhibition of the ATPase activity (Fig. 3), and 50% inhibition was observed at 310 nmol/mg of protein. Analogues of DCCD were studied to determine whether a more sensitive inhibitor could be identified. The results showed that among the various carbodiimides tested, DCCD was the most effective inhibitor in its class on a molar basis, closely followed by N,N'-disopropylcarbodiimide and N-ethyl-N'(3-dimethylaminopropyl)carbodiimide (metho-para-toluene sulfonate). 1-cyclohexyl-3-(2-morpholiny1-4-ethyl)carbodiimide HCI (MPCD), N-ethyl-N'(3-di-methylaminopropyl)carbodiimide (metho-para-toluene sulfonate) (EPICD), N,N'-disopropylcarbodiimide (DIPCD) indicated in the figure. Temperature was 22 °C.

**Fig. 2.** The effect of pH on ATPase activity in fragmented chromaffin granule membranes. Fragmented chromaffin granule membranes (1.1 mg) were incubated for 5 min in a reaction medium consisting of either 20 mM Mes (●—●) or 20 mM Tris-maleate (▲—▲) at the indicated pH. Total volume was 4.0 ml. The reaction was started by the addition of 5 mM MgATP (1:1). At various time points (0-5 min), 300-μl aliquots of the mixture were placed in sodium dodecyl sulfate (1% final concentration) and spun in an Eppendorf microcentrifuge for 5 min. The supernatants were assayed colorimetrically for P content as described under “Experimental Procedures.” Temperature was 22 °C.

**Fig. 3.** Dose-dependent effect of trimethyl tin chloride, DCCD, and other carbodiimides upon ATPase activities of fragmented chromaffin granule membranes. The experimental conditions were identical with those of Fig. 2 except that 20 mM Tris-maleate (pH 7.0) was present, together with the amounts of trimethyl tin chloride (TMT) and the carbodiimides DCCD, 1-cyclohexyl-3-(2-morpholiny1-4-ethyl)carbodiimide HCI (MPCD), N-ethyl-N'(3-di-methylaminopropyl)carbodiimide (metho-para-toluene sulfonate) (EPICD), or N,N'-disopropylcarbodiimide (DIPCD) indicated in the figure. Temperature was 22 °C.

**TABLE I**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Rate ATP hydrolysis*</th>
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<tr>
<td></td>
<td>nmol P_i/min/mg</td>
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<tr>
<td>Control</td>
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<td>100</td>
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<td>Effect of commonly used solvents</td>
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<tr>
<td>2.5% (v/v) ethanol</td>
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<td>Oligomycin (30 nmol/mg)</td>
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<td>DCCD (4 μmol/mg)</td>
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<td>Trimethyl tin (0.54 μmol/mg)</td>
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<tr>
<td>Effect of amine transport inhibitors</td>
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<td></td>
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<tr>
<td>Reserpine (0.2 μg/mg)</td>
<td>122</td>
<td>111</td>
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*ATPase activity was measured colorimetrically as the appearance of inorganic phosphate based on the method of Fiske and Subbarow (38), as described under “Experimental Procedures.”
chemical proton gradient in intact chromaffin granules was then investigated by measuring: 1) baseline ATPase activity, 2) ATPase activity after collapse of the ΔpH, and 3) ATPase activity after collapse of the Δψ.

When intact chromaffin granules are suspended in a sucrose medium at pH 6.8, an intravesicular pH of 5.5 can be measured by [14C]methylamine distribution (8) or 31P NMR techniques (9). This pH is independent of the presence of ATP. On the other hand, when ATP is added to isolated granules, the membrane potential, as measured by [3H]thiocyanate or Di-S-C3(5) (a membrane potential dye), increases from 0 to 60–80 mV, positive inside (13, 14, 45).

In the presence of 5 mM ATP, a linear rate of ATPase activity was observed (Fig. 4). Collapse of the ΔpH from 1.2 to 0.3 pH units with 50 mM NH4Cl resulted in a moderate increase in the ATPase activity (40%). On the other hand, collapse of the membrane potential from 60 to 0 mV with 50 mM thiocyanate produced a 120% increase in rates of ATP hydrolysis. Finally, the addition of FCCP, which permits H+ to equilibrate according to its electrochemical potential, resulted in a stimulation of the ATPase activity of over 300%.

The results indicate that the chromaffin granule ATPase is responsive to changes of either component of the electrochemical proton gradient, but is largely modulated by changes in Δψ.

Fig. 5 shows the relationship between rate of ATPase activity and the transmembrane potential, which was collapsed in a dose-dependent fashion with FCCP and which was monitored spectroscopically using a potential indicator. Under the conditions of the experiment, no measurable changes in the ΔpH could be detected (not shown). The results indicate that the rate of ATPase activity is in fact dependent on the magnitude of the membrane potential.

Stoichiometry of H+ Translocation and ATP Hydrolysis—The measurement of ATP-driven proton translocation in cell and organelle suspensions and the stoichiometry between ATP hydrolysis and H+ translocation are often difficult to quantitate and interpret because: 1) the hydrolysis of ATP itself results in a net pH change of the media at most values of pH and Mg2+ concentrations, 2) movement of H+ through other coupled transport systems often occurs, and 3) adenylate kinase activity occurs in most subcellular preparations.

In the measurements described, as previously established in other systems (42, 43), the accurate choice of pH and Mg2+ concentration in the medium (44) effectively prevented pH changes ascribable to ATP hydrolysis above. This was experimentally verified by measuring pH changes in the ATP-containing medium in which fragmented chromaffin granules or appyrase, instead of chromaffin ghosts, were dissolved. In either case, no pH changes were detectable. Contribution to the measurements by adenylate kinase activity was also excluded by the finding that negligible levels of AMP could be detected in our sample during the reaction through high pressure liquid chromatography and by the fact that virtually identical values were obtained in the presence or in the absence of 200 μM adenylate kinase inhibitor di(adenosine-5')pentaphosphate. Finally, chromaffin ghosts were utilized in all the experiments reported below because they are devoid of endogenous pH gradients and intragranular components which could engage in H+ exchange reactions during the

![Fig. 4. Effect of FCCP, thiocyanate (SCN-), and NH4+ on ATPase activity of chromaffin granules.](image-url)
course of the observations. For all of these reasons, any pH change of the medium should reflect actual translocation of H⁺ across the chromaffin ghost membrane. Fig. 6 shows a time-dependent pH change in the medium obtained by the continuous potentiometric monitoring of a fast responding pH electrode. The addition of ATP to the reaction mixture resulted in a large time-dependent alkalization of the external medium consistent with H⁺ translocation into the intravesicular space (Fig. 6A). The external pH reached a maximum alkalinity at approximately 120 s and thereafter slowly returned to the original pH. In the presence of FCCP (Fig. 6B) minimal H⁺ fluxes were observed; this experimental observation supported the notion that the pH changes reflected the generation and decay of a pH gradient across the membrane. Similarly, when the ATPase inhibitor trimethyl tin chloride (Fig. 6C) was included in the incubation medium, no pH change was observed. Included within the reaction mixtures was valinomycin (in order to prevent formation of diffusion potentials) and reserpine (to inhibit possible movement of endogenous catecholamines).

Under experimental conditions identical with those of Fig. 6A, ATP hydrolysis by chromaffin ghosts was measured following the release of ³²P from [γ-³²P]ATP, as described under "Experimental Procedures" (Fig. 7). The rate of ATP hydrolysis was approximately linear over the first 60-90 s. Fig. 8 shows that the H⁺/ATP ratio was obtained from a semilogarithmic plot of nmol of H⁺/nmol of ATP versus time in accordance with the relationship described by Mitchell and Moyle (42) and Thayer and Hinkle (43). The calculated H⁺/ATP ratio was 1.71 in this experiment. Values in other experiments ranged from 1.62 to 1.79 (1.66 ± 0.12).

**Discussion**

The purpose of this investigation was to provide important kinetic data not available in the literature on the H⁺ ATPase of chromaffin granules in situ and to demonstrate that the activity of this enzyme is effectively modulated by changes in the transmembrane H⁺ gradients across the granules. In addition, during the course of this study, a reinvestigation of a few properties of this enzyme, which have been published in the literature but have yielded a wide range of values and inconclusive information, was undertaken.

In order to provide reliable, or at least consistent values on ATPase activities under various conditions, a major emphasis of this study was placed on the use of (a) a highly purified and characterized preparation of chromaffin granules, from which very reproducible preparations of either resealed ghosts or fragmented membranes could be routinely made; (b) a standardized methodology for measuring ATPase specific activities coupled with a rigorous identification and definition of the factors influencing these activities, such as state of the membrane, substrate concentration, pH and temperature of the medium, etc.; and (c) a suitable experimental design allowing monitoring and/or control of H⁺ transmembrane gradients across granules and ghosts during the measurements of ATPase activities.

**Kinetics and Inhibitors**—The K_M of the ATPase for ATP (in the presence of a large, fixed Mg²⁺ concentration) was 69 µM. This value, which has not previously been determined, is far below the cytosolic ATP concentration (9). This suggests that ATPase activity in situ should not be affected by changes in cytosolic ATP concentrations in the physiologic range.

The V_max of the ATPase activity was consistently measured in the range 110-130 nmol of ATP/mg of protein/min. Literature values vary widely (25-34). This variability more than likely relates to the varying methods of preparation, the different temperatures under which the experiments were performed, and the inaccurate protein determination due to the unsuitability of the assays used (see above).

The measured pH optimum (pH 7.40) differs substantially from previous reports (11, 51), and compares favorably with another (27). Indirect evidence is accumulating which indicates that the F₁ portion of the ATPases is spatially located in the cytosolic side of the membrane (31). Since the presumed cytosolic pH is 7.4, as opposed to the pH 5.5 of the intravesicular space (8, 9), the ATPase would thus be functioning optimally in vivo.

Two specific inhibitors of the ATPase were studied (Fig. 3) and titrated over a wide range of concentrations. The first, DCCD, is known to inhibit the ATPase of mitochondria, chloroplasts, and *Escherichia coli*, and is thought to bind a proteolipid (52-53). Fifty per cent of the inhibition was observed at 310 nmol/mg of protein. No other substitution of DCCD produced a greater inhibition on a molar basis. The
second, trimethyl tin, is one of a group of structurally related agents which also act by binding to a protoporphyrin, probably near to but not identical with the site of FCCD binding (54). Fifty per cent inhibition was observed at 20 mmol/mg of protein, more than 1 order of magnitude smaller than that required for FCCD. The amount of these two inhibitors required for 50 or 90% inhibition of H⁺ ATPase activity is 2 to 5 times higher than that previously reported in the literature (11). This apparent discrepancy can be fully accounted for by catecholamine interference in some measurements of protein concentrations, and/or by the fact that ATPase specific activity, on a milligrams of protein basis, is much smaller in intact chromaffin granules than in fragmented membranes, which are devoid of intragranular proteins.

Two other inhibitors, oligomycin and aurovertin, specifically inhibit other H⁺ ATPases (55). None has any effect upon the chromaffin granule ATPase. These differences in the selectivity of agents whose mechanism of action is thought to relate to binding to the F₁ portion of the ATPase indicate at least the nonidentity of the mitochondrial and chromaffin granule ATPase. In addition, the absence of inhibition of the chromaffin granule ATPase activity further supports the notion that mitochondrial contamination is negligible.

Several other compounds have been reported to inhibit the ATPase activity of chromaffin granules. 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid and pyridoxal phosphate are nonspecific in that they have also been implicated as inhibitors of chloride permeability (56). Reserpine, a specific inhibitor of the catecholamine transporter, has had mixed reports on its ATPase inhibitory ability (57-59). The data in Table 1 supports the conclusion that reserpine at concentrations which totally inhibit catecholamine accumulation does not alter ATPase activity.

Regulation of ATPase Activity—Analysis of the rate of hydrolysis of the enzyme within the intact granule under various conditions permitted investigation of the factors which regulate its activity (Figs. 4 and 5). The experimental data suggests that the rate of the ATPase is relatively insensitive to the magnitude of the ΔpH. However, the ATPase is sensitive to manipulation of the ΔΨ over the range -90 to 40 mV, with a 5- to 6-fold increase in the rate of ATP hydrolysis. The large internal buffering capacity precludes short term proton fluxes affecting the ΔpH across the membrane; therefore, short term regulation of the ATPase can be achieved only through the electrical contribution.

H⁺/ATP Stoichiometry—The H⁺/ATPase ratio has been studied so far only through the application of very indirect methods. For example, Flatmark and Ingebretnsen (32) measured the H⁺/ATP ratio in chromaffin ghosts after a long incubation, in the presence of a high concentration of ATP and utilizing the distribution of a membrane permeable dye from which the proton flux was back-calculated. Njus et al. (60) calculated the H⁺/ATP ratio in intact granules which already possessed an endogenous pH gradient using large ATP concentrations, long incubation times, and regression analysis to calculate the back leak.

The measurements and calculations of the H⁺/ATP ratio described in this communication take advantage of an experimental protocol which has been described in detail by Mitchell and Moyle (45) and by Thayer and Hinkle (49), and successfully applied to similar measurements in mitochondria and sub mitochondrial particles. In our case the negligible contribution of (a) ATP hydrolysis alone on the observed pH changes in the medium during H⁺ accumulation, and (b) adenylate kinase in the calculation of the overall ratio, were experimentally verified. The coupling of H⁺ exchange to movement of other ions or metabolites was also excluded or largely minimized by the choice of experimental conditions. Among these were the use of chromaffin ghosts, which maintain low permeability towards various ions and are devoid of catecholamines, ATP, other metabolites, and H⁺ gradients endogenously present in intact granules. Furthermore, wide variation in extra and intravesicular ionic composition of the ghosts can be achieved during rescaling and resuspension with negligible change in the H⁺/ATP ratio.

The data reveals a H⁺/ATP ratio as high as 1.8, indicating that the H⁺/ATP ratio is most likely 2.0. It cannot be entirely excluded, however, that nonintegral proton translocation exists. For example, a functional grouping on the enzyme involved in proton translocation may have a pK₅ near the pH of the reaction medium, the nonintegral nature reflecting an average of two ongoing processes. Unfortunately, technical limitations preclude accurate measurements of the H⁺/ATP ratio over a wide pH range.

In conclusion, these data provide an overall characterization of the properties of chromaffin granule ATPase in situ, which, together with emerging structural studies, should afford the necessary basis for further experimentation towards the understanding of the physiological function and control of this enzyme. Such knowledge also has important wider implications for the investigation of biochemical mechanisms underlying uptake and/or storage of amines and peptides in various organelles which possess H⁺ ATPases similar to those of chromaffin granules (61-55), but which are more difficult to isolate in suitable purity and quantity.

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