Vol. 149, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1987 Pages 140-144

INTERNAL ANION BINDING SITE AND MEMBRANE POTENTIAL DOMINATE THE REGULATION OF PROTON PUMPING BY THE CHROMAFFIN GRANULE ATPase

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Received October 12, 1987

SUMMARY: Effects of anions and membrane potential on the reconstituted proton pump from chromaffin granules were investigated. When acetate was present inside of the vesicles, ATP-dependent proton uptake was absolutely dependent on external chloride. Without external chloride, however, substantial proton uptake was observed when chloride or sulfate was present inside of the vesicles. Inside negative membrane potential drove ATP-dependent proton uptake regardless of the anion species present inside or outside of the vesicles. It is concluded that the internal anion binding site and membrane potential regulate the proton pumping activity of the ATPase.

INTRODUCTION: The proton-ATPase of chromaffin granules is a member of proliferating class of proton pumps connected with the vacuolar system of eukaryotic cells. These enzymes function in a controlled acidification of organelles connected to the secretory pathway (1). Since the stoichiometry of the proton pumps is probably 2 H^+/ATP, at equilibrium, ΔpH of over three units should be formed (2,3). Yet the most common ΔpH across the membranes of these organelles is about 1.5 units, and a kinetic control must be imposed on this class of proton-ATPases in order to prevent them from over acidification of the organelles (4). The proton pumping activity of the vacuolar ATPases is enhanced by Cl^- ions and inhibited by nitrate (5-8). Anions were recognized as possible modulators of the acidification of the organelles, however, due to the lack of purified and properly reconstituted enzymes, it was difficult to study the mechanism of such regulation. Recently, we purified and reconstituted the proton-ATPase from chromaffin granule membranes (9). The effect of various anions, added from outside the vesicles on the ATP-dependent pump, was studied. Under these conditions, proton uptake was dependent on the addition of Cl^- or Br^-, while several other anions, such as sulfate were ineffective or even inhibitory. It was observed that nitrate inhibited only when present inside the vesicles. In this communication, we report on the effect of anions inside the reconstituted vesicles. It appears that various anions have different effects on proton pumping when present inside the vesicle than on the exterior. Membrane potential plays an important role in controlling the proton uptake activity of the enzyme.

0006-291X/87 $1.50

MATERIALS AND METHODS: 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 throughout the preparation (10,11,12). The membranes were suspended at protein concentration of 5 mg/ml in a solution containing 0.3 M sucrose, 10 mM MOPS (pH 7), 1 mM ATP, 25% glycerol, 2 μg/ml pepstatin A and 5 μg/ml leupeptin. The membranes were kept frozen at -85°C. The proton-ATPase was purified from the membranes as previously described and reconstituted by dilution followed by precipitation of the vesicles by centrifugation (9). For the experiments described in this work aliquots of 0.1 ml of the purified enzyme were diluted into 10 ml of a solution containing 20 mM MOPS (pH 7), 100 mM potassium salt of the specified anion and 0.5 mM dithiothreitol. The suspension was centrifuged at 200,000 g for 1 hr and the pellet was homogenized in 0.1 ml of the same solution. Samples of 20 μl were assayed for ATP-dependent proton uptake activity in 1 ml solution containing 20 mM MOPS (pH 7), 100 mM of the specified salt or 200 mM sucrose and 15 n mol of acridine orange. The reaction was initiated by the addition of 1 mM Mg-ATP and terminated by the addition of 1 n mol of FCCP. When specified 0.1 μg of valinomycin was added either before or after the addition of the Mg-ATP. Proton uptake was assayed by following the absorbance changes of acridine orange at 492-540 nm by an Aminco DW-2a spectromoter. Protein was estimated by the method of Schaffner and Weismann (13).

RESULTS AND DISCUSSION: One of the main characteristics of the vacuolar H^+-ATPases is its anion sensitivity (5-9). Purification of the chromaffin granules ATPase and the reconstitution of the enzyme enabled a detailed study of the effect of anions on the proton uptake activity of the enzyme (9). In the previous communication, we studied the effects of addition of anions to the reconstituted vesicles. It was observed that, when reconstituted without anions the proton uptake activity of the enzyme was absolutely dependent on the presence of chloride or bromide outside of the vesicles. Other ions such as sulfate or acetate either had no effect or even inhibited the activity in the presence of chloride. Nitrate stimulated the proton uptake activity from outside but inhibited it when present inside. This effect suggested the presence of an anion binding site inside the vesicles (9). In this report we prepared the reconstituted vesicles in the presence of various salts and investigated the effect of anions inside the vesicles on proton uptake activity.

Fig. 1 shows the ATP-dependent proton uptake in the vesicles reconstituted in the presence of acetate or chloride. The largest ATP-dependent proton uptake was observed when chloride was present inside and outside of the vesicles (Fig. 1-5). On the other hand, acetate cannot induce the ATP-dependent proton uptake activity when present inside the vesicles and the dependency on the chloride outside the vesicles was maintained even in the presence of 100 mM potassium and valinomycin (Fig. 1-1,3,4). Under similar conditions, sulfate fails to replace chloride for proton uptake activity of the enzyme (Fig. 1-2). These results show the absolute requirement of chloride for proton uptake activity when acetate is present inside of vesicles, confirming the previous result (9).

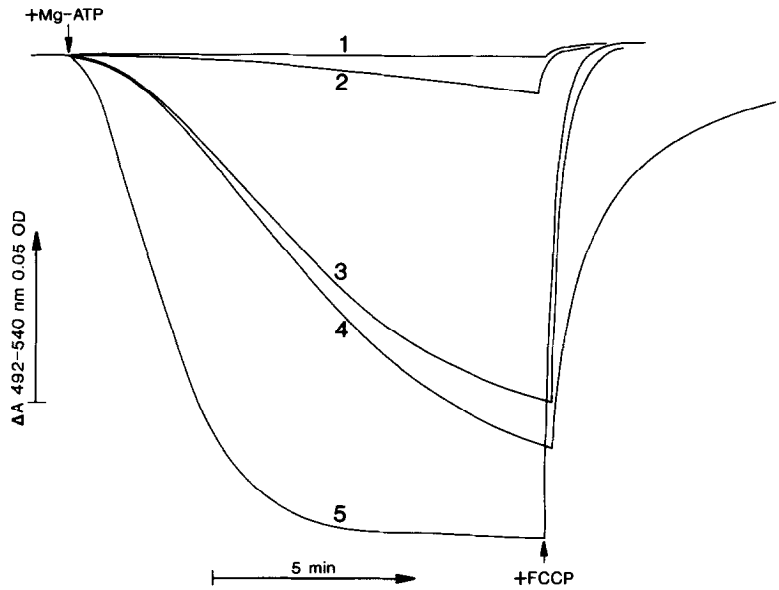

Figure 1. Effect of anions on the reconstituted H^+-pump activity. Reconstituted vesicles were prepared in the presence of K-acetate (1-4) or KCl (5), and ATP-dependent proton uptake was measured in the buffer containing K-acetate (1), K_2SO_4 (2), KCl (3,5) and choline chloride (4) in the presence of valinomycin. See details in "Materials and Methods". Reconstituted vesicles contained 2 μg protein per assay.

On the other hand, when the vesicles were formed in the presence of sulfate or chloride, ATP-dependent proton uptake was observed in the absence of chloride outside of the vesicles (table 1). Moreover, sulfate, phosphate and even acetate fail to inhibit the reaction from outside of the vesicles. Therefore, the anions which were present inside of the vesicles changed the anion sensitivity of the proton pumping activity of the enzyme. Some anions such as sulfate or phosphate induced the activity less than chloride. As is the case with nitrate inihibition (9), these anion effects suggest the presence of an anion binding site inside of the vesicles.

The effects of membrane potential on ATP-dependent proton uptake are also investigated. In the absence of valinomycin, little ATP-dependent proton uptake was observed even in the presence of chloride outside of the vesicles (table 1). This indicated that ΔpH formation across the vesicles is sensitive to membrane potential which is positive inside. In the absence of potassium outside the vesicles, the rate and extent of proton uptake were increased due to the formation of the negative membrane potential inside the vesicles (Fig. 1 and table 1). In the presence of potassium acetate inside and sucrose outside the vesicles, substantial ATP-dependent proton uptake was observed when valinomycin was added (table 1). Essentially the same effect is observed even with nitrate present inside the vesicles, which is expected to inhibit the ATP-dependent proton uptake from internal site (9) (not shown). Since

Table 1

Effect of anions inside and outside the vesicles on ATP-dependent proton uptake by reconstituted H^+-ATPase from chromaffin granules

Salt inside	Salt outside	Valinomycin (0.1 µg)	% of control
KCl	KCl	+	100
KCl	KCl	-	4
KCl	Sucrose	+	112
KCl	KPi	+	64
KCl	NaPi	+	144
KCl	K_2SO_4	+	96
KCl	K-acetate	+	56
K-acetate	K-acetate	+	0
K-acetate	KCl	+	20
K-acetate	Sucrose	+	19
K-acetate	KPi	+	0
K-acetate	K_2SO_4	+	1
K_2SO_4	K_2SO_4	+	31
K_2SO_4	KCl	+	80
K_2SO_4	Sucrose	+	86
K_2SO_4	Sucrose	-	10
K_2SO_4	K-acetate	+	38

The initial rate of acridine orange absorbance change was calculated as % of control in which KCl is present inside and outside of the vesicles. One hundred percent corresponded to $\Delta A_{492-540}$ of 0.042 OD per min. Reconstituted vesicles contained 2 µg protein per assay.

acetate itself (and also nitrate) cannot support the reaction, the membrane potential negative inside should have overruled the necessity for the presence of favorable anions inside or outside the vesicles.

It is concluded that both internal anion binding site and membrane potential are the dominant factors in the regulation of the ATP-dependent proton uptake of the reconstituted enzyme. Recently, it was shown that the ATPase is an allosteric enzyme (14). ADP changed the saturation curve of ATP from hyperbolic to sigmoidal shape and a non-hydrolyzable nucleotide, such as UTP, enhanced the ATP-dependent proton uptake activity. Further studies of these regulatory mechanisms and their interrelationship should provide a better understanding the mechanism of action of vacuolar H^+-ATPases.

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