Cold Inactivation of Vacuolar Proton-ATPases

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Incubation of the reconstituted H+-ATPase from chromaffin granules on ice resulted in inactivation of the proton-pumping and ATPase activities of the enzyme. Inactivation was dependent on the presence of Mg2+, Cl−, and ATP during the incubation at low temperature. Approximately 1 mM ATP, 1 mM Mg2+, and 200 mM Cl− were required for maximum inactivation. Incubation for about 10 min on ice was required to achieve 50% inactivation. A much smaller decline in activity was observed when the enzyme was incubated at room temperature with the same chemicals. Inactivation in the cold resulted in the release of five polypeptides from the membrane with apparent molecular masses of 72, 57, 41, 34, and 33 kDa on sodium dodecyl sulfate gels. Three of the polypeptides of 72, 57, and 34 kDa were identified as subunits of vacuolar H+-ATPases by antibody cross-reactivity. Similar results were obtained with several other vacuolar H+-ATPases including those from plant sources. It was concluded that the catalytic sector of the enzyme is released from the H+-ATPase complex by cold treatment, resulting in inactivation of the enzyme.

The vacuolar H+-ATPase was well established as a distinct family of proton pumps (1–5). However, recent studies on these proton pumps have revealed similarities between the vacuolar H+-ATPases and the eubacterial enzymes (6–10). The enzymes of these families are composed of several subunits, some of which are peripheral whereas others are integral membrane proteins. The proton-pumping activity of both families of enzymes is inhibited by NaN3-dicyclohexylcarbodiimide, and inhibition is concomitant with covalent binding of the agent onto a specific polypeptide denoted as a proteolipid (11–17). Recent sequencing data indicate a significant sequence homology among the proteolipids of these two families of proton pumps (10). Moreover, sequence homology occurs between the 70-kDa subunit of the vacuolar H+-ATPases and the β subunit of the eubacterial enzymes (9), both of which are believed to contain the active site of the respective enzymes (6, 9). Similarly, sequence analysis of the 57-kDa subunit of the yeast vacuolar H+-ATPase shows sequence homologies to the α and β subunits of the eubacterial type (F-ATPase) enzymes.1

The catalytic mechanisms of these two types of proton pumps appear to be similar; they lack a phosphoenzyme intermediate and involve a process denoted as single site catalysis (6, 7). An important difference between the vacuolar and the eubacterial enzymes is that the latter can synthesize ATP at the expense of the proton-motive force, but the former operates exclusively as a proton pump (18). Another difference is that the vacuolar enzymes require chloride for their proton-pumping activity (19).

An unusual property of the eubacterial H+-ATPases is their sensitivity to cold (20). In this paper we describe conditions under which the H+-ATPase from chromaffin granules and other vacuolar proton pumps is inactivated by cold treatment and present information on the nature of proteins, likely ATPase subunits, that are released from the membranes.

EXPERIMENTAL PROCEDURES

Materials—Most of the chemicals were purchased from Sigma. [γ-32P]ATP was obtained from Amersham Corp. and purified on a Dowex 1–Cl column as previously described (21). Bovine adrenal glands, kidney, and brain were obtained from a local slaughterhouse. Rats (Wistar, male, 200–250 g body weight) were obtained from Charles River Breeding Laboratories, Inc. Red beets (Beta vulgaris L.) and tomatoes were purchased from a local supermarket.

Analytical Methods—Published procedures were used for determination of protein concentrations (22, 23), assay of ATPase activity by following the release of 32P from [γ-32P]ATP (24), ATP-dependent proton uptake (19), SDS–gel electrophoresis (24), and immunoblotting (25).

Preparations—Chromaffin granule membranes were prepared from bovine adrenal glands as previously described (19). The membranes were frozen in liquid nitrogen and stored at −85 °C. The H+-ATPase was purified and reconstituted as previously described (6, 19). The reconstituted enzyme was either used fresh or stored at −85 °C. The specific ATPase activity of the reconstituted enzyme was 3.5 units/mg of protein. Microsomes from bovine kidney medulla were prepared as described by Gluck and Al-Awqati (26). Crude clathrin-coated vesicles (omitting the last step of sucrose-D2O gradient centrifugation (27)) and synaptic vesicles from rat brain (28) were prepared according to published procedures. Microsomes containing vacuolar H+-ATPase from red beet and tomato were prepared as previously described (28, 30). All of these membrane preparations were frozen in liquid nitrogen and stored at −85 °C. Antibodies against the 72- and 57-kDa subunits of the chromaffin granule H+-ATPase were prepared following electroelution of the subunits from SDS gels as described previously (25).

RESULTS

Vacuolar H+-ATPases are unstable enzymes, and therefore it took a long time to discover the conditions by which they could be purified. The purification time of the enzyme from chromaffin granules had to be reduced to less than 10 h in order to preserve its capacity to be reconstituted into an ATP-dependent proton pump (19). The ATPase activity of the enzyme following reconstitution was much more stable than that of the soluble enzyme. The stability under most conditions was greater at low temperatures; however, as shown in Fig. 1 incubation of the reconstituted enzyme at 0 °C in the

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1 H. Nelson, S. Mandiyan, and N. Nelson, submitted for publication.

2 The abbreviations used are: SDS, sodium dodecyl sulfate; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylenediaminetriacetate acid; ATPγS, adenosine 5′-O-(thiotriphosphate).
Cold Inactivation of Vacuolar H\(^{+}\)-ATPase

**Fig. 1.** Cold inactivation of the H\(^{+}\)-ATPase from chromaffin granule membranes. Reconstituted H\(^{+}\)-ATPase (0.15 mg of protein/ml) was incubated in buffer containing 20 mM MOPS-Tris (pH 7.0), 0.5 mM DTT, 0.3 M NaCl, 5 mM ATP, 5 mM MgCl\(_2\), 5 mM creatine phosphate, and 20 \(\mu\)g of creatine kinase at room temperature (\(\bullet\)) or at 0 \(^\circ\)C (\(\bigcirc\)). Aliquots (20 \(\mu\)l) were taken at the indicated time intervals, and the ATPase activities were measured in buffer containing 20 mM MOPS-Tris (pH 7.0), 0.1 M KCl, 1 mM MgCl\(_2\), and 1 mM \(\gamma^{32}P\)ATP as described under "Experimental Procedures."

**Fig. 2.** Effect of Mg\(^{2+}\) and ATP on the cold inactivation of the chromaffin granule H\(^{+}\)-ATPase. Reconstituted H\(^{+}\)-ATPase (0.3 mg of protein/ml) was incubated at 0 \(^\circ\)C in buffer containing 20 mM MOPS-Tris (pH 7.0), 0.5 mM DTT, and 0.3 M NaCl (\(\bullet\)); the same buffer plus 5 mM MgCl\(_2\) (\(\bigcirc\)); the same buffer plus 5 mM ATP (\(\triangle\)); and the same buffer plus 5 mM MgATP (\(\times\)). Aliquots (20 \(\mu\)l) were taken at the indicated time intervals, and the ATPase activities were measured as described in the legend to Fig. 1.

presence of Mg\(^{2+}\), ATP, and NaCl caused a rapid inactivation of its ATPase activity. A similar decrease in the ATP-dependent proton uptake activity was observed (not shown). While incubation at 0 \(^\circ\)C for less than 10 min was sufficient to cause 50% inhibition, more than 1 h was required to get similar inhibition at room temperature. As shown in Figs. 2 and 3, Mg\(^{2+}\), ATP, and Cl\(^{-}\) were required for the cold inactivation to take place. Omission of any one of these from the reaction largely prevented the cold inactivation process. About 50 \(\mu\)M ATP and 50 \(\mu\)M Mg\(^{2+}\) were required for half-maximal inhibition, and 1 mM MgATP together with 0.2 M NaCl gave maximal inhibition.

Nitrate is a well known inhibitor of vacuolar H\(^{+}\)-ATPases; however, under most conditions the inhibition is reversible, and upon removal of the agent most of the proton uptake and ATPase activities of the enzyme were restored (31). Fig. 3 depicts the effect of chloride and nitrate on the cold inactivation process. About 0.15 M Cl\(^{-}\) was required to get 50% inactivation, and only 0.02 M nitrate was sufficient to give the same effect. This inhibition could not be reversed by the removal of the salt. Table I shows the effect of nitrate in the presence and absence of MgATP on the proton uptake activity of various vacuolar membranes incubated at 0 \(^\circ\)C. Although in the absence of MgATP very little inhibition was observed, inclusion of MgATP during incubation in the cold caused marked inhibition of the proton-pumping activity of the var-

**Table I**

| Salt (M) | ΔA/min | MgATP | ΔMgATP | % of control |
|---------|--------|-------|--------|-------------|
| Chromaffin granules | 0.0324 | 0.0008 | | 2 |
| Clathrin-coated vesicles | 0.1200 | 0.0066 | | 6 |
| Synaptic vesicles | 0.0650 | 0.0022 | | 3 |
| Kidney microsomes | 0.0336 | 0.0002 | | 6 |
| Red beet vacuoles | 0.1330 | 0.0136 | | 10 |
| Tomato vacuoles | 0.0138 | 0.0024 | | 17 |
Cold Inactivation of Vacular H⁺-ATPase

Reconstituted H⁺-ATPase (0.15 mg/ml) was incubated at 0 °C for 30 min in buffer containing 20 mM MOPS-Tris (pH 7.0), 0.5 mM DTT, 0.3 mM NaCl, 1 mM MgCl₂, and the listed chemicals. Then aliquots (20 μl) were assayed for ATPase activity as described in the legend to Fig. 1. The residual nucleotide analogues used during the preincubation did not influence the ATPase activity under the standard assay conditions.

| Addition (1 mM) | Activity % of control |
|----------------|----------------------|
| No addition    | 100                  |
| ATP            | 30                   |
| ADP            | 54                   |
| AMP            | 100                  |
| GTP            | 101                  |
| ATPγS          | 31                   |
| 2-Deoxy-ATP    | 39                   |
| GTP            | 49                   |
| ITP            | 58                   |
| UTP            | 90                   |

TABLE IV

Effect of various nucleotides on cold inactivation of H⁺-ATPase from chromaffin granules

| Addition (1 mM) | Activity % of control |
|----------------|----------------------|
| No addition    | 100                  |
| ATP            | 30                   |
| ADP            | 54                   |
| AMP            | 100                  |
| GTP            | 101                  |
| ATPγS          | 31                   |
| 2-Deoxy-ATP    | 39                   |
| GTP            | 49                   |
| ITP            | 58                   |
| UTP            | 90                   |

These results show the effect of various nucleotides on the cold inactivation of the enzyme. While AMP had no effect, all the other nucleotides increased the activity with the exception of ADP, which had no significant effect. The most effective nucleotides were ATP, ADP, and AMP, followed by GTP and ATPγS. The least effective nucleotide was UTP.

TABLE II

Effect of anions on the cold inactivation of H⁺-ATPase from chromaffin granules

Reconstituted H⁺-ATPase (0.2 mg/ml) was incubated at 0 °C for 30 min in buffer containing 20 mM MOPS-Tris (pH 7.0), 0.5 mM DTT, 0.3 mM NaCl, 1 mM MgCl₂, and the listed salts in the presence or absence of 5 mM MgATP. Then aliquots (20 μl) were assayed for measurement of the ATPase activity. The ATPase activity in the presence of NaCl without Mg-ATP was taken as 100%.

| Salt      | -MgATP | +MgATP |
|-----------|--------|--------|
| NaCl      | 100    | 47     |
| NaBr      | 98     | 28     |
| Na₂SO₄   | 84     | 52     |
| NaF       | 102    | 56     |
| NaSCN     | 94     | 20     |
| NaN₂O₃   | 76     | 20     |

TABLE III

Effect of divalent cations on cold inactivation of reconstituted H⁺-ATPase

Reconstituted H⁺-ATPase (0.15 mg/ml) was incubated at 0 °C for 30 min in buffer containing 20 mM MOPS-Tris (pH 7.0), 0.5 mM DTT, 0.3 mM NaCl, 5 mM ATP, and listed cations or divalent cations. Then aliquots (20 μl) were assayed for ATPase activity.

| Addition (1 mM) | Activity % of control |
|----------------|----------------------|
| No addition    | 100                  |
| EDTA           | 106                  |
| EGTA           | 106                  |
| Ca²⁺           | 36                   |
| Mg²⁺           | 93                   |
| Mn²⁺           | 60                   |
| Co²⁺           | 40                   |
| Zn²⁺           | 52                   |

These results show the effect of various divalent cations on the cold inactivation of the enzyme. The most effective cation was Mg²⁺, followed by Ca²⁺ and Mn²⁺. The least effective cation was Zn²⁺.

DISCUSSION

The discovery of F₁, the catalytic sector of the mitochondrial H⁺-ATPase, was concomitant with the observation that this enzyme is cold-sensitive (35). Later on it was shown that polypeptides are 72, 57, 41, 34, and 33 kDa. The 72- and 57-kDa polypeptides correspond to the subunits denoted as II and III (19). Polypeptides at the same position on SDS gels as those released from the chromaffin granule enzyme were detected in purified H⁺-ATPases from kidney and clathrin-coated vesicles, and those of 41, 34, and 33 kDa had been previously overlooked in the preparation from chromaffin granules (19, 32–34). However, they were not detected in plant and fungal preparations (12–15).

Fig. 5 shows that cold treatment of salt-washed red beet tonoplasts liberated polypeptides migrating in similar positions in SDS gels as those of chromaffin granules. The apparent molecular masses of the released polypeptides were: A, 69 kDa; B, 55 kDa; C, 44 kDa; and E, 35 kDa. The same results were obtained with tomato microsomes. Similarly, cold treatments of salt-washed chromaffin granule membranes, kidney microsomes, clathrin-coated vesicles, and synaptic vesicles released polypeptides of identical size to those released from the purified enzyme from chromaffin granules (not shown). Moreover, antibodies raised against the 72-, 57-, and 34-kDa polypeptides of chromaffin granule cross-reacted with polypeptides of corresponding molecular weights from all the above sources. The identity of the 33-kDa polypeptide was verified by amino acid sequencing. This suggests not only that the mechanism of cold inactivation is similar in various vacuolar H⁺-ATPases but also that they have a similar subunit structure. Fig. 6 shows an SDS gel of fractions from glycerol gradient centrifugation of a supernatant containing the polypeptides released during cold inactivation of the reconstituted H⁺-ATPase from chromaffin granules. The various polypeptides migrated as two distinct fractions of higher mass than each individual subunit. The heavier band (fractions 6–8) migrated to a position corresponding to a protein complex of 400–500 kDa, judged from the migration of the bacterial H⁺-ATPase to the same position. Except for polypeptide C (41 kDa) all the polypeptides maintained their relative concentrations, as in the supernatant after cold inactivation.

Y. Moriyama, M. Miedel, and N. Nelson, unpublished data.
Cold Inactivation of Vacuolar $H^+\text{-ATPase}$

**FIG. 4.** Release of hydrophilic sector from the $H^+\text{-ATPase}$ complex during cold inactivation. Cold inactivation of the reconstituted $H^+\text{-ATPase}$ was carried out as described in Fig. 1 at 0 °C for 1 h. Then the reconstituted $H^+\text{-ATPase}$ was sedimented by ultracentrifugation at 250,000 x $g$ for 1 h at 4 °C. The pellet was suspended at the original volume in 20 mM MOPS-Tris (pH 7.0) and 0.5 mM DTT. Proteins in the supernatant were concentrated by ammonium sulfate precipitation at 50% saturation. Aliquots were mixed with SDS sample buffer and electrophoresed on 10% acrylamide gels. Lane 1, released proteins (supernatant); lane 2, pellet of the cold-inactivated $H^+\text{-ATPase}$; lane 3, control reconstituted $H^+\text{-ATPase}$. On the left the letters A-E indicate the proposed nomenclature of the subunits of the hydrophilic sector, and on the right the apparent molecular weight of the subunits are given.

several other $H^+\text{-ATPases}$ of the eubacterial type exhibited the same property (20, 36, 37). Usually the isolated catalytic sector is much more cold-sensitive (20); however, in the presence of salts the membrane-bound enzyme also underwent cold inactivation (38, 39). Salt-induced inactivation of the chloroplast $H^+\text{-ATPase}$ was studied in detail (40). High concentrations of salts were required to inactivate the photophosphorylation and ATPase activities of isolated chloroplasts. The treatment released some of the enzyme subunits from the membrane, and the catalytic sector was disintegrated (39). Inclusion of ATP during the incubation in the cold protected against the inactivation (40).

The cold inactivation of the vacuolar $H^+\text{-ATPases}$ described in this paper resembles that of the eubacterial enzymes (F-ATPases of eubacteria, chloroplasts, and mitochondria) in its requirement for salts. However, much lower salt concentrations were required for inactivating the vacuolar enzyme, and the salt specificity was somewhat different. While monovalent cations have little effect on the extent of the cold inactivation, anions are required to produce an effect. Nitrate and thiocyanate are the most effective anions, followed by bromide, chloride, sulfate, and fluoride. The first two are known as inhibitors of the proton-pumping activity of the vacuolar enzymes, and the latter two promote this activity. Therefore, it is likely that the effect of anions on the cold inactivation is expressed through one or two specific anion binding sites on the enzyme.

The effect of Mg$^{2+}$ and nucleotides on the cold inactivation of the vacuolar $H^+\text{-ATPase}$ is opposite to their effect on the eubacterial enzymes. It was widely demonstrated that ATP stabilizes the catalytic sector (F$_i$) of the various eubacterial enzymes (20, 41). In chloroplasts the presence of MgATP almost fully protected membrane-bound enzyme against cold inactivation in the presence of 0.75 M sodium bromide (40). On the other hand, both magnesium and ATP were required to inactivate the vacuolar enzymes. It is interesting to note that various divalent cations were effective in the same order for the protection of the chloroplast enzyme against cold

**FIG. 5.** Release of polypeptides from vacuolar membrane vesicles by cold inactivation. Red beet vacuoles (3 mg/ml) were suspended in buffer containing 20 mM MOPS-Tris, (pH 7.0), 0.5 mM DTT, and 0.3 M NaCl. Aliquots (200 µl) were incubated at 0 °C for 1 h with or without 5 mM MgATP, then centrifuged in a Beckman Airfuge at 30 p.s.i. for 1 h. Supernatants were incubated in SDS dissociation buffer and electrophoresed on 10% polyacrylamide-SDS gel. The gel was stained with Coomassie Brilliant Blue. Lane 1, chromaffin granule $H^+\text{-ATPase}$; lane 2, supernatant of cold-treated membranes without MgATP; lane 3, supernatant of cold-treated membranes in the presence of MgATP. The letters A-C and E indicate the relation to homologous polypeptides of the chromaffin granule $H^+\text{-ATPase}$.
inactivation (40) as for cold inactivation of the vacuolar enzymes. The same is true for the effect of the various nucleotides on the inactivation or protection of the two systems. The order of potency in cold inactivation of the vacuolar H+ -ATPase was maintained in the protection against salt inactivation of the chloroplast enzyme. It is apparent that the MgATP acted in both systems via one of the nucleotide binding sites of the respective enzymes.

Cold inactivation of the membrane-bound eubacterial and vacuolar H+ -ATPases resulted in the dissociation of the peripheral part from the membrane. This phenomenon suggests some clues to the structure of the vacuolar enzymes. Rea et al. (8) showed that chaotropic agents liberate the 70- and 60-kDa polypeptides from tonoplast membranes. Similar observations were reported for vacuolar enzymes from other plants and fungal sources (42). Recently this notion was strongly supported by studies of the H+ -ATPase of clathrin-coated vesicles (43, 44). It was concluded that these subunits, like those of the eubacterial enzymes, are peripheral and are not integral membrane proteins. The observation that cold inactivation specifically liberated polypeptides (subunits A–E) from the H+ -ATPase fully supports this conclusion. Moreover, we observed that polypeptides with molecular weights similar to those of vacuolar enzymes from mammalian sources were liberated from plant vacuoles. The identity of the 69- and 55-kDa polypeptides liberated from red beet vacuoles as homologous to the 72- and 57-kDa polypeptides of the chromaffin granule enzyme was confirmed by a strong cross-reactivity of the corresponding antibodies (data not shown). This suggests that the basic subunit structures of the mammalian and the plant enzymes are identical, and the cold treatment revealed additional subunits that were not detected in enzyme preparations of plant vacuoles. It is tempting to suggest that the protein-liberated form of the membrane by the cold treatment is the catalytic sector of the vacuolar H+ -ATPase. We are now attempting to stabilize the ATPase activity of the liberated sector.

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