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Depolymerization of solubilized gastric ($H^+ + K^+$)-ATPase by *n*-octylglucoside or cholate

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We have previously shown that an active ($H^+ + K^+$)-ATPase can be extracted from gastric apical membranes using *n*-octylglucoside (Soumarmon, A., Grelac, F. and Lewin, M.J.M. (1983) Biochim. Biophys. Acta 732, 579–585). This extract contained an holomeric enzyme of 390–420 kDa and contained 68% of the K^+-stimulated ATPase specific activity originally present. We demonstrate here that inactivation, induced during a more classically designed protocol, is associated with the appearance of smaller, polymorphic structures with molecular mass of 330–360 and 240–250 kDa estimated using molecular sieve chromatography and glycerol gradients. This suggests that ($H^+ + K^+$)-ATPase solubilization by *n*-octylglucoside is a complex process involving first extraction of the enzyme as an active polymer, with subsequent depolymerication and inactivation of this polymer. Depolymerization was specifically studied by treating the large holomeric *n*-octylglucoside-extracted ($H^+ + K^+$)-ATPase with increasing concentrations of either *n*-octylglucoside or cholate. Detergent-induced changes were characterized by centrifugation on glycerol gradients. Progressive displacement of ATPase activity into three different peaks at 32%, 26% and 20% glycerol was found with increasing detergent concentrations. *n*-Octylglucoside inhibited enzyme activities and was more deleterious for phosphatase than for ATPase activity. Moreover, it induced the dissociation of phosphatase and ATPase distribution profiles. At concentrations of 0.2 to 1.15%, cholate induced the displacement of the glycerol gradient profiles but no loss of activities and no dissociation of phosphatase and ATPase profiles. Higher concentrations of this detergent (2.5%) also inactivated the ATPase concomitantly with the appearance of a protein peak with no related activity at 16–18% glycerol. From this study we suggest that solubilization of gastric ($H^+ + K^+$)-ATPase can be achieved through the extraction of a polymer by *n*-octylglucoside and through subsequent depolymerization using cholate. We suggest that the different sizes correspond to monomers, dimers, trimers and perhaps tetramers. The monomers were apparently inactive under present test conditions.

Introduction

Located on the apical membrane of the secreting parietal cell, ($H^+ + K^+$)-ATPase is widely thought to be involved in gastric H^+ secretion. This enzyme has been reported to be a membraneous polymer of 95 kDa subunits [1]. Its monomers, isolated after sodium dodecylsulfate membrane treatment, are inactive and solubilization of an active form of ($H^+ + K^+$)-ATPase has long been unsuccessful. It is still a matter of debate whether the minimal functional structure is monomeric, dimeric, trimeric or tetrameric [1–5]. Solu-

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bilization of an active $(H^+ + K^+)$-ATPase was achieved using *n*-octylglucoside [6,7] and the extracted enzyme was a large polymer which elicited transport capacity after reconstitution on liposomes [8].

To gain further insight into structure-function relationship of the enzyme, it will be necessary to isolate, separate and reconstitute $(H^+ + K^+)$-ATPase subunits in an undenaturated state. As a first approach, the present study is an attempt to better understand *n*-octylglucoside-ATPase inactivation during solubilization. Preliminary results were presented in a congress report [7].

Materials and Methods

Materials

p-Nitrophenyl phosphate, dithiothreitol, ATP (magnesium salt), glycerol, cholic acid, phospho*enol*pyruvate and Hepes buffer were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Cholate was recrystallized before use. Tris buffer was purchased from Merck (Darmstadt, F.R.G.), *n*-octylglucoside and pyruvate kinase (glycerol solution) from Boehringer (F.R.G.).

Methods

Preparation of gastric vesicles. Microsomal fractions were prepared in sucrose buffer (sucrose 250 mM, Hepes 40 mM buffered at pH 7.3 with Tris powder, dithiothreitol 2 mM) from fresh hog stomachs by differential centrifugation as previously described [9]. The gastric membranes were purified by discontinuous sucrose gradients [6]. The band of material at the interface of the 8.3–30% sucrose layers was collected and used in this study.

Assay procedures. Proteins were determined according to Bradford [10] using bovine serum albumin as standard. ATPase activity was measured as previously described [6] in the presence of 2 mM ATP-Mg, 4 mM phospho*enol*pyruvate, 1 unit/ml pyruvate kinase, 40 mM Hepes-Tris (pH 7–7.2) with or without 20 mM KCl. Phosphatase activity was measured as previously described [6] in the presence of 5 mM *p*-nitrophenyl phosphate, 5 mM $MgCl_2$, 40 mM Hepes-Tris (pH 6.9) with or without 20 mM KCl. Phosphorylation was measured as previously described [6] in the presence of 5 μM [γ-^{32}P]ATP, 2 mM $MgCl_2$ plus or minus 20 mM KCl. 10-s incubations were performed at 0–4°C and stoppped by precipitation in acid.

Solubilization of ATPase activity. Gastric membranes (10–15 mg/ml) were treated at 0°C with *n*-octylglucoside 1.5% for 15–30 min. These extracts (whole extract) were subsequently either centrifuged for 1 h at 40000 rpm (70 Ti rotor, Beckman) to obtain a $100000 \times g$ supernatant or diluted 20-fold in detergent-free sucrose buffer and centrifuged for 45 min at 40000 rpm (70 Ti rotor, Beckman) to obtain a pellet containing '*n*-octylglucoside-extracted $(H^+ + K^+)$-ATPase'. The pellet was resuspended in 0.5% *n*-octylglucoside, treated with 0.5–1.9% *n*-octylglucoside or 0.25–1.15% cholate at 0°C for 10–30 min, and centrifuged on glycerol gradients.

Glycerol gradients. Gradients were made as previously described [6] using successive layers of 18, 20, 23, 26, 29, 32, 35, 38 and 41% glycerol prepared in sucrose buffer which contained either 0.5% *n*-octylglucoside (series A and B) or 0.1% cholate (series C). Membrane extracts (1 to 1.35 ml) were layered on top of the gradient (15.65 ml) and centrifuged at 40000 rpm for 2 h in a Beckman 70 Ti rotor.

Sephacryl columns. Column (1 cm × 60 cm) was packed with Sephacryl S400 gel (Pharmacia Fine Chemicals) and equilibrated with 50 mM Tris-HCl (pH 7.4), 0.2% cholate and 100 mM Na_2SO_4 prior elution of the membrane extracts.

Results

I. Solubilization of gastric membranes with n-octylglucoside

Gastric membranes were solubilized using *n*-octylglucoside and proteins present in the 'whole extract' or the '$100000 \times g$ supernatant' were separated by glycerol gradients or Sephacryl gel chromatographies.

11. Glycerol gradients. Whereas native gastric membranes centrifuge in tight pellets, 95–100% of the proteins present in the whole extract and the $100000 \times g$ supernatant were recovered within the glycerol gradients. Proteins in the whole extract were broadly distributed in the gradient from 15 to 32% glycerol (Fig. 1). Proteins of the $100000 \times g$ supernatant, representing 65–76% of the whole

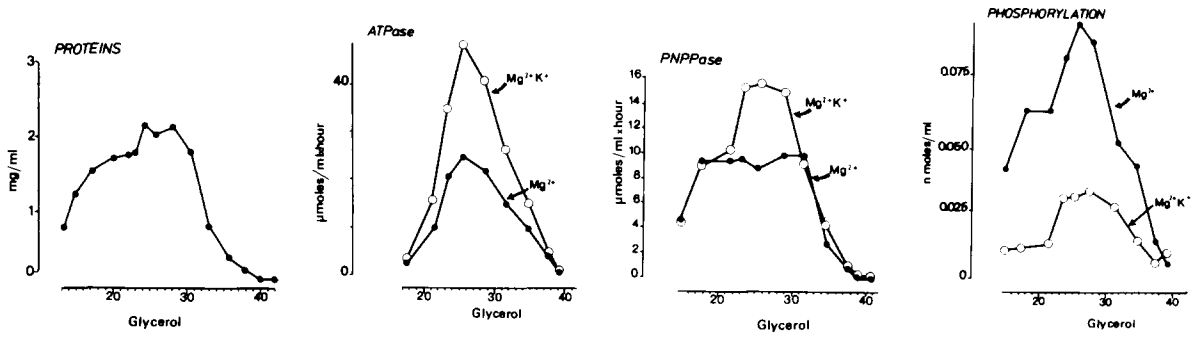

Fig. 1. Proteins, ATPase, *p*-nitrophenylphosphatase (pNPPase) and phosphorylation profiles of a whole extract on glycerol gradients. Gastric membranes (11.9 mg/ml) were treated with 1.5% *n*-octylglucoside for 30–60 min at 0 °C, loaded on top of glycerol gradients and centrifuged for 2 h at 40000 rpm. Gradients were collected and activities measured as described in Materials and Methods. This profile is representative of a series of four gradients.

extract proteins, remained in the lightest gradient fractions, with a peak at 18% glycerol (Fig. 2).

In the whole extract, K^+-stimulated ATPase was present in one broad peak, whose maximum was at 26% glycerol (Fig. 1). This peak accounted for 45–52% of activity present in the native-membranes. Both K^+-stimulated phosphatase and K^+-sensitive phosphorylation had broader distributions with several apparent peaks; one at 18–20% glycerol; a major peak at 26% glycerol, which overlapped that of ATPase activity and a third minor peak at 30–34% glycerol. The distribution of phosphatase and phosphorylation activities more closely paralleled the distribution of total proteins than did that of ATPase activity.

K^+-dependent ATPase in the 100000 × *g* supernatant was present in a single peak which was maximal between 21 and 24% glycerol according to the preparations tested (Fig. 2). This peak accounted for only 12–16% of the total ATPase activity present in the original membrane preparations. The profile of distribution was strikingly

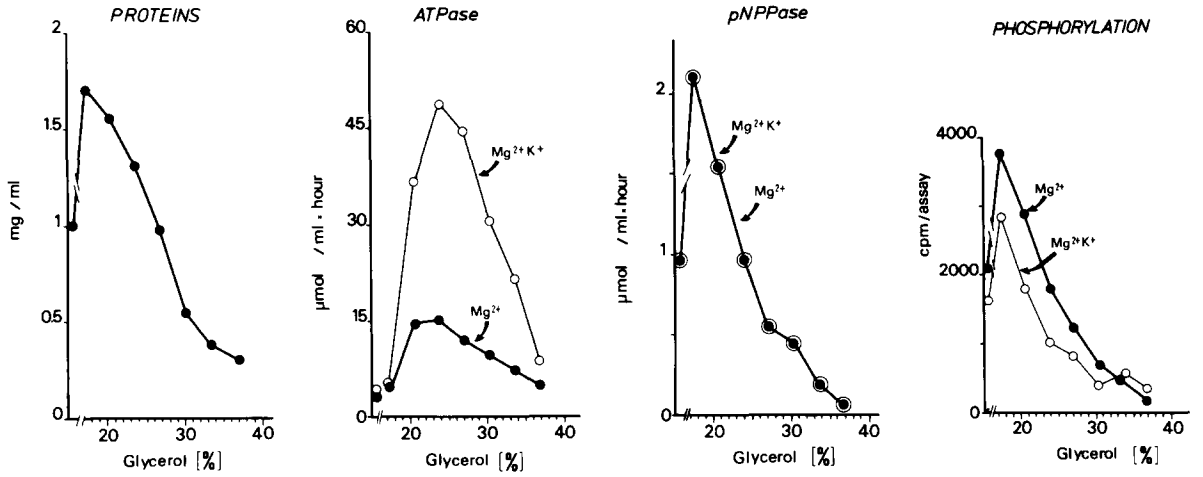

Fig. 2. Proteins, ATPase, *p*-nitrophenylphosphatase (pNPPase) and phosphorylation profiles of a 100000 × *g* supernatant on glycerol gradients. Gastric membranes (3.2 mg/ml) were treated with 1.5% *n*-octylglucoside for 30 min at 0 °C and centrifuged for 1 h at 40000 rpm. The supernatant was collected, loaded on top of a glycerol gradient and centrifuged for 2 h at 40000 rpm. Gradients were collected and the activities were measured as described in Materials and Methods, except phosphorylation which was measured in the presence of [γ-^{32}P]ATP tracer only (no unlabelled ATP) to increase the sensitivity of the assay. These profiles are representative of a series of three gradients.

different from those of phosphatase and phosphorylation activities, which were both present in a single peak with a maximum at 18% glycerol. Phosphatase and phosphorylation activities were quite low and their distributions more closely resembled that of total proteins than did the distribution of ATPase activity.

Recoveries of ATPase activity in the glycerol gradient varied between 93 and 112% (100% activity was defined as the activity of sample loaded onto the gradient). Recovery of phosphatase activity was 97 to 108% for the whole extract but could be as much as 195 to 225% for the $100\,000 \times g$ supernatant whose initial activity was low (see Ref. 6). This suggests that glycerol did not induce inactivation of these enzymes and could even partly counteract *n*-octylglucoside inhibition.

12. Sephacryl columns. The presence of ATPase activities of heterogeneous sizes in the detergent-treated fractions was confirmed by chromatography on Sephacryl S400. Maximal ATPase activity of the whole extract corresponded to a protein of 420 kDa. However, the elution profile was broad, not symmetrical and overlapped that of phosphatase which dissociated into one major peak at 420 kDa and another at 330 kDa. This suggested that two forms of ATPase might also exist. A third minor peak of phosphatase was also found at 220 kDa (Fig. 3).

As previously published [7], ATPase from the $100\,000 \times g$ supernatant was present in one large peak which corresponded to a 330–360 kDa structure, smaller than that observed in the whole extract. Recovery of ATPase activity was low ($< 10\%$) and its K^+-sensitivity was seen only after reconstitution in liposomes. However, reconstitution of K^+-sensitivity was demonstrable only for ATPase activity present within this peak [11]. Phosphatase profile was dissociated from that of ATPase with a peak maximal at 210–250 kDa.

Sephacryl chromatography of the most active fraction collected from a glycerol gradient of whole extract (the 26% glycerol fraction) showed two peaks of ATPase activity at 370 and 235 kDa. Phosphatase had a similar distribution, but its major peak was situated at 235 kDa whereas that of ATPase was at 370 kDa. The profile of total proteins had a peak at 370 kDa and slowly decreased towards the smallest sizes with an additional peak at 135 kDa. These findings suggest that the structure centrifuging at 26% glycerol corresponds to a molecular mass of 370 kDa. This is in agreement with the estimation made from glycerol gradient centrifugation of soluble proteins under the same conditions [6].

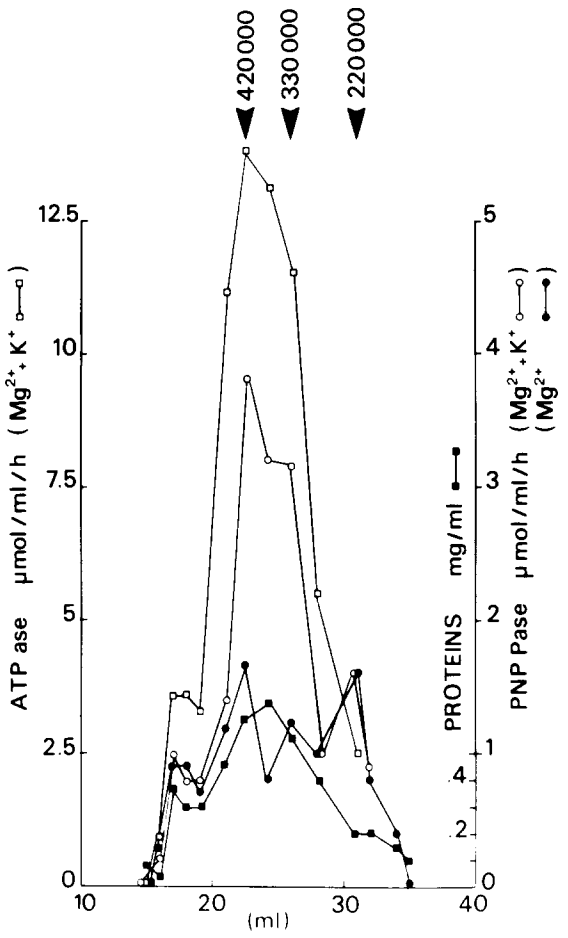

Fig. 3. ATPase, *p*-nitrophenylphosphatase (pNPPase) and protein profiles of a whole extract following molecular sieve chromatography. Hog gastric membranes (12.3 mg protein/ml, 0.8 ml) were treated with 1.5% *n*-octylglucoside and whole extract was loaded on the column and eluted with 50 mM Tris-HCl (pH 7.4), 0.2% cholate and 100 mM and Na_2SO_4. Activities of ATPase and phosphatase were assayed by adding 10 µl of the samples in 1 ml of incubation medium (ATPase assay) or 10 µl in 0.45 ml of incubation assay (phosphatase assay) at 37°C.

These results suggest that during the process of membrane solubilization by *n*-octylglucoside, different structures are obtained which can be iso-

lated either by chromatography or glycerol gradients. Moreover, the occurrence of the lightest structures (in the 100 000 × *g* supernatant) is associated with the largest loss of activity. This led us to think that major inactivation by *n*-octylglucoside occurs during depolymerization.

II. Treatment of n-octylglucoside extracted ($H^+ + K^+$)-ATPase by n-octylglucoside or cholate

n-Octylglucoside-extracted ($H^+ + K^+$)-ATPase was obtained by treating gastric membranes with 1.5% *n*-octylglucoside and by rapidly diluting this mixture to stop detergent action. This extract contained 68% of the native ATPase specific activity; ATPase, phosphatase and phosphorylation activities centrifuged in the same band at 30–32% glycerol. Extracted ATPase was then re-treated with 0.5–1.9% *n*-octylglucoside or 0.2–1.15% cholate.

As expected from the previous results, treatment with *n*-octylglucoside modified the distribution of proteins in the glycerol gradients, by producing lighter structures. K^+-stimulated ATPase activity was maximal at 32% glycerol following treatment with 0.25–0.5% *n*-octylglucoside [6], after treatment with 0.75% *n*-octylglucoside, two peaks at 32 and 27% glycerol were present; with 1.35% *n*-octylglucoside, a major peak was at 27%

TABLE I

ACTIVITIES OF ATPase AND PHOSPHATASE ON GLYCEROL GRADIENTS

Gastric membranes were solubilized with *n*-octylglucoside as described in Fig. 4. Extracted ATPase was then treated with *n*-octylglucoside (series A) or cholate (series B and C) using the concentrations specified in the table. Samples were centrifuged on 18–40% glycerol gradients. This table shows specific activity of extracted K^+-ATPase prior to detergent retreatment; specific activity of K^+-ATPase in the glycerol peak and localization of this peak (specified by the percentage of glycerol). Specific activities are expressed as μmol P_i/h per mg of protein. Other values are percentage of total initial activity recovered after detergent-induced depolymerization and glycerol gradients (100% being the total extracted-ATPase activity. Inactivation by *n*-octylglucoside in series A was not due to a loss of activity during glycerol gradient because the yield of total K^+-ATPase recovery were 97, 94, 102 and 110%, respectively (100% being the loaded sample). Inactivation of phosphatase in series B was mostly due to *n*-octylglucoside in the gradient because recoveries of total K^+-phosphatase in the gradient were 107, 102, 105, 83 and 27%, respectively (100% being the loaded sample).

Treatment with	K^+-ATPase spec. act.	Peak % glyc.	ATPase recovery			Phosphatase recovery		
			Mg^{2+}	$Mg^{2+} + K^+$	K^+	Mg^{2+}	$Mg^{2+} + K^+$	K^+
Series A [a]	44							
n-Octylglucoside								
0%	53	32	100	100	100	100	100	100
0.75%	28	26	40	52	64	164	28	17
1.35%	21	26	36	41	46	74	9	3
1.9%	23	20	25	29	33	33	2	0
Series B [a]	43							
Cholate								
0%	43	32	100	100	100	100	100	100
0.25%	44	31	81	99	115	213	114	106
0.5%	54	27	79	85	92	226	112	103
0.8%	62	22	114	107	100	200	84	75
0.95%	29	21	40	50	60	146	25	15
Series C [b]	64							
Cholate								
0%	65	32	100	100	100	100	100	100
0.25%	68	32	91	107	113	111	98	97
0.45%	59	27	129	119	115	86	92	93
1.15%	77	22	155	129	120	128	90	85

[a] Glycerol gradients were prepared with 0.5% *n*-octylglucoside.
[b] Glycerol gradients were prepared with 0.1% cholate.

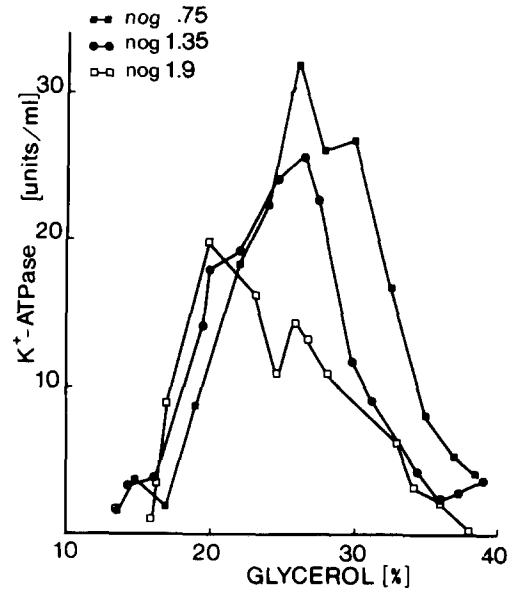

Fig. 4. K^+-stimulated ATPase centrifugation profiles of extracted ATPase following treatment with *n*-octylglucoside. Gastric membranes (13.2 mg/ml) were solubilized with 1.5% *n*-octylglucoside for 20 min at 0°C, diluted 15-fold in 2 mM dithiothreitol, 40 mM Hepes-Tris (pH 7.3) and centrifuged for 1 h at 40000 rpm. Pellets were suspended and treated with *n*-octylglucoside at the concentrations specified on the figure. Samples were then loaded on the glycerol gradients (18–40%) which contained 0.5% *n*-octylglucoside. After 2 h of centrifugation, fractions were collected and assayed for ATPase activity with and without 20 mM KCl. K^+-dependent specific activities of the peaks of ATPase are given in Table I.

glycerol and minor one at 20% was seen; and after treatment with 1.9% *n*-octylglucoside, the 20% glycerol peak became preponderant. However, as previously noted, a marked and progressive decrease in ATPase activity was found. The lightest structures showed little activity (Table I, Fig. 4). Phosphatase activity was strongly decreased by the addition of *n*-octylglucoside which also inhibited its K^+ sensitivity (Table I). The lightest K^+-insensitive phosphatasic structure centrifuged at 16% glycerol, lighter than ATPase activity.

As seen with *n*-octylglucoside, cholate shifted glycerol gradient profiles of protein, ATPase and phosphatase towards lighter densities. Cholate was more efficient than *n*-octylglucoside; 0.80% cholate was sufficient to induce a shift in the distribution of proteins similar to that elicited by 1.9% *n*-octylglucoside (data not shown).

However, up to 1.15% concentrations of cholate, did not inactivate K^+-stimulated ATPase activity (Figs. 5 and 6, Table I). After treatment with 0.95–1.15% cholate, K^+-stimulated ATPase activity was maximal at 20–22% glycerol. Recovery of phosphatase activity was almost as good as that of ATPase if only cholate was present (Table I, series C). In this case, phosphatase distributed in the gradient in the same manner as ATPase.

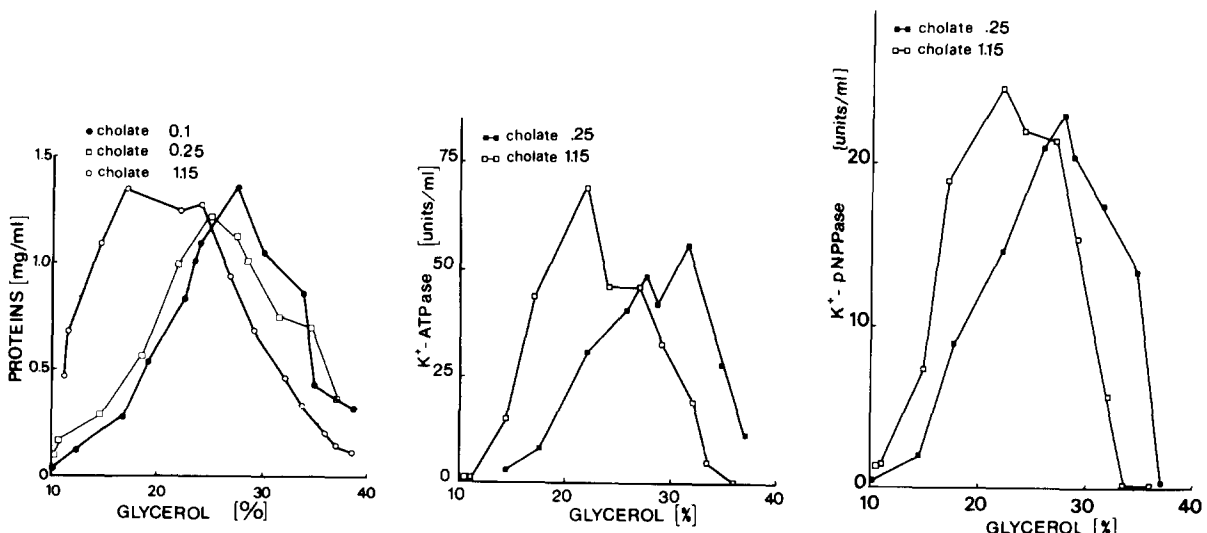

Fig. 5. Proteins, K^+-stimulated ATPase and K^+-stimulated *p*-nitrophenylphosphatase (pNPPase) centrifugation profiles of extracted ATPase following treatment with cholate. Gastric membranes (8.5 mg of protein/ml) were solubilized with 1.5% *n*-octylglucoside, diluted 15-fold and centrifuged for 1 h at 40000 rpm. Pellets were suspended and treated with cholate at the concentrations specified on the figure. Samples were then centrifuged on glycerol gradients which contained 0.1% cholate. After 2 h of centrifugation, fractions were collected. Specific activities of the peaks of ATPase are given in Table I.

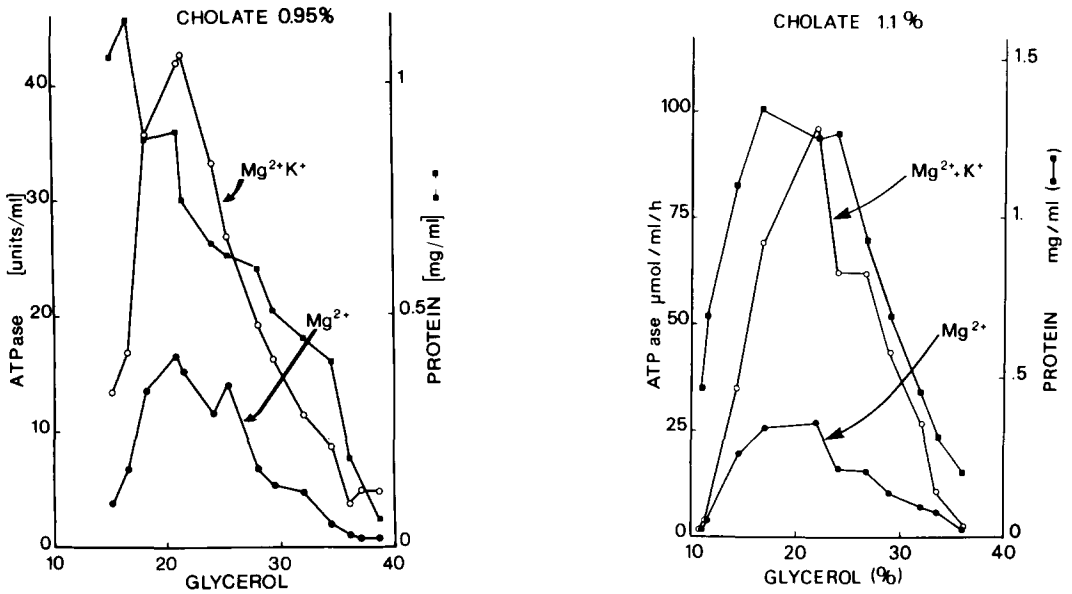

Fig. 6. Protein, Mg^{2+}-ATPase and ($Mg^{2+} + K^+$)-ATPase profiles of extracted holomeric ATPase following treatment with cholate and centrifugation on glycerol gradients. Gastric membranes (13.2 and 12.4 mg of protein/ml) were solubilized with 1.5% *n*-octylglucoside for 20 min at 0 °C, diluted 15-fold in 2 mM dithiothreitol, 40 mM Hepes-Tris (pH 7.3) and centrifuged for 1 h at 40000 rpm. Pellets were suspended at 13.2 and 10.1 mg of protein/ml and treated with 0.95% and 1.1% cholate, respectively. Treated materials were loaded on top of the gradients which contained 0.5% *n*-octylglucoside (sample with 0.95% cholate) or 0.1% cholate (sample with 1.1% cholate).

Fig. 6 illustrates a particular feature which was repeatedly found when high concentrations of cholate of *n*-octylglucoside were used. A protein peak appeared on top of the gradient, in very light fractions (16% glycerol). This peak was not associated with an enzyme activity, suggesting that the corresponding structure was inactive. Increasing cholate concentration up to 2.5% inhibited all activity.

Discussion

Solubilization of an active ($H^+ + K^+$)-ATPase has, so far, only been achieved using *n*-octylglucoside. The most active soluble form was a large polymer of 390–420 kDa. In this study, we demonstrate that treatment with *n*-octylglucoside for longer times decreased activity and created smaller polymorphic structures. Loss of activity was particularly important for phosphatase. Differential recovery of the two subparts of ($H^+ + K^+$)-ATPase activity is not a new concept, having been described during enzyme inactivation with thimerosal or phospholipase [12,13]. However, this study demonstrates that the inhibition of ($H^+ + K^+$)-ATPase activity is associated with the appearance of smaller protein structures. Relevance of such data to the function of the ATPase will require more information on structure-activity relationships.

The present data suggest that solubilization of ($H^+ + K^+$)-ATPase can be dissociated into two steps: the extraction of a large polymer and its subsequent depolymerization. Although *n*-octylglucoside is, at present, the only detergent successful for extraction, our study demonstrates that cholate, recently used for ($H^+ + K^+$)-ATPase reconstitution [8] is preferable for depolymerization because it decreased the inactivation (up to 1.15% detergent). It should be noted that when cholate-treated samples were centrifuging on *n*-octylglucoside-containing gradients, a marked decrease of phosphatase activity was found due to a loss of activity during centrifugation. This demonstrated that phosphatase inactivation by *n*-octylglucoside was not due to depolymerization itself, which could be obtained to a similar degree but without inactivation, using cholate but to a inhibi-

tory effect of *n*-octylglucoside on the enzyme.

As previously found [6], glycerol medium allows a good recovery of soluble ATPase activities. It also enables separation of proteins as efficiently as that seen with molecular sieve chromatography in the range of 250 to 450 kDa molecular masses. Elution on Sephacryl gels was, however, deleterious to enzyme activity. Recoveries were especially low in fractions containing the lightest structures. Moreover, in most cases, reconstitution with phospholipid was required to recover K^+-sensitivity of the ATPase following molecular sieve chromatography. Therefore, fractionation of ATPase activity on glycerol gradients was preferable.

The shift of $(H^+ + K^+)$-ATPase distribution induced by cholate or *n*-octylglucoside treatment could be interpreted as the appearance or disappearance of successive forms of enzyme. Thus, subtraction of profiles should emphasize the existence of specific entities. Such an analysis of *n*-octylglucoside-treated profiles suggests the presence of three K^+-ATPase structures at 30–31% glycerol, 24–26% and 20–21%. These were associated with protein peaks, at 31, 24 and 19–20% glycerol, however, a fourth protein peak at 16–18% glycerol was also present. Using the same analysis for cholate treated samples demonstrated that K^+-stimulated ATPase could be separated into three peaks at 32–34%, 26–27% and 20% glycerol whose location was very close to those found using *n*-octylglucoside. A fourth peak of protein was also found at 16% glycerol. Phosphatase activity was associated with all ATPase peaks. However, it was unclear whether the fourth 16% glycerol peak of protein was truly inactive in cholate-treated samples. This uncertainty was due in part of the low resolution achieved in that zone of the glycerol gradients and the question could not be solved by the use of molecular sieve chromatography because of the low activity of eluted fractions. Attempts to improve resolution of gradients by modifying glycerol densities have so far been unsuccessful.

Our results suggest that, in our conditions, soluble $(H^+ + K^+)$-ATPase can exist as four substructures, the smallest one being devoid of ATPase activity. Thus we suggest that the largest complex isolated after *n*-octylglucoside treatment of the membrane corresponds to a 390–420 kDa tetramer, the others to a 360–370 kDa trimer and to a 210–235 kDa dimer. The 16% glycerol protein peak might correspond to a 135–150 kDa monomer. All forms would be surrounded by a core of phospholipids and detergents. The tetramers, trimers and dimers would be enzymatically active, whereas the monomers would be inactive. However, it is possible that the monomers are not denatured and it remains to be tested whether they will be active in a phospholipidic environment. It should also be noted that the so-called tetramers, could be an aggregate of trimers surrounded by a large core of phospholipids or detergents. These suggestions make no assumption concerning the homogeneity or the heterogeneity of the subunits. Further experiments will be required to clarify this point and to investigate the transport capacity of the different structures after reconstitution [8].

Extension of these results to the understanding of previous unsuccessful attempts to solubilize $(H^+ + K^+)$-ATPase suggests that *n*-octylglucoside has been more effective because of its relatively low capacity to depolymerize the ATPase. Moreover, direct solubilization with cholate was probably unsuccessful because the concentrations used immediately led to production of the most depolymerized and inactive form.

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