Interaction of Anions and ATP with the Coated Vesicle Proton Pump†

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ABSTRACT: ATP-driven proton transport in intact clathrin-coated vesicles requires the presence of a permeant anion, such as Cl^-, to provide charge compensation during the electrogenic movement of protons. Using the purified (H^+)-ATPase from clathrin-coated vesicles in both the detergent-solubilized and reconstituted states, we have studied the direct effects of anions on the activity of this enzyme. Both proton transport and ATP hydrolysis by the purified enzyme are independent of the presence of Cl^-. In addition, proton transport does not occur even at high Cl^- concentrations unless K^+ and valinomycin are present to dissipate the membrane potential generated. These results indicate that the anion channel which provides for Cl^- flux in intact coated vesicles is not a component of the purified (H^+)-ATPase. Inhibition of ATPase activity is observed in the presence of I^-, NO_3^-, or SO_4^{2-}, with 50% inhibition occurring at 350 mM I^-, 50 mM NO_3^-, or 40 mM SO_4^{2-}. The presence of ATP lowers the concentration of I^- required for 50% inhibition from 350 mM to 100 mM and increases the maximal inhibition observed in the presence of NO_3^- from 65% to 100%. Two separate mechanisms appear to be responsible for anion inhibition of the (H^+)-ATPase. Thus, I^- and high concentrations of NO_3^- (in the presence of ATP) cause inhibition by dissociation of the (H^+)-ATPase complex, while SO_4^{2-} and NO_3^- (in the absence of ATP) cause inhibition without dissociation of the complex, suggesting the existence of an inhibitory anion binding site on the enzyme. We have also investigated the interactions of ATP with the purified (H^+)-ATPase. The dependence of ATPase activity on ATP concentration reveals the presence of two sites with K_m values of 83 (±15) μM and 790 (±70) μM. These sites are responsible for 27% (±5%) and 73% (±6%) of the maximal ATPase activity, respectively. Saturation of the high-affinity site results in increased proton transport in reconstituted vesicles, while saturation of the low-affinity site causes inhibition of proton transport by 80%, suggesting a decreased stoichiometry of protons transported per ATP hydrolyzed at high ATP concentrations. Dissociation of the (H^+)-ATPase by I^- is promoted by binding of ATP to a site with a K_d of 150–200 nM. Thus, our results are consistent with the existence of at least three distinct classes of ATP binding sites on the coated vesicle (H^+)-ATPase.

Acidification of vacuolar compartments in eukaryotic cells is accomplished by a unique class of ATP-driven proton pumps present in the membranes of these intracellular organelles (Forgac, 1988). A feature common to virtually all of these systems is the dependence of acidification on the presence of a permeant anion, such as Cl^- (see Discussion). These results have been interpreted to indicate that ATP-driven proton transport requires the presence of an anion channel to provide charge compensation during the electrogenic movement of protons across the membrane. In the present paper, we address the question of whether an anion channel activity resides in some component of the purified proton-translocating adenosinetriphosphatase [(H^+)-ATPase][1] from clathrin-coated vesicles and investigate the direct effects of anions on the purified enzyme.

The coated vesicle (H^+)-ATPase contains nine subunits of molecular weight 17 000–100 000 (Arai et al., 1987b; Xie & Stone, 1986), and we have identified the function of two of the subunits in this complex. Thus, the 73 000-dalton subunit has been shown to contain a nucleotide binding site required for activity (Arai et al., 1987b) while the 17 000-dalton subunit is involved in DCCD-inhibitable proton conductance (Arai et al., 1987a). Labeling studies on the plant vacuolar (H^+)-ATPase suggest that the 58 000-dalton subunit may also possess a nucleotide binding site (Manolson et al., 1985). The subunits of the coated vesicle (H^+)-ATPase are present in a stoichiometry of three copies each of the 73 000- and 58 000-dalton subunits, six copies of the 17 000-dalton subunit, and one copy each of the remaining six polypeptides (Arai et al., 1988). This structure is remarkably similar to that observed for the F_1F_0 class of (H^+)-ATPases [for a review, see Amzel and Pedersen (1983)] and together with recent sequence data on the vacuolar proton pumps (Bowman, B. J., et al., 1988; Bowman, E. J., et al., 1988; Zimniak et al., 1988) suggests a common evolutionary origin for the F_1F_0 and vacuolar (H^+)-ATPases (Arai et al., 1988). Give the presence of multiple nucleotide binding subunits and the close homology with the F_1F_0 class of (H^+)-ATPases, which show complex nucleotide binding properties (Senior & Wise, 1983; Cross, 1981), we have investigated the interactions of the purified coated vesicle (H^+)-ATPase with ATP. We present evidence suggesting the existence of at least three distinct classes of ATP

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[1] Abbreviations: (H^+)-ATPase, proton-translocating adenosinetriphosphatase; $C_{12}E_9$, poly(oxyethylene) 9-lauryl ether; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonate; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; SDS, sodium dodecyl sulfate.

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binding sites which are distinguishable by their affinity and by their effects on the activity and structure of the purified (H^+)-ATPase.

Materials and Methods

Materials. Calf brains were obtained fresh from a local slaughterhouse. Phosphatidylcholine and phosphatidylserine were obtained as chloroform solutions from Avanti Polar Lipids, Inc., and stored at −20 °C. $C_{12}E_9$, cholesterol, cholic acid, and the disodium salt of ATP (grade II) were purchased from Sigma Chemical Co., and both cholic acid and cholesterol were recrystallized prior to use. Acridine orange was obtained from Eastman Kodak while p-nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indoyl phosphate (toluidine salt), and affinity-purified goat anti-mouse IgG conjugated to alkaline phosphatase were obtained from Bio-Rad.

Assays. ATPase activity was measured by a continuous spectrophotometric assay as previously described (Forgac et al., 1983). Unless otherwise indicated, the assay mixture contained 1.0 mM ATP, 2.0 mM $MgSO_4$, 1.6 mM phosphoenolpyruvate, 0.25 mg of NADH/mL, 20 μg of pyruvate kinase/mL, and 10 μg of lactate dehydrogenase/mL. In addition, either 0.05% $C_{12}E_9$, 20 μg of phosphatidylcholine/mL, and 10 μg of phosphatidylserine/mL or 2.3 μM valinomycin and 2.5 μg of FCCP/mL were added as indicated to prevent inhibition of ATPase activity due to the generation of an electrochemical gradient of protons across the reconstituted vesicle membrane.

Proton transport in reconstituted vesicles was measured by acridine orange fluorescence quenching using a Perkin-Elmer LS-5 spectrofluorometer as previously described (Arai et al., 1987b). Unless otherwise indicated, the assay medium contained 2 μM acridine orange and 2.3 μM valinomycin. Valinomycin was present to prevent the generation of a membrane potential during proton uptake by reconstituted vesicles. Protein concentration was determined either by the method of Lowry et al. (1951) or, for samples containing $C_{12}E_9$, by the method of Schaffman and Weissman (1973).

Purification and Reconstitution of the (H^+)-ATPase from Clathrin-Coated Vesicles. Clathrin-coated vesicles were prepared from calf brain as previously described (Forgac & Cantley, 1984). Vesicles were stripped of their clathrin coat by dilution (40-fold) into 5 mM Tris (pH 8.5), 150 mM sucrose, and 0.5 mM EGTA followed by incubation for 1 h at 23 °C and sedimentation for 1 h at 100000*g*. Stripped vesicles displayed a 10-fold higher specific activity for the (H^+)-ATPase relative to intact coated vesicles and were employed in the dissociation experiments to avoid complication due to the clathrin coat.

The (H^+)-ATPase was solubilized from stripped vesicles and purified by density gradient sedimentation as previously described (Arai et al., 1987b). Briefly, stripped vesicles at 1.0 mg of protein/mL were incubated with 1.0% $C_{12}E_9$ and 0.4 mg of phosphatidylcholine/mL in solubilization buffer [50 mM NaCl, 30 mM KCl, 20 mM HEPES (pH 7.0), 10% glycerol, and 0.2 mM EGTA] for 5 min at 23 °C followed by 20 min on ice. The solubilized mixture was sedimented at 100000*g* for 1 h and the pellet discarded. The solubilized (H^+)-ATPase (0.5 mL) was then loaded on an 11-mL 15–30% glycerol gradient prepared in solubilization buffer containing 0.02% $C_{12}E_9$ and 8 μg of phosphatidylcholine/mL and sedimented in a Beckman SW-41 rotor using a Beckman L7-55 ultracentrifuge for 16 h at 38000 rpm. The gradients were fractionated from the bottom to give 18–20 fractions and the fractions assayed for ATPase activity as described above. The purified (H^+)-ATPase sediments as a complex of molecular weight 700000–750000 (Arai et al., 1988), has a specific activity of 7–8 μmol of ATP min^{-1} (mg of protein)$^{-1}$ at 37 °C (corresponding to a 100-fold purification relative to intact coated vesicles), and contains nine polypeptides of molecular weights 100000, 73000, 58000, 40000, 38000, 34000, 33000, 19000, and 17000 (Arai et al., 1987b). We have previously demonstrated that this preparation is reconstitutively active and that this set of polypeptides forms a single macromolecular complex by their immunoprecipitation using any of a series of monoclonal antibodies which recognize the native enzyme (Arai et al., 1987b).

Reconstitution of the purified (H^+)-ATPase was carried out as previously described (Arai et al., 1987b). Briefly, to 0.5 mL of the purified enzyme (4 μg of protein/mL) were added the following: 100 μL of 5% cholate, 4 mg of phosphatidylcholine/mL, 2 mg of phosphatidylserine/mL, 150 μL of 10% cholate, 4 mg of cholesterol/mL, and 7 mg of phosphatidylcholine/mL. Following incubation for 5 min at 23 °C and 20 min on ice, the mixture was dialyzed for 3 days against five changes of 200 volumes of solubilization buffer using Spectrapor 2 dialysis tubing with a molecular weight cutoff of 12000–14000. For reconstituted vesicles prepared in the absence of Cl^-, solubilization buffer containing no Cl^- but with the glycerol concentration increased to maintain the same osmolarity was employed during dialysis. The pH of this modified buffer was adjusted with KOH (final potassium concentration approximately 10 mM) to provide sufficient intravesicular K^+ to dissipate the pump-generated membrane potential on addition of valinomycin.

Anion-Dependent Inhibition of (H^+)-ATPase Activity. The effects of anions on the activity of the purified (H^+)-ATPase were determined by one of two methods. In the first, various concentrations of the Na^+ or K^+ salt of the anion were added directly to the assay mixture containing the purified, reconstituted enzyme in the presence of 0.05% $C_{12}E_9$, 20 μg of phosphatidylcholine/mL, and 10 μg of phosphatidylserine/mL, and the effect on activity was measured. The same behavior was observed in all cases whether the Na^+ or K^+ salt was employed. In the second method, reconstituted vesicles were incubated for 60 min at 4 °C with the indicated concentrations of the appropriate salt and then diluted 10-fold into the assay mixture containing detergent plus phospholipid as above. By comparison of the activity under these two conditions, the reversibility of the inhibitory effects of I^-, NO_3^-, and SO_4^{2-} could be tested (Figure 3).

Anion-Dependent Dissociation of the 73000-Dalton Subunit of the Coated Vesicle (H^+)-ATPase. We previously demonstrated that 0.5 M KI causes dissociation of the 73000-dalton subunit of the coated vesicle (H^+)-ATPase (Arai et al., 1988). To determine whether this dissociation showed the same concentration dependence as inhibition of activity and to test whether other inhibitory anions also caused dissociation of the 73000-dalton subunit, the following experiments were performed. Coated vesicles from which the clathrin coat had been stripped as described above were incubated in solubilization buffer containing various concentrations of the K^+ salt of the appropriate anion for 1 h at 4 °C at a protein concentration of 0.38 mg of protein/mL. Where indicated, 5 mM ATP was also present. The vesicles were then removed by sedimentation at 100000*g* for 1 h, and the supernatant was dialyzed against solubilization buffer containing 0.02% $C_{12}E_9$ to remove the dissociating anion. Half the volume of 3-fold-concentrated Laemmli sample buffer was added, and the vesicle pellet was also dissolved in Laemmli sample buffer such that the final volumes of the supernatant and dissolved pellet were identical.

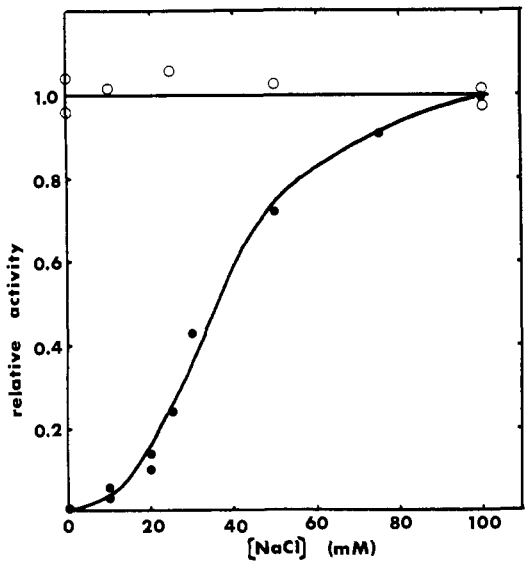

FIGURE 1: Cl^- concentration dependence of ATP hydrolysis by the purified (H^+)-ATPase and proton pumping in intact coated vesicles. (○) ATPase activity of the purified (H^+)-ATPase (0.4 μg of protein) was measured in the presence of 0.05% $C_{12}E_9$, 20 μg of phosphatidylcholine/mL, 10 μg of phosphatidylserine/mL, and the indicated concentrations of NaCl as described under Materials and Methods. The specific activity of the purified (H^+)-ATPase was 7–8 μmol of ATP min^{-1} (mg of protein)$^{-1}$ at 37 °C. (●) ATP-dependent proton transport in intact coated vesicles (100 μg of protein) was measured in the presence of the indicated concentrations of NaCl using acridine orange fluorescence quenching as described under Materials and Methods.

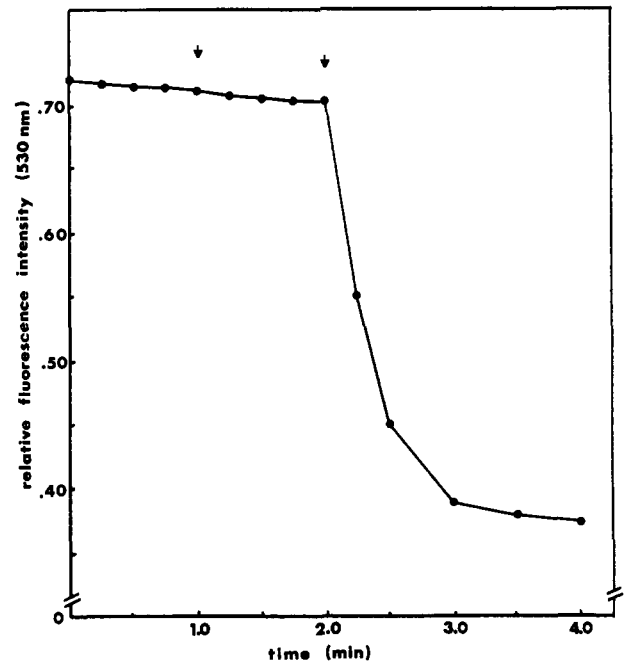

FIGURE 2: Dependence of proton transport in reconstituted vesicles on valinomycin. Reconstituted vesicles (0.8 μg of protein) prepared in solubilization buffer containing 50 mM NaCl and 30 mM KCl were equilibrated with 2 μM acridine orange, and at the first arrow 1 mM ATP and 2 mM $MgSO_4$ were added while at the second arrow 2.3 μM valinomycin was added. Acridine orange fluorescence quenching was monitored as described under Materials and Methods using an excitation wavelength of 490 nm and an emission wavelength of 530 nm. Addition of valinomycin in the absence of Mg^{2+} and ATP had no effect on the fluorescence intensity at 530 nm (data not shown).

Equal volumes of each sample were then run on an 8% acrylamide gel by the method of Laemmli (1970) and blotted to nitrocellulose overnight at 4 °C.

After being blotted, the paper was washed with 20 mM Tris (pH 7.5)/500 mM NaCl for 10 min at room temperature and soaked for 60 min in the same buffer containing 1.5% gelatin. After being blocked, the blot was washed twice with buffer containing 0.05% Tween-20 and then soaked for 2 h at room temperature in a 1:100 dilution of the monoclonal antibody TG3.2-F1 in buffer containing 0.5% gelatin and 0.05% Tween-20. The monoclonal antibody TG3.2-F1 is specific for the 73 000-dalton subunit of the coated vesicle (H^+)-ATPase and is capable of recognizing this polypeptide on Western blot and of immunoprecipitating the native (H^+)-ATPase complex as previously described (Arai et al., 1987b, 1988). The preparation and screening of monoclonal antibodies specific for the coated vesicle (H^+)-ATPase were carried out as previously described (Arai et al., 1987b).

The blot was then washed twice in Tween-containing buffer followed by soaking in a solution containing a goat anti-mouse IgG–alkaline phosphatase conjugate (Bio-Rad) plus 0.5% gelatin and 0.05% Tween-20 for 1 h at room temperature. After being washed in Tween-containing buffer twice and buffer alone once, the blot was developed by incubation with 0.03% *p*-nitro blue tetrazolium chloride and 0.015% 5-bromo-4-chloro-3-indoyl phosphate (toluidine salt) in 0.1 M sodium bicarbonate (pH 9.8)/1 mM $MgCl_2$ for 30 min. Development was stopped by washing with water, and the blot was air-dried.

RESULTS

Figure 1 shows the dependence on Cl^- concentration of both proton transport in intact clathrin-coated vesicles and ATPase activity of the purified, detergent-solubilized (H^+)-ATPase. As can be seen, while proton transport in coated vesicles is absolutely dependent on the presence of Cl^-, the ATPase activity of the purified enzyme is unchanged over the range of 0–100 mM Cl^-. To determine whether the Cl^- dependence of the pump was manifested during ATP-driven proton transport, we tested the ability of the reconstituted (H^+)-ATPase to carry out proton uptake in the presence of 80 mM Cl^- but in the absence of valinomycin. Figure 2 demonstrates that no ATP-driven proton transport is observed in reconstituted vesicles until valinomycin is added to dissipate the membrane potential established. In addition, we have observed that proton transport in the presence of valinomycin occurs normally even in the absence of Cl^- (data not shown). In contrast, intact coated vesicles show normal acidification in the absence of valinomycin provided Cl^- is present (Figure 1). These results indicate that the (H^+)-ATPase has no absolute requirement for Cl^- and that no component of the purified enzyme is capable of acting as a Cl^- channel in the reconstituted system. This in turn suggests that the Cl^- requirement of proton transport in intact coated vesicles is due to the presence of a structurally distinct anion channel present in the native membrane.

Table I shows the effects of various anions on the ATPase activity of the purified, detergent-solubilized (H^+)-ATPase. Although the activity in the absence of anions or in the presence of 100 mM Cl^-, PO_4^{3-}, or Br^- is nearly the same, inhibition of activity is observed in the presence of 100 mM I^-, SO_4^{2-}, or NO_3^-. The concentrations required for 50% inhibition of ATPase activity are 350 mM I^-, 40 mM SO_4^{2-}, or 50 mM NO_3^- (Figure 3). Inhibition by I^- and NO_3^- was not reversed on 10-fold dilution of the enzyme into the assay mixture while inhibition by SO_4^{2-} was almost completely reversible (Figure 3). Also shown in Figure 3 is the effect of ATP on anion-dependent inhibition of the (H^+)-ATPase. In the presence of ATP, the concentration of I^- required for 50%

Table I: Effect of Anions on (H^+)-ATPase Activity of the Purified Enzyme[a]

anion (100 mM)	rel (H^+)-ATPase act.	anion (100 mM)	rel (H^+)-ATPase act.
none	1.00 (±0.06)	I^-	0.46 (±0.06)
Cl^-	0.95 (±0.04)	SO_4^{2-}	0.36 (±0.07)
PO_4^{3-}	0.90 (±0.04)	NO_3^-	0.19 (±0.05)
Br^-	0.80 (±0.2)		

[a] Reconstituted vesicles (0.4 μg of protein) prepared in the absence of Cl^- were added to an assay mixture containing 100 mM Na^+ salt of the indicated anion, and the ATPase activity was measured as described under Materials and Methods. Activity was measured in the presence of 0.05% $C_{12}E_9$, 20 μg of phosphatidylcholine/mL, and 10 μg of phosphatidylserine/mL to avoid generation of an electrochemical gradient of protons during ATP hydrolysis by the reconstituted enzyme. The specific activity of the enzyme employed was 7–8 μmol of ATP min^{-1} (mg of protein)$^{-1}$ at 37 °C.

inhibition was lowered from 350 mM to 100 mM while the maximal inhibition observed by NO_3^- was increased from 65% to 100%.

We previously demonstrated that in the presence of 0.5 M KI, the 73 000-dalton subunit dissociated from the (H^+)-ATPase complex in the absence of detergent, indicating that this polypeptide is a peripheral membrane protein (Arai et al., 1988). To test whether inhibition of ATPase activity by anions is due to dissociation of the active complex, intact vesicles from which the clathrin coat has been dissociated were treated with various concentrations of I^-, NO_3^-, and SO_4^{2-} in the absence and presence of ATP, and dissociation of the 73 000-dalton subunit was monitored by Western blot analysis using a monoclonal antibody (TG3.2-F1) specific for this polypeptide (Arai et al., 1988). As can be seen by comparing Figures 3 and 4, inhibition of ATPase activity by I^- is closely correlated with dissociation of the 73 000-dalton subunit, both in the absence and in the presence of ATP. By contrast, SO_4^{2-} causes inhibition of ATPase activity without dissociation of the 73 000-dalton subunit, while NO_3^- causes dissociation only at high concentrations (>100 mM) and only in the presence of ATP. These results indicate that anions are capable of inhibiting the (H^+)-ATPase by two separate mechanisms. Thus, I^- and high concentrations of NO_3^- (in the presence of ATP) cause inhibition of activity by dissociation of the (H^+)-ATPase complex while SO_4^{2-} and NO_3^- (in the absence of ATP) inhibit activity without dissociation of the 73 000-dalton subunit. The latter result suggests the existence of an inhibitory binding site for anions on the purified enzyme.

Because of the observed effect of ATP in promoting dissociation of the (H^+)-ATPase complex and in light of the presence of multiple nucleotide binding subunits [see Discussion and Arai et al. (1988)], we have investigated the interaction of ATP with the purified enzyme. Figure 5 shows the dependence of ATPase activity of the purified, reconstituted (H^+)-ATPase on ATP concentration. As can be seen, the results are consistent with the existence of two sites with K_m values of 83 (±15) μM and 790 (±70) μM which are responsible for 27% (±5%) and 73% (±6%) of the maximal ATPase activity, respectively. Figure 6 shows the ATP dependence of proton transport by the reconstituted (H^+)-ATPase. Surprisingly, while saturation of the high-affinity site leads to increased proton transport, saturation of the low-affinity site causes inhibition of proton transport by 80%. The same result is observed for proton transport in intact coated vesicles, indicating that inhibition is not a property unique to the reconstituted enzyme. This result suggests that high concentrations of ATP cause a decrease in the stoichiometry of protons transported per ATP hydrolyzed. Figure 7 shows

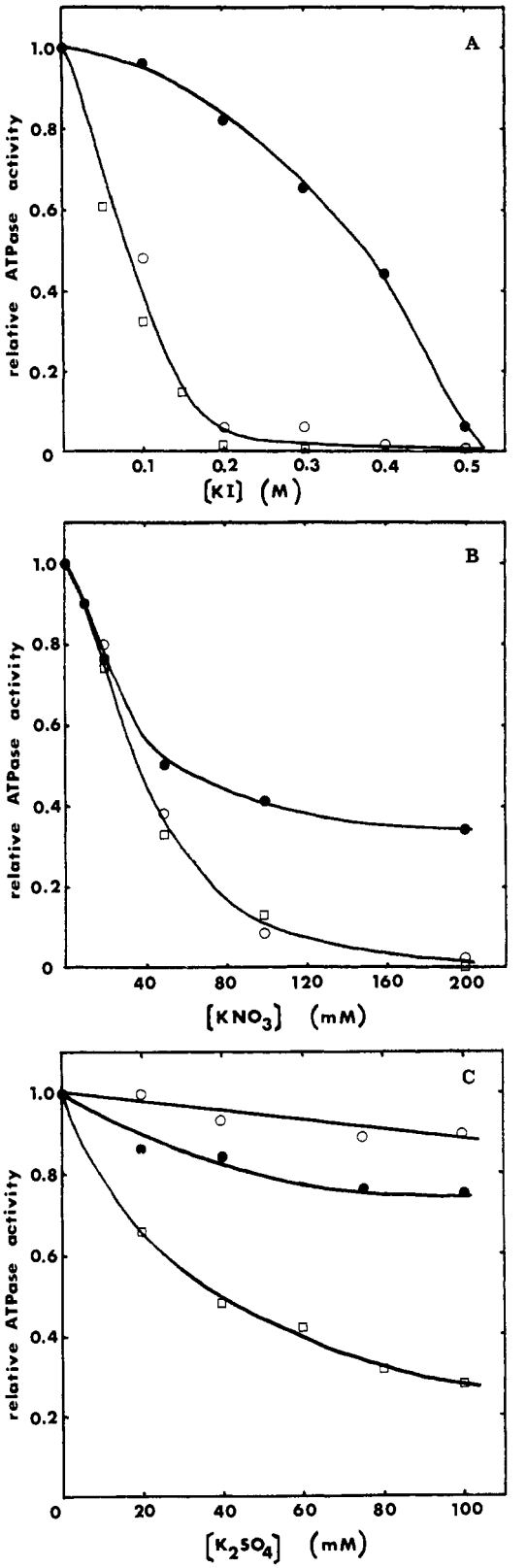

FIGURE 3: Anion-dependent inhibition of the purified (H^+)-ATPase. Reconstituted vesicles containing the purified (H^+)-ATPase (0.4 μg of protein) either were added directly to the assay mixture containing the indicated concentrations of anions (□) or were preincubated for 1 h at 4 °C with the indicated anions either in the presence (○) or in the absence (●) of 5 mM ATP and then diluted 10-fold into the assay mixture without the inhibitory anion. ATPase activity was measured as indicated under Materials and Methods in the presence of 0.05% $C_{12}E_9$, 20 μg of phosphatidylcholine/mL, and 10 μg of phosphatidylserine/mL to avoid generation of an electrochemical gradient of protons during ATP hydrolysis by the reconstituted (H^+)-ATPase. Shown are the effects of I^- (A), NO_3^- (B), and SO_4^{2-} (C).

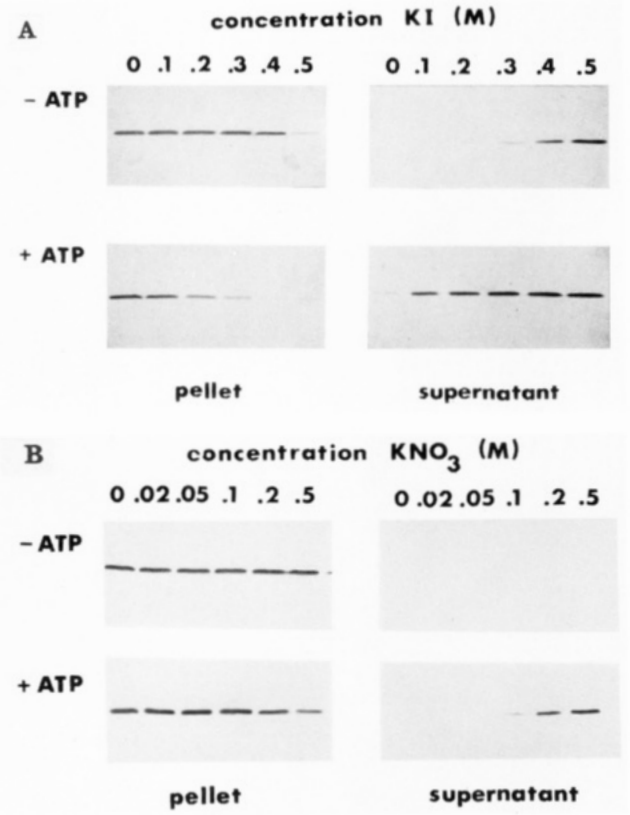

FIGURE 4: Anion-dependent dissociation of the 73 000-dalton subunit. Stripped vesicles (0.38 mg of protein/mL) were incubated with the indicated concentrations of either KI (A) or KNO_3 (B) in the absence or presence of 5 mM ATP for 1 h at 4 °C. The vesicles were then removed by sedimentation for 1 h at 100000g, and the supernatant was dialyzed against solubilization buffer containing 0.02% $C_{12}E_9$ to remove the dissociating anion. Half the volume of 3-fold-concentrated Laemmli sample buffer was added to the dialyzed supernatant, and the pellet was dissolved in Laemmli sample buffer such that the final volumes of the supernatant and pellet were identical. Equal volumes of each sample were run on an 8% acrylamide gel, and Western blotting using the monoclonal antibody TG3.2-F1 specific for the 73 000-dalton subunit and a goat anti-mouse IgG–alkaline phosphatase conjugate was carried out as described under Materials and Methods.

the ATP dependence of inhibition of the (H^+)-ATPase by 0.15 M I^- [a concentration which causes minimal inhibition of the (H^+)-ATPase in the absence of ATP (Figure 3)]. As can be seen, ATP promotes inhibition of the enzyme by I^- with a K_d of 150–200 nM. This affinity is considerably higher than that of either of the two sites described above and suggests the existence of a third distinct nucleotide binding site on the (H^+)-ATPase complex.

Discussion

Acidification of a variety of intracellular compartments has been shown to be dependent on the presence of a permeant anion, including clathrin-coated vesicles (Xie et al., 1983), lysosomes (Schneider, 1983), Golgi-derived vesicles (Glickman et al., 1983), and multivesicular bodies (van Dyke et al., 1985). Because ATP-dependent proton transport across vacuolar membranes is electrogenic (Forgac, 1988), these results have been interpreted as a requirement for a passive anion conductance to provide charge compensation during proton movement. Inhibition of proton transport by the covalent anionic inhibitor DIDS has also been used in support of the existence of a conductive anion channel (Yamashiro et al., 1983; Schneider, 1983; Xie et al., 1983), although these studies are complicated by the ability of this reagent to directly inhibit the proton pump itself (Xie et al., 1983). In the case of clathrin-coated vesicles, the effects of DIDS on proton transport and ATP–P_i exchange could be separated by prereaction with the reversible sulfhydryl reagent DTNB (Xie et al., 1983). These studies provided no indication, however, of whether anion conductance activity might be associated with one of the subunits of the (H^+)-ATPase.

The proton pump of clathrin-coated vesicles is composed of nine subunits of molecular weights 17 000–100 000 (Arai et al., 1987b; Xie & Stone, 1986). Although we have dem-

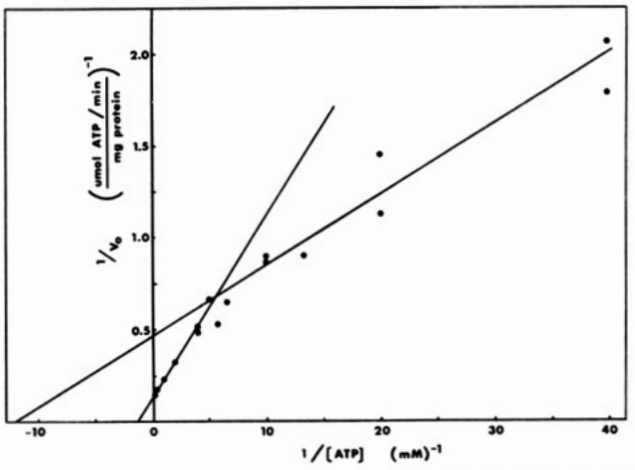

FIGURE 5: ATP concentration dependence of ATPase activity of the purified, reconstituted (H^+)-ATPase. ATPase activity of the reconstituted (H^+)-ATPase (0.4 μg of protein) was measured as a function of ATP concentration over the range of 25 μM to 5 mM as described under Materials and Methods. The assay mixture contained $MgSO_4$ at a concentration 1 mM greater than the ATP concentration such that essentially all of the ATP was present as the MgATP complex. Assays were performed in the presence of 2.3 μM valinomycin and 2.5 μg of FCCP/mL to prevent inhibition of ATPase activity due to generation of an electrochemical gradient of protons across the vesicle membrane. The lines shown represent a least-squares analysis assuming two sites and give K_m values of 83 (±15) μM and 790 (±70) μM and V_{max} values of 2.1 (±0.4) and 5.8 (±0.5) μmol of ATP min^{-1} (mg of protein)$^{-1}$ [the sum of the maximal velocities for the two sites corresponds to the observed maximal velocity of 7–8 μmol of ATP min^{-1} (mg of protein)$^{-1}$]. The errors represent the standard deviations calculated as previously described (Forgac, 1980).

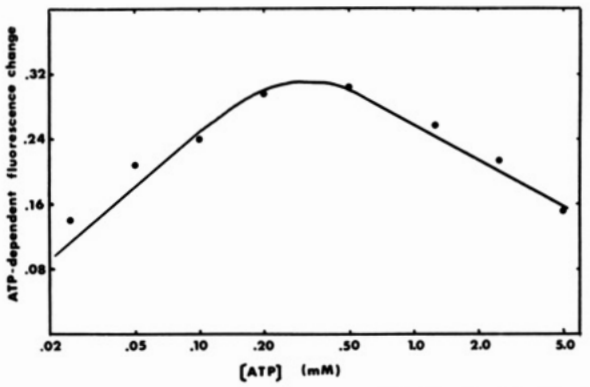

FIGURE 6: ATP concentration dependence of proton transport by the purified, reconstituted (H^+)-ATPase. Proton transport by reconstituted vesicles (0.4 μg of protein) was measured by acridine orange fluorescence quenching as described under Materials and Methods except that the assay was initiated by addition of reconstituted vesicles in order to correct for any fluorescence change due to direct interaction between acridine orange and ATP. The assay mixture contained 2.3 μM valinomycin to provide for charge compensation during ATP-driven proton uptake. As in Figure 5, the $MgSO_4$ concentration exceeded the ATP concentration by 1.0 mM. The points represent the experimental data while the curve was calculated assuming that binding of ATP to a site with a K_m of 83 μM causes activation of proton pumping while binding of ATP to a site with a K_m of 790 μM causes inhibition of proton pumping by 80%.

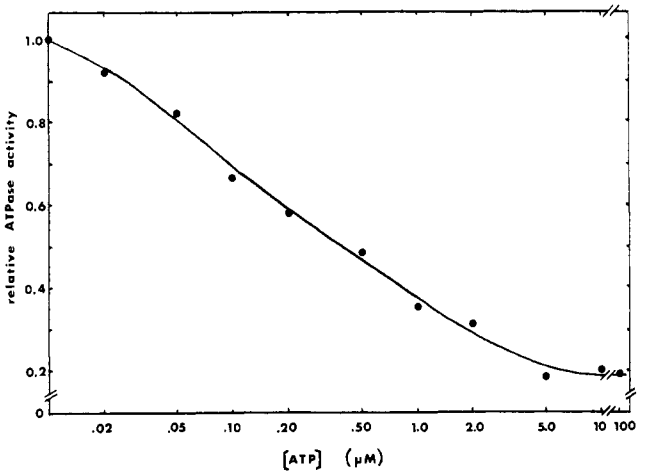

FIGURE 7: ATP concentration dependence of KI inhibition of the purified (H^+)-ATPase. Reconstituted vesicles (0.4 μg of protein) were incubated in solubilization buffer containing 150 mM KI for 1 h at 4 °C in the presence of the indicated concentrations of ATP. ATPase activity was then measured on dilution of the vesicles 10-fold into solubilization buffer containing 2.5 μM valinomycin and 2.5 μg of FCCP/mL. The activity is expressed relative to that observed in the presence of 150 mM KI but in the absence of ATP during the preincubation which, as indicated by Figure 3, corresponds to approximately 90% of the activity observed for vesicles incubated in the absence of both KI and ATP. The presence of ATP alone (in the absence of KI) did not cause any significant loss of ATPase activity. As in Figure 5 and 6, the $MgSO_4$ concentration was maintained at 1.0 mM greater than the ATP concentration.

onstrated that the 73 000-dalton subunit possesses a nucleotide binding site required for activity (Arai et al., 1987b) and that the 17 000-dalton subunit is responsible for DCCD inhibition of proton transport (Arai et al., 1987a), the function of the remaining subunits remains uncertain. Asano et al. (1987) have recently suggested that the gastric (H^+, K^+)-ATPase possesses a Cl^- channel activity. It therefore seemed important to test whether any component of the purified (H^+)-ATPase from coated vesicles could act as a conductive anion channel. In addition, the purified proton pump from chromaffin granules has been reported to be absolutely dependent on the presence of Cl^- (Moriyama & Nelson, 1987a), and both the (H^+)-ATPases from plant vacuoles (Wang & Sze, 1985) and chromaffin granules (Moriyama & Nelson, 1987a) are sensitive to inhibition by NO_3^-. It was therefore also necessary to test the direct effects of anions on the activity of the purified (H^+)-ATPase from coated vesicles.

Figure 1 shows that while proton transport in intact coated vesicles is absolutely dependent on the presence of Cl^-, the ATPase activity of the purified, detergent-solubilized (H^+)-ATPase is insensitive to Cl^- over the concentration range of 0–100 mM. This Cl^- independence contrasts with the Cl^- dependence of the chromaffin granule (H^+)-ATPase (Moriyama & Nelson, 1987a) and suggests that the coated vesicle pump is fully functional in the complete absence of Cl^-. To test whether any component of the purified (H^+)-ATPase can act as a Cl^- channel, the ability of ATP to support proton transport in reconstituted vesicles in the presence of Cl^- but in the absence of valinomycin was tested (Figure 2). The results indicate that ATP-driven proton transport in reconstituted vesicles is absolutely dependent on the presence of valinomycin and that the purified (H^+)-ATPase does not possess Cl^- channel activity. Thus, the Cl^- dependence of proton transport in intact coated vesicles derives from a Cl^- channel structurally distinct from the (H^+)-ATPase.

Although the purified (H^+)-ATPase shows no dependence on Cl^-, inhibition by the anions I^-, SO_4^{2-}, and NO_3^- is observed (Table I). In addition, the presence of ATP increases the sensitivity of the (H^+)-ATPase to inhibition by I^- and NO_3^- (Figure 3). Anion-dependent inhibition of the purified (H^+)-ATPase appears to occur by two distinct mechanisms. Thus, I^- and NO_3^- (in the presence of ATP) cause inhibition by dissociation of the 73 000-dalton subunit which, in the dissociated state, possesses no ATPase activity. The ability of ATP to promote this dissociation suggests that binding of ATP to the enzyme causes the complex to exist in a looser, more readily dissociated conformation. In contrast, SO_4^{2-} and NO_3^- (in the absence of ATP) cause inhibition without dissociation of the 73 000-dalton subunit, suggesting the existence of an inhibitory anion binding site on the purified (H^+)-ATPase. The coated vesicle pump again differs in the concentration of NO_3^- required for 50% inhibition (40–50 mM) from the vacuolar (H^+)-ATPases present in chromaffin granules and plant vacuoles, which require 3–4 mM (Moriyama & Nelson, 1987a) and 8 mM (Wang & Sze, 1985), respectively. Because NO_3^- inhibition of the purified pump is not reversible (Figure 3), we have tested to see whether NO_3^- (in the absence of ATP) may cause dissociation of some other subunit from the (H^+)-ATPase complex. Glycerol gradients run on the purified (H^+)-ATPase in the presence and absence of 200 mM NO_3^- reveal no change in either the sedimentation behavior or the subunit composition (data not shown), indicating that NO_3^- in the absence of ATP causes inhibition of activity without dissociation of the (H^+)-ATPase complex. The fact that PO_4^{2-} does not cause inhibition of activity suggests that the anion binding site responsible for SO_4^{2-} and NO_3^- inhibition is unlikely to be the catalytic site.

Aside from nucleotide specificity, little information concerning the nucleotide binding properties of the vacuolar proton pumps has been reported. We have demonstrated that the 73 000-dalton subunit of the coated vesicle proton pump shows ATP-protectable labeling by the covalent inhibitors NBD-Cl and NEM (Arai et al., 1987b), providing evidence that this subunit possesses a nucleotide binding site required for activity, possibly the catalytic site. Similar ATP-protectable labeling of a 70 000-dalton polypeptide has also been reported for the vacuolar proton pumps from *Neurospora* (Bowman et al., 1986), plants (Mandala & Taiz, 1986; Randall & Sze, 1987), yeast (Uchida et al., 1986), and chromaffin granules (Moriyama & Nelson, 1987b). In addition, the 60 000-dalton subunit of the plant vacuolar (H^+)-ATPase is labeled by the photoactivated ATP analogue Bz-ATP (Manolson et al., 1985). These results are consistent with the primary sequence data available (Bowman, B. J., et al., 1988; Bowman, E. J., et al., 1988; Zimniak et al., 1988) which indicate that both the 70 000- and 60 000-dalton polypeptides, like the α and β subunits of F_1 with which they are homologous, are nucleotide binding proteins. We have recently demonstrated that there are three copies each of the 73 000- and 58 000-dalton subunits present in the purified (H^+)-ATPase complex from coated vesicles (Arai et al., 1988). Assuming each polypeptide possesses a single nucleotide binding site, this result suggests that the native complex contains as many as six nucleotide binding sites. It was therefore of interest to determine the affinities of the ATP binding sites responsible for ATP hydrolysis, proton transport, and ATP-dependent anion dissociation.

The dependence of ATPase activity of the purified (H^+)-ATPase on ATP concentration is consistent with the existence of two kinetically distinct sites (Figure 5). Binding of ATP to a site with a K_m of 83 (±15) μM is responsible for 27% (±5%) of the maximal activity while binding to a second site

of K_m 790 (±70) μM is responsible for the remaining 73% (±6%) activity. The dependence of proton transport by the reconstituted (H^+)-ATPase on ATP concentration is also consistent with the existence of two ATP binding sites (Figure 6). In this case, however, only the high-affinity site is involved in activation of proton transport while binding to the low-affinity site causes inhibition of proton pumping by 80%. The same behavior is observed for proton transport in intact coated vesicles, indicating that the inhibition observed at high concentrations of ATP is not unique to the reconstituted (H^+)-ATPase. These results indicate that there is a decrease in the stoichiometry of protons transported per ATP hydrolyzed at high ATP concentrations, suggesting a structural change in the interactions beween the ATP-hydrolyzing and proton-conducting portions of the pump.

We have suggested that a similar change in the tightness of coupling between proton transport and ATP hydrolysis occurs on detergent solubilization of the pump, thus accounting for the insensitivity of the detergent-solubilized (H^+)-ATPase to DCCD and the restoration of sensitivity on removal of detergent (Arai et al., 1987a). That proton transport and ATP hydrolysis remain at least partially coupled at high ATP concentrations is indicated by the observation that valinomycin and FCCP continue to stimulate ATP hydrolysis by the reconstituted enzyme under these conditions (data not shown).

When the ATP dependence of I^--dependent inhibition of the (H^+)-ATPase is determined, a K_d of 150–200 nM ATP is observed (Figure 7). This value is approximately 500-fold lower than the high-affinity site involved in activation of proton transport and ATPase activity (70–100 μM) and suggests the existence of a third class of ATP binding site(s) on the coated vesicle (H^+)-ATPase. Binding to this very high affinity site appears to cause a conformational change in the (H^+)-ATPase such that the complex is more readily dissociated by anions, again suggesting a looser quaternary structure. This site, however, should be saturated under all physiological ATP concentrations. It should be noted that while our observations are consistent with the existence of three classes of nucleotide binding sites distinguishable on the basis of their affinity, they do not exclude the possibility that cooperativity between binding sites occurs [as has been suggested for the yeast vacuolar proton pump by Uchida et al. (1988)], or necessarily imply that ATP binding is occurring to three structurally distinct sites on the protein.

It is of interest to compare these results with those obtained for other (H^+)-ATPases of the vacuolar and F_1F_0 class. Both the plant vacuolar (H^+)-ATPase (Wang & Sze, 1985) and the kidney plasma membrane (H^+)-ATPase (Gluck & Caldwell, 1987) have been reported to have a single K_m value for ATPase activity of 0.25 and 0.15 mM, respectively, although the ATP dependence of proton transport was not reported. Data presented on the chromaffin granule (H^+)-ATPase gave a K_m for ATP hydrolysis of approximately 80 μM (Moriyama & Nelson, 1987b), but the activity at ATP concentrations greater than 250 μM was not tested and the ATP dependence of proton transport was not determined. Evidence for a high-affinity nucleotide binding site on both the chromaffin granule and yeast vacuolar (H^+)-ATPase with a K_d value of ≤1 μM has been obtained from labeling studies using [α-^{32}P]ATP (Moriyama & Nelson, 1987b) and 8-azido[α-^{32}P]-ATP (Uchida et al., 1988), and this site may be related to the highest affinity ATP binding site reported in the current study.

Extensive data also exist which indicate the presence of multiple nucleotide binding sites on the isolated F_1F_0 complex [for reviews, see Senior and Wise (1983) and Cross (1981)]. Thus, positive cooperativity is detected between catalytic sites with K_m values of 30 and 150 μM (Cross et al., 1982), and binding to a very high affinity site ($K_a = 10^{12}$ M^{-1}) has also been identified (Grubmeyer et al., 1982). In general, the catalytic sites are thought to residue on the β subunit, although evidence also exists for nucleotide binding to the α subunit (Senior & Wise, 1983). Given the structural similarities which exist between the vacuolar and F_1F_0 classes of (H^+)-ATPase (Arai et al., 1988), a similar complexity in nucleotide interactions is not surprising. No evidence for a decreased transport stoichiometry by the F_1F_0 ATPases at high ATP concentrations has been reported, however, suggesting that this property may be unique to the vacuolar class. The identity of the subunits in the coated vesicle (H^+)-ATPase responsible for each of the classes of ATP binding sites, as well as the physiological importance of these sites, remains to be determined.

Registry No. ATP, 56-65-5; ATPase, 9000-83-3; H^+, 12408-02-5; I^-, 20461-54-5; NO_3^-, 14797-55-8; SO_4^{2-}, 14808-79-8.

References

Amzel, L. M., & Pedersen, P. L. (1983) *Annu. Rev. Biochem. 52*, 801–824.

Arai, H., Berne, M., & Forgac, M. (1987a) *J. Biol. Chem. 262*, 11006–11011.

Arai, H., Berne, M., Terres, G., Terres, H., Puopolo, K., & Forgac, M. (1987b) *Biochemistry 26*, 6632–6638.

Arai, H., Terres, G., Pink, S., & Forgac, M. (1988) *J. Biol. Chem. 263*, 8796–8802.

Asano, S., Inoie, M., & Takeguchi, N. (1987) *J. Biol. Chem. 262*, 13263–13268.

Bowman, B. J., Allen, R., Wechser, M. A., & Bowman, E. J. (1988) *J. Biol. Chem. 263*, 14002–14007.

Bowman, E. J., Mandala, S., Taiz, L., & Bowman, B. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 48–52.

Bowman, E. J., Tenney, K., & Bowman, B. J. (1988) *J. Biol. Chem. 263*, 13994–14001.

Cross, R. L. (1981) *Annu. Rev. Biochem. 50*, 681–714.

Cross, R. L., Grubmeyer, C., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12101–12105.

Forgac, M. (1980) *J. Biol. Chem. 255*, 1547–1553.

Forgac, M. (1988) *Physiol. Rev.* (in press).

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

Forgac, M., Cantley, L., Wiedenmann, B., Altstiel, L., & Branton, D. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1300–1303.

Glickman, J., Croen, K., Kelly, S., & Al-awquati, Q. (1983) *J. Cell Biol. 97*, 1303–1308.

Gluck, S., & Caldwell, J. (1987) *J. Biol. Chem. 262*, 15780–15789.

Grubmeyer, C., Cross, R. L., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12092–12100.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

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

Mandala, S., & Taiz, L. (1986) *J. Biol. Chem. 261*, 12850–12855.

Manolson, M. F., Rea, P. A., & Poole, R. J. (1985) *J. Biol. Chem. 260*, 12273–12279.

Moriyama, Y., & Nelson, N. (1987a) *J. Biol. Chem. 262*, 9175–9180.

Moriyama, Y., & Nelson, N. (1987b) *J. Biol. Chem. 262*, 14723–14729.

Randall, S. K., & Sze, H. (1987) *J. Biol. Chem. 262*, 7135–7141.

Schaffner, W., & Weissman, C. (1973) *Anal. Biochem. 56*, 502-514.
Schneider, D. (1983) *J. Biol. Chem. 258*, 1833-1838.
Senior, A. E., & Wise, J. G. (1983) *J. Membr. Biol. 73*, 105-124.
Uchida, E., Ohsumi, Y., & Anraku, Y. (1988) *J. Biol. Chem. 263*, 45-51.
van Dyke, R. W., Hornick, C. A., Belcher, J., Scharschmidt, B. F., & Havel, R. J. (1985) *J. Biol. Chem. 260*, 11021-11026.
Wang, Y., & Sze, H. (1985) *J. Biol. Chem. 260*, 10434-10443.
Xie, X. S., & Stone, D. K. (1986) *J. Biol. Chem. 261*, 2492-2495.
Xie, X. S., Stone, D. K., & Racker, E. (1983) *J. Biol. Chem. 258*, 14834-14838.
Yamashiro, D. J., Fluss, S. R., & Maxfield, F. R. (1983) *J. Cell Biol. 97*, 929-934.
Zimniak, L., Dittrich, P., Gogarten, J. P., Kibak, H., & Taiz, L. (1988) *J. Biol. Chem. 263*, 9102-9112.

High-Resolution NMR Studies of Fibrinogen-like Peptides in Solution: Interaction of Thrombin with Residues 1-23 of the Aα Chain of Human Fibrinogen†

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ABSTRACT: The interaction of the following human fibrinogen-like peptides with bovine thrombin was studied by use of one- and two-dimensional NMR techniques in aqueous solution: Ala(1)-Asp-Ser-Gly-Glu-Gly-Asp-Phe(8)-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg(16)-Gly(17)-Pro-Arg(19)-Val(20)-Val-Glu-Arg (F10), residues 1-16 of F10 (fibrinopeptide A), residues 17-23 of F10 (F12), residues 1-20 of F10 (F13), residues 6-20 of F10 with Arg(16) replaced by a Gly residue (F14), and residues 6-19 of F10 with Arg(16) replaced by a Leu residue (F15). At pH 5.3 and 25 °C, the Arg(16)-Gly(17) peptide bonds of both peptides F10 and F13 were cleaved instantaneously in the presence of 0.6 mM thrombin, whereas the cleavage of the Arg(19)-Val(20) peptide bonds in peptides F12, F13, and F14 took over 1 h for completion. On the basis of observations of line broadening, fibrinopeptide A was found to bind to thrombin. While resonances from residues Ala(1)-Glu(5) were little affected, binding of fibrinopeptide A to thrombin caused significant line broadening of NH and side-chain proton resonances within residues Asp(7)-Arg(16). There is a chain reversal within residues Asp(7)-Arg(16) such that Phe(8) is brought close to the Arg(16)-Gly(17) peptide bond in the thrombin-peptide complex, as indicated by transferred NOEs between the aromatic ring protons of Phe(8) and the $C^{\alpha}H$ protons of Gly(14) and the $C^{\gamma}H$ protons of Val(15). A similar chain reversal was obtained in the *isolated* peptide F10 at a subzero temperature of -8 °C. The titration behavior of Asp(7) in peptide F13 does not deviate from that of the reference peptide, *N*-acetyl-Asp-NHMe at both 25 and -8 °C, indicating that no strong interaction exists between Asp(7) and Arg(16) or Arg(19). Peptides with Arg(16) replaced by Gly and Leu, respectively, i.e., F14 and F15, were also found to bind to thrombin but with a different conformation, as indicated by the absence of the long-range NOEs observed with fibrinopeptide A. Residues Asp(7)-Arg(16) constitute an essential structural element in the interaction of thrombin with fibrinogen.

The specific removal of fibrinopeptides A and B by thrombin exposes complementary polymerization sites near the N-termini of the Aα and Bβ chains (Laudano & Doolittle, 1980) located in the central domain of the soluble plasma protein fibrinogen (Telford et al., 1980), an event that initiates the spontaneous polymerization of the resulting fibrin monomer into the insoluble fibrin clot (Scheraga, 1983, 1986, and references cited therein). Much has been learned in recent years about the mechanism of the interaction of thrombin with fibrinogen, especially about the cleavage of the Arg-Gly peptide bond in the Aα chain of human fibrinogen (Scheraga, 1983, 1986, and references cited therein). Among other things, by use of an active-site mapping approach, it has been shown that residues Asp(7) and Phe(8), which are located 10 and 9 residues away, respectively, from the thrombin cleavage site, influence the effectiveness of the binding of synthetic peptide substrates to thrombin (Meinwald et al., 1980; Marsh et al., 1982, 1983). These kinetic data help explain the observations

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