The purified proton ATPase of chromaffin granules contains five different polypeptides denoted as subunits I to V in the order of decreasing molecular weights of 115,000, 72,000, 57,000, 39,000, and 17,000, respectively. The purified enzyme was constituted as a highly active proton pump, and the binding of N-ethylmaleimide and nucleotides to individual subunits was studied. N-ethylmaleimide binds to subunits I, II, and IV, but inhibition of both ATPase and proton pumping activity correlated with binding to subunit II. In the presence of ADP, the saturation curve of ATP changed from hyperbolic to a sigmoid shape, suggesting that the proton ATPase is an allosteric enzyme. Upon illumination of the purified enzyme in the presence of micromolar concentrations of 8-azido-ATP, a(235)ATP, or a(32)P]ATP subunits I, II, and IV were labeled. However, at concentrations of a(32)P]ATP below 0.1 μM, subunit II was exclusively labeled in both the purified and reconstituted enzyme. This labeling was absolutely dependent on the presence of divalent cations, like Mg2+ and Mn2+, while Ca2+, Co2+, and Zn2+ had little or no effect. About 0.2 mM Mg2+ was required to saturate the reaction even in the presence of 50 mM a(32)P]ATP, suggesting a specific and separate Mg2+ binding site on the enzyme. Nitrate, sulfate, and thiocyanate at 100 mM or N-ethylmaleimide and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole at 100 μM prevented the binding of the nucleotide to subunit II. The labeling of this subunit was effectively prevented by micromolar concentrations of three phosphonucleotides including those that cannot serve as substrate for the enzyme. It is concluded that a tightly bound ADP on subunit II is necessary for the activity of the enzyme.

The function of vacuolar ATPases is to pump protons into organelles of the vacuolar system of eukaryotic cells. This activity is vital for the function of several organelles, such as lysosomes, endosomes, and neurosecretory granules (1). The function of vacuolar ATPases is to pump protons into organelles of the vacuolar system of eukaryotic cells.

EXPERIMENTAL PROCEDURES

Materials—Most of the chemicals were purchased from Sigma [3H]N-ethylmaleimide (40 mCi/mmol) was obtained from Du Pont-New England Nuclear. a(32)P]ATP, γ(32)P]ATP, and a(235)ATP were obtained from Amersham. 8-Azido-α-[32P]ATP was obtained from ICN Radiochemicals. Hydroxylapatite was from Bio-Rad. Pepstatin A was solubilized in dimethyl sulfoxide as a 2 mg/ml solution, and leupeptin as a 5 mg/ml ethanolic solution.

Analytical Methods—Published procedures were used for determination of protein concentrations (13, 14), purification of γ(32)P]ATP and assay of ATPase activity (15), SDS-gel electrophoresis, and fluorography in the presence of Amplify (16). ATP-dependent proton uptake was assayed by following the absorbance changes of acridine orange at 492-540 nm by Aminco DW-2A spectrophotometer as previously described (17, 18). Binding of nucleotides to individual subunits was assayed by UV illumination of unmodified or azido-modified nucleotides (19, 20). The reaction was performed in a 50-μl reaction mixture containing 10 mM MOPS/Tris (pH 7.0), 50 mM KCl, 2 mM MgCl2, about 2 μg of the reconstituted enzyme, and the labeled nucleotide at the specified concentration and specific activity. The reaction mixture was placed in flat bottom plastic test tubes, on ice, and at a 5-cm distance from a UV lamp (UVP Inc. 0.90A) without filter. After a 10-min illumination, the reaction was terminated by the addition of 10 μl of concentrated dissociation buffer to give a final concentration of 2% SDS and 0.5 M-mercaptoethanol. Samples of 30 μl were electrophoresed in the presence of SDS, and, following fluorography for 24 h without fluorography for 24 h, were exposed to x-ray film.

Preparations—Chromaffin granule membranes were prepared from bovine adrenal glands as previously described, with the protease inhibitors pepstatin A at 2 μg/ml and leupeptin at 5 μg/ml present during all of the purification steps (12, 18, 21). The membranes were sonicated, and the membranes were sonicated as previously described, and usually the enzyme was reconstituted by dilution immediately following the purification or after thawing the purified enzyme frozen at liquid nitrogen. Typically, 1 ml of the purified enzyme was diluted to 40 ml of solution containing 20 mM MOPS/Tris (pH 7.0), 100 mM KCl, and 0.5 mM DTT, and the

1 The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; NEM, N-ethylmaleimide; SDS, sodium dodecyl sulfate; MOPS, 3-(N-morpholino)propanesulfonic acid; DTT, dithiothreitol; DIDS, 4,4'-dithiothiocyanostilbene-2,2'-disulfonic acid; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; EGTA, (ethylenebis(oxyethylenenitrilo)tetraacetic acid; AMP-PNP, adenosine 5'- (p-chlorophenyl) triphosphate; NDB-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole.
Correlation among the inhibition of proton uptake and ATPase activity and labeling the various subunits by $^{14}$C-NEM. Samples of reconstituted enzyme containing 4 µg of protein were incubated at room temperature for 10 min in a 50-µl solution containing 20 mM MOPS/Tris (pH 7.0), 100 mM KCl, and various concentrations of $^{14}$C-NEM (for labeling) or cold NEM (for ATPase and ATP-dependent proton uptake assays). For labeling, the specific activity of $^{14}$C-NEM was kept at 2 µCi/µmol. The reaction was terminated by the addition of 1 mM DTT, and aliquots were taken for measurements of ATPase activity, ATP-dependent proton uptake activity, and SDS-gel electrophoresis. Right, labeling of the different subunits by the following NEM concentrations: lane 1, 5 µM; lane 2, 10 µM; lane 3, 15 µM; lane 4, 20 µM; lane 5, 30 µM; lane 6, 50 µM; lane 7, 75 µM; lane 8, 100 µM; lane 9, 150 µM. The amount of $^{14}$C-NEM incorporated at 150 µM NEM corresponded to about 2 eq for subunit I, 4 eq for subunit IV, and 6 eq for subunit II. Left, inhibition of ATPase activity (├──┤) and ATP-dependent proton uptake activity (△──△). The control 100% activities were 4 pmol of Pi released/min/mg of protein for the ATPase activity and initial rate of acridine orange absorbance changes of 0.015 A at 492-540 nm/min for proton uptake activity. The relative labeling of the various subunits was obtained from x-ray film depicted in the right part of this figure by the method of Scissa (35). The intensity of 150 µM NEM for each subunit was taken as 100%.

RESULTS

One of the main characteristics of vacuolar ATPases is inhibition of their activity by NEM (1). In isolated chromaffin granules, the proton pumping activity was found to be much more sensitive to the alkylation agent than the ATPase activity (11, 22, 23). A high affinity binding site for NEM was recently identified on the $M_i = 72,000$ subunit of the enzyme while sites with lower affinity were detected on the $M_i = 115,000$ and 39,000 subunits (12). Fig. 1 shows that the labeling of the $M_i = 72,000$ polypeptide correlates well with inhibition of both the proton pumping and ATPase activities of reconstituted vesicles. At saturation, about 2 eq of NEM were bound to the $M_i = 72,000$ polypeptide per one enzyme molecule. Fig. 2 shows that prolonged incubation of the reconstituted enzyme with oxidized glutathione caused a complete inhibition of the proton uptake activity of the enzyme. This effect could be reversed by the addition of DTT. Moreover, the binding of glutathione protected against inhibition by NEM (not shown). This experiment indicates that a reduced —SH outside the vesicles is required for the proton pumping activity of the enzyme.

Several nucleotides can protect against inhibition by NEM. Table I depicts the effects of added nucleotides during the incubation of the reconstituted enzyme with NEM. Inclusion of AMP or Mg$^{2+}$ had no effect on the inhibition by NEM. However, high Mg$^{2+}$ + AMP concentrations slightly prevented the inhibition. Mg$^{2+}$ + ADP was the most effective in protecting against NEM inhibition. The observation that protection by ATP was much more pronounced in the pres-
Binding Sites on Proton ATPase from Chromaffin Granules

Fig. 2. Inhibition of ATP-dependent proton uptake by oxidized glutathione is reversible. Reconstituted enzyme containing 2.3 pg of protein was incubated at room temperature for 2 h with 10 mM oxidized glutathione. The ATP-dependent proton uptake activity was assayed by measuring acridine orange absorbance changes as described under “Experimental Procedures”. Trace 1, control without glutathione. Trace 2, 1 mM DTT was added after the incubation with glutathione. Trace 3, no addition after the incubation with glutathione. Where specified, 1 mM Me-ATP or 1 μM FCCP was added.

FIG. 3. Effect of nucleotides on the labeling of the Mr = 72,000 subunit by [14C]NEM. The reaction conditions were as described in Fig. 1, except that the enzyme (2.2 pg of protein) was incubated with 50 μM [14C]NEM for 5 min at room temperature. Lanes 1 and 5, without nucleotides; lane 2, 2 mM Mg2+ATP was added; lane 3, 2 mM Me-ADP; lane 4, 2 mM Mg2+AMP.

The effects of nucleotides on the labeling by [14C]NEM indicated the possibility that more than one nucleotide binding site is present on the enzyme. Fig. 4 shows that the presence of ADP modifies the dependence of ATPase activity on ATP concentrations. The data strongly suggest that the enzyme is an allosteric enzyme and more than one nucleotide binding site may be operating. A possible involvement of a regulatory site was also suggested by the effect of UTP and CTP on the proton pumping activity of the enzyme. While these nucleotides could not catalyze proton uptake by themselves, they stimulated proton pumping catalyzed by low concentrations of ATP up to 3-fold (data not shown). Further support for the existence of more than one nucleotide binding site was obtained by labeling of the enzyme by 8-azido-ATP and α-[35S]ATP. As depicted in Fig. 5, UV illumination of the reconstituted enzyme in the presence of α-[35S]ATP-labeled subunits I, II, and IV. The presence of Mg2+ was required for the labeling, and inclusion of EDTA abolished it (not shown).

The presence of 1 mM cold ATP completely prevented the labeling. The presence of NEM or NBD-Cl or DIDS decreased

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**TABLE I**

Protection against NEM inhibition by nucleotides

Reconstituted enzyme containing 3 μg of protein was incubated at room temperature for 10 min in a 25-μl solution containing 20 mM MOPS/Tris (pH 7.0), 100 mM KCl, 100 μM NEM, and the listed nucleotides. Then, 1 mM DTT was added, and the sample was diluted into a 1-ml solution containing 20 mM MOPS/Tris (pH 7.0), 100 mM KCl, and 0.1 μg of valinomycin. After addition of acridine orange, the reaction was started by the addition of 1 mM Me-ATP, and it was terminated by the addition of 1 μM FCCP. The initial rates of acridine orange absorbance changes were calculated as percent of control of preincubation without NEM.

| Treatment            | % of control |
|----------------------|--------------|
| NEM                  | 4            |
| NEM + 2 mM Mg2+      | 4            |
| NEM + 2 mM AMP       | 20           |
| NEM + 2 mM Mg2+AMP   | 65           |
| NEM + 0.2 mM ADP     | 87           |
| NEM + 2 mM Mg2+ADP   | 97           |
| NEM + 2 mM Mg2+AMP-PMP| 51         |
| NEM + 2 mM ATP       | 29           |
| NEM + 2 mM Mg2+ATP   | 83           |

ence of Mg2+ suggests that the ADP formed during the incubation accounted for at least part of the proteic effects of ATP. Fig. 3 shows the effect of ATP and ADP on the labeling of the Mr = 72,000 subunit by [14C]NEM. The experiment was carried out under conditions identical with those in Table I. Both ADP and ATP prevented only about 50% of the labeling of this subunit, but fully protected against inactivation by NEM. This observation suggests that modification of only one of the two sulfhydryl groups on the Mr = 72,000 polypeptide can cause a complete inhibition of the ATP-dependent proton uptake activity of the enzyme. However, the possibility that the residual label is due to a population of enzyme that was not properly reconstituted cannot be excluded.
the labeling by the nucleotide, but DCCD had no effect. Similar results were obtained when α-[32P]β-azido-ATP was used instead of α-[35S]ATP.

When α-[32P]ATP at concentrations below 0.1 μM was used for the labeling of the purified or reconstituted enzyme, mostly subunit II was labeled (Fig. 6). At higher concentrations, subunits I and IV were also labeled (not shown). This phenomenon enabled a thorough study of a single nucleotide binding site on the enzyme. The presence of a divalent cation was necessary for the labeling of subunit II. Mg2+ and Mn2+ were the best, and Ca2+, Co2+, and Zn2+ had much smaller effects. Even though the ATP concentration was only 50 nM, the Mg2+ saturation appears to be about 0.2 mM. This observation may suggest the presence of a cation binding site on the enzyme and that Mg2+ was not required merely for the formation of Mg2+-ATP as a substrate. Fig. 7 shows the effect of anions on the labeling subunit II by α-[32P]ATP. While organic anions like acetate had no effect, anions that are required for the proton uptake activity of the enzyme, such as Cl− and Br−, inhibited the labeling. Much stronger inhibition was obtained by anions which were shown to inhibit the proton uptake activity of the enzyme. The nitrate, nitrite, thiocyanate, and sulfate nearly prevented the labeling of subunit II. This may suggest the presence of the anion binding site on subunit II, but long distance effects of conformational changes cannot be excluded. Fig. 8 shows that pretreatment with NEM or NBD-Cl competed with the binding of α-[32P]ATP on subunit II, and NEM at 50 μM and NBD-Cl at 10 μM decreased the labeling by about 90%. This experiment is consistent with the protection against inhibition by NEM in the presence of nucleotides. [14C]NBD-Cl labeled all of the ATPase subunits, and it may act as a sulfhydryl reagent with this enzyme rather than a tyrosine reagent in the eubacterial type proton ATPases.

The effect of various nucleotides on the labeling of subunit II by α-[32P]ATP is depicted in Fig. 9. Phosphate and AMP at a concentration of 50 μM had little effect. Pyrophosphate at the same concentration inhibited about 80% of the labeling. ATP, GTP, and AMP-PNP at 1 μM inhibited up to 90% of the labeling, and similar effects were obtained by 10 μM ADP and CTP. The very high affinity for nucleotide binding and the binding of CTP which cannot serve as a substrate for the enzyme, may suggest that the nucleotide binding site revealed on subunit II by UV illumination of α-[32P]ATP is a regulatory site rather than catalytic site of the enzyme.

DISCUSSION

Several lines of evidence indicated that proton pumping into chromaffin granules is not tightly coupled to the ATPase activity of the enzyme catalyzing these reactions. It was observed that much higher concentrations of NEM or nitrate are required for inhibiting the ATPase activity of chromaffin granules than those required to inhibit proton uptake (11, 12, 22, 23). The presence of chloride markedly enhanced the proton uptake activity, while marginally affecting the ATPase activity of the same preparation (12, 24). Sulfate concentrations, which strongly inhibit proton uptake, do not affect the ATPase activity. The ATPase activity of the purified enzyme was inhibited by NEM via two distinct affinities with Ki values in the micro- and millimolar ranges (11). The loose coupling between ATPase and proton pumping may serve as a regulatory mechanism for the limited acidification required in the various organelles. The data presented in Fig. 1 show that, in reconstituted vesicles, the proton uptake and the
ATPase activities were inhibited in a parallel fashion by treatment with NEM. Moreover, treatment with up to 50 \( \mu \)M \(^{14}\)C NEM exclusively labeled the \( M_2 = 72,000 \) polypeptide, while inhibiting the proton uptake and ATPase activities by about 50%. The extent of inhibition correlated with labeling of the \( M_2 = 72,000 \) subunit by \(^{14}\)C NEM, an observation that may implicate this subunit in the proton uptake and even the ATPase activities of the enzyme. An alternative explanation for this phenomenon may be that NEM binds a site close to the active site of the enzyme and by so doing directly competes with the binding of the substrate onto the enzyme. The latter explanation is supported by the observation that Mg\(^{2+}\) + ADP protected against inhibition by NEM (see Table I). If this is the case, a complete elimination of NEM binding by Mg\(^{2+}\) + ADP would be expected. However, the experiment depicted in Fig. 3 did not substantiate this suggestion. The most probable explanation for this phenomenon may be the presence of two sulfhydryl groups on the \( M_2 = 72,000 \) polypeptides having the same high affinity to NEM, but only one of them is responsible for the inhibition of the enzyme.

The saturation curve for substrate may give some indication on the catalytic mechanism of the enzyme. Thus, the first indication that eucaryote type H\(^+\)-ATPases have more than one nucleotide binding site was obtained by observing the changes of the ATP saturation curve with the chloroplastic H\(^+\)-ATPase (25). As shown in Fig. 4, for the ATPase activity of the reconstituted enzyme from chromaffin granules, ADP changed the saturation curve of ATP from a hyperbolic to a sigmoid shape. The apparent reaction order changed from 1.0 in the absence of ADP to 1.6 in the presence of ADP, for both the ATPase and the initial rates of proton uptake activities of the enzyme (not shown). This suggests that more than a single nucleotide binding site is operating in the proton ATPase from chromaffin granules. This suggestion is supported by the labeling of three subunits by UV illumination of 8-azido-ATP, \( \alpha\)-\(^{32}\)P ATP, and high concentrations of \( \alpha\)-\(^{32}\)P ATP. When the same treatment was applied to the enzyme at submicromolar concentrations of \( \alpha\)-\(^{32}\)P ATP, subunit II was exclusively labeled. The labeling was absolutely dependent on the presence of divalent cations like Mg\(^{2+}\) and preincubation of the enzyme with Mg\(^{2+}\) + ATP at room temperature increased the labeling of the \( M_2 = 72,000 \) polypeptide. This effect together with the strong competition of ADP and the protection against inactivation by NEM suggests that ADP tightly bound to the \( M_2 = 72,000 \) subunit is required for the catalytic activity of the enzyme. This notion is supported by the observation that preincubation with ADP markedly enhanced the proton uptake activity of the reconstituted enzyme (not shown).

It is quite difficult to ascribe a precise function for the nucleotide binding site on subunit II. The extremely high affinity to the nucleotides tested, revealed by the competition for labeling by \( \alpha\)-\(^{32}\)P ATP (Fig. 9), may suggest a regulatory or mechanistic function for this binding site. Similar tight binding of slow turnover ADP was observed in the chloroplastic ATPase (26, 27). The regulatory function of this binding site is supported by the strong competition of nucleotides that cannot serve as substrates for the enzyme (see Fig. 9). Those nucleotides also enhanced the proton uptake activity of the enzyme in the presence of low ADP concentrations. Therefore,
it is suggested that the nucleotide binding site on the M₁ = 72,000 polypeptide should be occupied in order for the enzyme to be catalytically active. This effect may mimic the single site catalsys and the cooperativity that was recently discovered in the mitochondrial proton ATPase; however, several other explanations may apply for the same set of observations described in this work (28-34).

The location of the active site and the mechanism of proton uptake by the vacuolar ATPases is yet to be discovered. Several questions should be solved before we are able to get a more definite idea on the function of each individual subunit in this class of ATPases.

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FIG. 8. Effect of NEM and NBD-Cl on the labeling of subunit II by α-[32P]ATP. The experiment was carried out as described in Fig. 6 except that 2 mM MgCl₂ was present in all the assays. Lanes I and II, no addition; lane 2, 1 μM NEM; lane 3, 10 μM NEM; lane 4, 50 μM NEM; lane 5, 100 μM NEM; lane 6, 500 μM NEM; lane 7, 1 μM NBD-Cl; lane 8, 10 μM NBD-Cl; lane 9, 100 μM NBD-Cl; lane 10, 1% ethanol. NBD-Cl was added as an ethanolic solution. The final ethanol concentration did not exceed 1%. Purified ATPase containing 2.5 μg of protein was present in each reaction.

FIG. 9. Effect of nucleotides on the labeling of subunit II by α-[32P]ATP. The experiment was carried out as described in Fig. 6 except that 2 mM MgCl₂ was present in all the assays. Lanes I and II, no addition; lane 2, 1 μM ATP; lane 3, 10 μM ATP; lane 4, 1 μM GTP; lane 5, 10 μM GTP; lane 6, 1 μM CTP; lane 7, 10 μM CTP; lane 8, 1 μM ADP; lane 9, 10 μM ADP; lane 10, 1 μM AMP-PNP; lane 11, 10 μM AMP-PNP; lane 12, 50 μM AMP; lane 13, 50 μM pyrophosphate; lane 14, 50 μM phosphate; lane 15, 0.1 μM ATP; lane 17, 0.5 μM ATP; lane 18, 1 μM ATP; lane 19, 5 μM ATP.
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