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[^{3}H]Bafilomycin as a probe for the transmembrane proton channel of the osteoclast vacuolar H$^+$-ATPase

Jan P. Mattsson *, David J. Keeling

Department of Cell Biology, Astra Hässle AB, Karragatan 5, S-431 83 Mölndal, Sweden

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Abstract

Bone resorption by the osteoclast is dependent on acidification of the bone surface by a vacuolar type H$^+$-ATPase (V-ATPase) present in the ruffled membrane of the actively resorbing cell. V-ATPases are a highly conserved family of proton pumps consisting of two functional complexes: a cytoplasmic catalytic sector (V_C) and a membrane bound proton channel (V_B). Bafilomycin A_1, a macrolide antibiotic, is a highly potent inhibitor of V-ATPases, and inhibits bone resorption in vitro in isolated rat calvariae. In order to investigate the binding of bafilomycin to the osteoclast V-ATPase, we used a tritiated bafilomycin which had been prepared by acetylating the 21-hydroxyl group of bafilomycin A_1. Osteoclast ruffled membrane vesicles were prepared from purified chicken osteoclasts by differential centrifugation and proton transport in these vesicles was shown to be inhibited by [^{3}H]bafilomycin ($IC_{50} \approx 2$ nM). Control membrane vesicles or membrane vesicles partially inhibited with [^{3}H]bafilomycin were solubilized and separated by centrifugation on 15–30% glycerol gradients. V-ATPase activity and reconstitutable proton transport activity could be recovered in high density fractions of the gradient. However, the peak of [^{3}H]bafilomycin radioactivity (> 70% of total radioactivity in the gradient) was present in a single peak at lower density. Antibodies against subunits of V_C (70, 56 and 40 kDa) reacted only in fractions containing the peak V-ATPase activity whereas an antibody to the 39 kDa subunit of V_B reacted both with fractions containing the peak V-ATPase activity but also, and more strongly, in fractions containing the peak [^{3}H]bafilomycin. The fractions in the control gradient corresponding to the peak of [^{3}H]bafilomycin were reconstituted into liposomes and shown to mediate passive bafilomycin A_1-inhibitable proton conductance. SDS-PAGE followed by autoradiography, indicated that the bafilomycin was not covalently bound to the V-ATPase or the proton channel. Quantification of V_B by [^{3}H]bafilomycin binding or by antibody staining suggested an excess of the free proton channel to that of the intact holoenzyme. A corresponding amount of free catalytic sector could not be found in any fraction throughout the isolation procedure of the V-ATPase from the initial homogenate. Thus, in conclusion, bafilomycin inhibits the V-ATPase by binding tightly but non-covalently to the proton channel region of the V-ATPase which appears to be present in excess over the intact holoenzyme in the osteoclast. The possible role of an excess of the proton channel subcomplex in the osteoclast is discussed.

Keywords: Bafilomycin; ATPase, H$^+$-; Osteoclast; Vacuolar ATPase; Proton channel

1. Introduction

Vacuolar H$^+$-ATPases (V-ATPases) are heteromultimeric proton pumps responsible for the acidification of a variety of intracellular organelles in the eukaryotic cell, including clathrin-coated vesicles, lysosomes and chromaffin granules [1]. In some cells, such as the osteoclast and the renal intercalated cell, V-ATPases are found at high density in the plasma membrane where they are involved in acid transport out of the cell [2–5]. In the active osteoclast, V-ATPase is polarized to a region of the plasma membrane, known as the ruffled membrane, which faces the bone surface [2,3]. Protons are secreted into the compartment formed between the bone attachment site, the ruffled membrane and the bone surface, and this acidity combined with the release of proteolytic enzymes leads to bone resorption [6].

Recently we partially purified the chicken osteoclast ruffled membrane V-ATPase and suggested that this acid pump contains subunits of apparent molecular mass 116-, 71-, 57-,40-, 39-, 33- and 17-kDa subunits [4]. This is very

Abbreviations: $C_{12}E_9$, polyoxyethylene 9-lauryl ether; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)-*N'*-(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholine)ethanesulfonic acid; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gelelectrophoresis; EGTA, (ethylenebis(oxyethylenenitrilo))tetraacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid.

* Corresponding author. Fax: +46 31 7763761; e-mail: jan.mattsson@hassle.se.astra.com.

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similar to the basic subunit structure reported for other V-ATPases that have been characterized [1]. The exact subunit composition is not known for any V-ATPase and the number of polypeptides found to be present in preparations from different sources show some variation. It is not yet clear if these variations represent genuine subunits, contaminants or regulatory proteins. However, evidence suggests that the V-ATPase subunits are grouped into two distinct functional complexes: a catalytic hydrophilic sector (V_C) containing the 71-, 57-, 40- and 33-kDa subunits and a transmembranous proton channel (V_B) containing the 116-, 39- and 17-kDa subunits [4,7,8].

One of the major functional characteristics of the V-ATPases is their sensitivity to a group of macrolide antibiotics, the bafilomycins [9]. Bafilomycin A_1, specifically inhibits V-ATPases at nanomolar concentrations whereas at least micromolar concentrations are needed to inhibit other types of ATPases. We [4,10] and others [11], have shown that bafilomycin A_1 inhibits the osteoclast H^+-ATPase at nanomolar concentrations and also inhibits bone resorption in vitro in mouse calvariae. The mechanism of action and the binding site(s) of bafilomycin to the V-ATPase are poorly understood [12,13]. Recently, however, Crider et al. [8] showed that the isolated transmembranous complex is a functional proton conductance which is highly sensitive to bafilomycin A_1. In order to get more insight into the mechanism of action of bafilomycin, we have investigated the binding of a tritiated bafilomycin to the osteoclast H^+-ATPase.

2. Experimental procedures

2.1. Materials

Acridine orange was obtained from Kodak; phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine from Avanti Polar Lipids (Birmingham, AL); cholic acid was obtained from Calbiochem. Bafilomycin A_1, isolated from *Streptomyces* sp., was purified by Astra Hässle AB, Mölndal, Sweden. The tritiated bafilomycin (108 kBq/nmol, 99% radiochemical purity) was prepared by Astra Hässle AB by acetylating the 21-hydroxyl group of bafilomycin. The clathrin-coated vesicle H^+-ATPase [14], the antibodies to the 70-, 40-, and 39-kDa subunits [15,16]), were generous gifts from Prof. Dennis Stone, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas. The antibody to the 56 Da subunit (against the B2 isoform) was prepared as described [17]. All other chemicals were obtained from Sigma.

2.2. Isolation of osteoclast vacuolar H^+-ATPase

Osteoclasts were isolated from egg laying hens after 14-days on a calcium-deprived diet as previously described [4] with the exception that only one serum gradient was used. A final osteoclast purity of approx. 1:15 (osteoclast/contaminating cells) was obtained. Due to the large size of the osteoclast (50–100 μm) compared to the smaller contaminating cells it is estimated that greater than 75% of the plasma membranes were derived from osteoclasts in this preparation.

Osteoclast plasma membrane vesicles containing the ruffled membrane were prepared as described by Blair et al. [18]. Briefly, osteoclasts were resuspended in isolation buffer (5 mM Tris-HCl, pH 7.0, 1 mM $NaHCO_3$, 250 mM sucrose, 1 mM EGTA and 1 mM DTT) and then disrupted by nitrogen cavitation in a cell disruption bomb (40 atm for 25 min). The homogenate (H) was centrifuged at $1000 \times g$ for 5 min to obtain a supernatant (S1) and a pellet (P1). S1 was centrifuged at $6000 \times g$ for 15 min giving a supernatant (S2) and a pellet (P2). S2 was centrifuged for 40 000 × for 45 min generating the final supernatant (S3) and pellet (P3). P3, containing the membrane vesicles, was resuspended in isolation buffer and frozen at −70°C. The osteoclast membrane vesicle preparation used in the present study was previously characterized by cell surface labelling which indicated that at least 25% of the total membranes and 50% of the vesicles in the preparation were derived from the osteoclast ruffled membrane [18]. In the experiment shown in Fig. 1, membrane vesicles were prepared directly from medullary bone of egg laying hens without first purifying the osteoclasts [19].

The vacuolar H^+-ATPase present in the osteoclast membrane vesicles was further purified as described [4], with some modifications. Briefly, membrane vesicles were diluted to ≈ 3 mg protein/ml in isolation buffer (5 mM Tris-HCl, pH 7.0, 250 mM sucrose, 1 mM EGTA and 10 mM DTT). The membranes were then solubilized by incubation in the presence of 1.5% $C_{12}E_9$ for 1 h 15 min at 5°C followed by centrifugation at $90\,000 \times g$ for 45 min at 4°C. The supernatant was removed and layered onto a 3.5 ml 15–30% (v/v) glycerol gradient prepared in buffer A (20 mM Tris-Mes, pH 7.0, 0.05% $C_{12}E_9$, 10 mM DTT and 1 mM EGTA). The gradients were centrifuged at 485 000 $\times g$ for 5 h 30 min, and fractions were collected from the bottom of the gradient. In some experiments, fractions containing bafilomycin A_1-sensitive ATPase activity were pooled, concentrated in Centrikon 10 concentrators and centrifuged on a second 15–30% glycerol density gradient. Where indicated, membrane vesicles or the partially purified ATPase were preincubated with [^{3}H]bafilomycin or ethanol (control) for 5 min prior to separation as described above. Radioactivity in fractions was measured by liquid scintillation spectroscopy in a Wallac Microbeta scintillator counter (Wallac, Finland). For autoradiography, fractions were separated by SDS-PAGE using 12.5% polyacrylamide gels. After electrophoresis, the gels were incubated in fixing solution (isopropanol/acetic acid/H_2O; 25:10:65) for 30 min at room temperature, followed by 30

min incubation in amplifier (Amplify, Amersham). The gels were then dried under vacuum and then exposed to film for up to 5 weeks at −70°C.

The osteoclast V-ATPase was dissociated into V_C and V_B complexes using cold inactivation [19,20]. Medullary bone membrane vesicles, prepared as described above, were incubated in dissociation solution (400 mM KI, 5 mM ATP (pH 7.0 with Tris), 5 mM $MgSO_4$, 1 mM DTT, 0.5 mM EDTA) for 1 h on ice, followed by centrifugation at 100 000 × *g* for 1 h at 4°C. The pellet was resuspended in isolation buffer (5 mM Tris, pH 7.0, 1 mM EGTA and 250 mM sucrose) and both the resuspended pellet and the supernatant were incubated in the presence of 1.5%$C_{12}E_9$ and 10 mM DTT for 1 h 15 min at 5°C. After centrifugation at 90 000 × *g* for 45 min, the supernatants were loaded onto 15–30% glycerol density gradients and centrifugation and fractionation was performed as described above.

2.3. SDS-PAGE and Western blot analysis

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli [21] using 12.5% polyacrylamide gels. After electrophoresis, the gel was stained with 0.125% Coomassie blue R-250 (Sigma). For Western blot analysis, protein fractions were separated on 12.5% SDS-polyacrylamide gels and then transferred electrophoretically to a nitrocellulose membrane by blotting at 100 V for 1 h using a Bio-Rad Trans Blot Cell. After transfer, the membrane was washed in rinse buffer (TBS) consisting of 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA and 0.75% Tween 20 for 15 min at room temperature followed by incubation in TBS containing 5% nonfat dry milk for 2 h at room temperature or overnight at 5°C. After a 2 × 15 min wash in TBS the membrane was incubated for 2 h with the primary antibody at appropriate dilution (1:2000 for the antibodies to the 70 and 40 kDa subunits, 1:5000 for the antibody to the 56 kDa subunit and 1:10 000 for the antibody to the 39 kDa subunit). The membrane was then washed in TBS and bound antibody detected using horseradish peroxidase-linked anti-rabbit immunoglobulin (1:4000 dilution) and the ECL-detection kit (Amersham).

2.4. ATPase activity measurement

ATPase activity was measured in a total volume of 250 μl by the release of inorganic phosphate from ATP. Protein fractions (10 μl) were preincubated in the presence of 6 μl 0.5 mg/ml phosphatidylserine for 10 min at room temperature. 221.5 μl reaction mixture (50 mM Tris-Mes, pH 7.0, 60 mM KCl and 5 mM magnesium gluconate) was added followed by the addition of 2.5 μl bafilomycin A_1 (50 nM, final concentration) or 2.5 μl DMSO (control). After 5 min pre-incubation at room temperature, the reaction was initiated by the addition of 10 μl 62.5 mM Na_3ATP (pH 7.0 with Tris base). After 30 min incubation at 37°C, the reaction was terminated by the addition 25 μl of 50% TCA and released P_i was determined as described by Yoda and Hokin [22]. Bafilomycin A_1-sensitive ATPase (V-ATPase) activity was defined as the ATPase activity inhibited by 50 nM bafilomycin A_1.

2.5. Reconstitutions and measurement of proton transport

Liposomes composed of phophatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol at a mass ratio of 43:28:2:27 were prepared at a concentration of 50 mg/ml by sonication as described by Xie et al. [23] with the exception that the lipids were sonicated to clarity (≈ 3 × 10 min).

Reconstitution of the H^+-ATPase into liposomes was performed using the freeze-thaw, cholate dilution method [4]. To measure ATP-dependent proton transport, osteoclast membrane vesicles or reconstituted fractions were diluted into a 1.6 ml assay buffer containing 5 mM Hepes-Tris, pH 7.5, 120 mM KCl, 2.5 mM magnesium gluconate and 1 μM acridine orange. Proton transport was monitored by measurement of acridine orange fluorescence (Ex. = 492, Em. = 528) quenching in a Shimadzu spectrofluorimeter [10]. Reactions were measured at room temperature and were initiated by the addition of 1 mM Na_3ATP (pH 7.0 with Tris base) followed by valinomycin (1 μM, final concentration) and terminated after 2 min by the addition of 6 mM NH_4Cl. Amount of acidification was taken to be the difference between the fluorescence before and after the addition of NH_4Cl and is expressed as fluorescence units (F.U.). In the experiments illustrated in Fig. 1, medullary bone membrane vesicles (30 μg protein/ml) were added to an assay medium containing 120 mM KCl, $MgCl_2$, acridine orange. Bafilomycin A_1 or the [^{3}H]bafilomycin was added at the indicated concentration, with DMSO or ethanol, respectively in the control samples, and the samples were preincubated for 10 min at room temperature. Reactions were initiated by the addition of 1 mM ATP and the decrease in acridine orange absorbance at 490 nm was monitored in a microplate reader (Molecular Devices, CA, USA). The initial rate of acidification, taken to be the initial decrease in acridine orange absorbance, was determined from the slope of the absorbance trace after the addition of ATP [10].

To test for the presence of passive proton conductance activity, fractions were reconstituted into liposomes using the method described by Crider et al. [8]. Briefly, 100 μl protein fraction and 60 μl liposomes were mixed with 30 μl reconstitution solution (6% sodium cholate, 30% glycerol and 740 mM KCl) followed by the addition of 5 μl of a solution containing 0.6 M DTT and 60 mM $CaCl_2$. The mixture was frozen in liquid N_2, thawed for 1 h at room temperature and then diluted into 7 ml dilution buffer (150 mM KCl, 20 mM Tricine, pH 7.5, 3 mM $MgCl_2$ and 0.5 mM EDTA). After incubation for 10 min at room temperature, the proteoliposomes were pelleted by centrifugation

at 100 000 × *g* for 1 h at 15°C and then resuspended in 50 μl dilution buffer. To activate the proton conductance, 10 μl of the proteoliposomes were mixed with 4 μl 0.5 M Mes and incubated for 1 h at room temperature. Proton conductance activity in the proteoliposomes was measured using acridine orange fluorescence as described above with the exception that the proteoliposomes were diluted into a 1.6 ml assay buffer containing 20 mM Tricine, pH 7.5, 150 mM NaCl, 3 mM $MgCl_2$, 0.5 mM EDTA, 1 μM acridine orange and 5 nM bafilomycin A_1 or DMSO (control). Reactions were initiated by the addition of 1 μM valinomycin.

2.6. Protein determination

Protein was determined by the Amido-Schwartz method [24], with bovine serum albumin as a standard.

3. Results and discussion

The inhibitory effect of bafilomycin A_1 or its tritiated 21-acetyl analogue ([^{3}H]bafilomycin) on ATP-dependent proton transport in medullary bone membrane vesicles was similar (Fig. 1). At 30 μg protein/ml, IC_{50} values were 1.2 nM and 2.0 nM for bafilomycin A_1 and [^{3}H]bafilomycin, respectively. Thus, acetylation of bafilomycin A_1 had only a marginal effect on its inhibitory potency. Similar to previous reports for bafilomycin A_1 [9,19], the inhibitory effect of [^{3}H]bafilomycin was inversely related to the amount of V-ATPase preparation present in the assay (result not shown).

To investigate the binding of the [^{3}H]bafilomycin to the V-ATPase, osteoclast membrane vesicles (≈ 2.5 mg protein/ml) were either incubated in the presence of ethanol (control) or [^{3}H]bafilomycin. At this protein concentration it was found that 280 nM [^{3}H]bafilomycin resulted in about 60% inhibition of both proton transport and bafilomycin A_1-sensitive ATPase activity (Table 1). After incubation, the membranes were then treated with 1.5% $C_{12}E_9$, centrifuged and proteins in the supernatant separated on a 15–30% glycerol density gradient. As illustrated in Fig. 2, V-ATPase and reconstitutable ATP-dependent H^+-transport could be recovered from high density fractions of the gradient. This was true for both the control and the [^{3}H]bafilomycin pre-treated samples. Consistent with the 60% inhibition of the starting material, the gradient on which [^{3}H]bafilomycin pre-treated proteins had been loaded contained 29 ± 1% of the bafilomycin A_1-sensitive ATP-

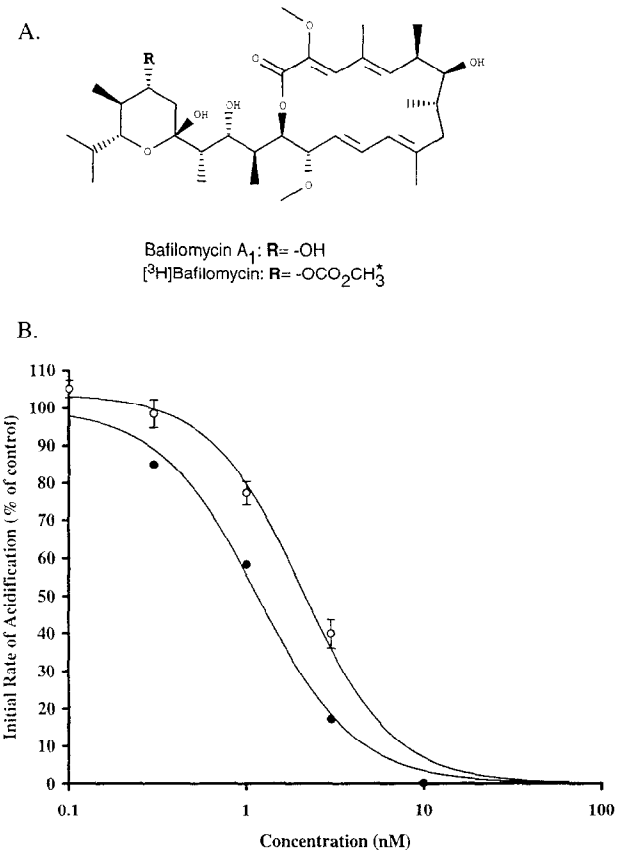

Fig. 1. Comparison of the inhibitory effects of bafilomycin A_1 and [^{3}H]bafilomycin on proton transport in bone membrane vesicles. (A) The structures of bafilomycin A_1 and its tritiated 21-acetyl analogue ([^{3}H]bafilomycin). The acetyl group was tritiated (*). (B) ATP-dependent proton transport in bone membrane vesicles was measured as described under Section 2 in the presence or absence of the indicated concentrations of bafilomycin A_1 (closed circles) or [^{3}H]bafilomycin (open circles). The initial rate of acidification in the control samples was set to 100%. Values are the mean ± range of two determinations from a representative experiment. Error bars smaller than the symbol are not shown.

Table 1
Solubilization and glycerol gradient separation of osteoclast membrane vesicles in the presence or absence [^{3}H]bafilomycin

Fraction	V-ATPase activity (nmol min^{-1})			Acidification (F.U.)		
	control	[^{3}H]Baf.	(%)	control	[^{3}H]Baf	(%)
Osteoclast membranes	152 ± 31	64 ± 18	42 ± 15	370	139	38
Solubilized membranes	56 ± 17	23 ± 10	41 ± 22	1400	300	21
Gradient fraction	89 ± 2	26 ± 0.5	29 ± 1	815 ± 43	415 ± 72	51 ± 9

Bafilomycin A_1-sensitive ATPase activity (V-ATPase) and ATP-dependent acidification in fractions obtained during solubilization and separation of osteoclast membranes which had been pre-treated with ethanol (control) or [^{3}H]bafilomycin as described in the legend to Fig. 2 were measured as detailed under Section 2. Values are the mean ± S.E. of two or three determinations from a representative experiment except for the values of acidification in osteoclast membranes and solubilized membranes which are one determination from a representative experiment. Acidification in solubilized fractions were measured after reconstitution into liposomes as described in Section 2.

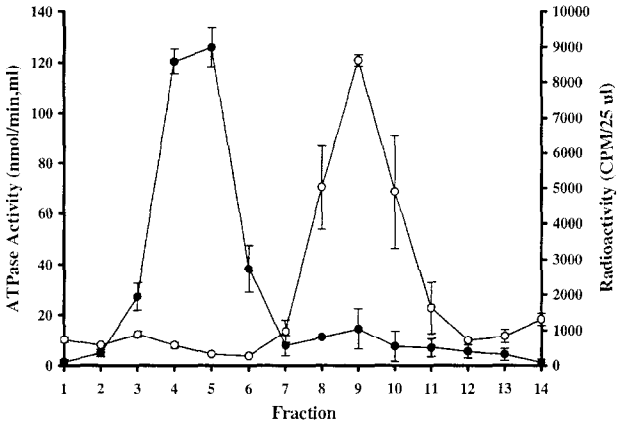

Fig. 2. Distribution of bafilomycin A_1-sensitive ATPase activity and [^{3}H]bafilomycin radioactivity after separation of solubilized osteoclast membrane vesicles on a glycerol gradient. Osteoclast membrane vesicles were incubated in the presence or absence (control) of [^{3}H]bafilomycin and then treated with 1.5% $C_{12}E_9$. Solubilized proteins were centrifuged on a 15–30% glycerol density gradient and fractions were collected from the bottom (Fraction 1 = 30% glycerol) to the top (Fraction 14). ATPase activity and radioactivity were measured in the fractions as described in Section 2. Bafilomycin A_1-sensitive ATPase activity in fractions from the control gradient (closed circles) and radioactivity in fractions from the [^{3}H]bafilomycin gradient (open circles) are displayed. Values are the mean ± range of two determinations on separate gradients.

ase seen on the control gradient and 51 ± 9% of total reconstitutable ATP-dependent proton transport activity (Table 1). Thus, the inhibitory effect of the [^{3}H]bafilomycin had not been lost during solubilization and isolation on the glycerol gradient, suggesting either tight binding to the ATPase or an irreversible mechanism. SDS-PAGE showed the presence of the expected V-ATPase polypeptides of apparent molecular masses 72, 56, 42, 39, 32 and 16 kDa in fractions containing the V-ATPase (Fig. 3). A 100–120 kDa peptide which is reported to be present in a number of

Table 2
Distribution of radioactivity in fractions during solubilization and separation of [^{3}H]bafilomycin pre-treated osteoclast membrane vesicles

	Total radioactivity (cpm) × 10^{-4}	Recovery (%)
Osteoclast membranes	31 ± 0	100
Solubilized membranes	31 ± 1	100 ± 3
Gradient, fr 1–14	33 ± 1	106 ± 3
Gradient, fr 3–6	2 ± 0	6 ± 0
Gradient, fr 8–11	23 ± 1	74 ± 3

Radioactivity in fractions obtained after solubilization and separation of osteoclast membranes treated with [^{3}H]bafilomycin as described in the legend to Fig. 2 was measured as described under Section 2. Values are the mean ± range of two determinations from a representative experiment.

V-ATPase preparations could not be seen in the fractions correlating with the peak V-ATPase using Coomassie staining. However, we have previously reported that problems can occur detecting this subunit in preparations of the osteoclast V-ATPase [4] and the 100–120 kDa subunit of V-ATPase from other sources is reported to be unstable and sensitive to proteinase digestion [25]. There was no detectable difference in the polypeptide patterns or intensity of Coomassie staining between gradient fractions containing [^{3}H]bafilomycin pre-treated proteins and the control gradient fractions, indicating that bafilomycin does not induce loss of known V-ATPase subunits.

Despite the fact that [^{3}H]bafilomycin was recovered quantitatively from the glycerol gradient and that [^{3}H]bafilomycin-treated ATPase remained inhibited after glycerol gradient separation (Tables 1 and 2), no peak of radioactivity was found in fractions containing the intact V-ATPase (Fig. 2). Instead, a peak of radioactivity (≈ 74% of the total radioactivity applied, Table 2) was present in lower density fractions of the gradient (Fig. 2). This radioactivity did not represent free bafilomycin since

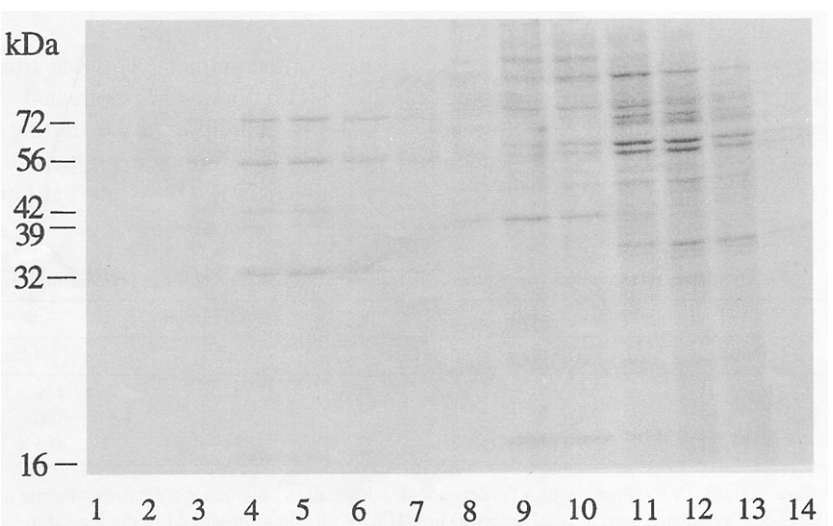

Fig. 3. SDS-PAGE of fractions from glycerol density gradient separation of solubilized osteoclast membrane vesicles. Fractions (10 μl of fractions 1–14) from the experiment described in the legend to Fig. 2 were separated by SDS-PAGE using 12.5% polyacrylamide gels. The figures to the left indicate the apparent molecular weight of the major bands seen in fraction 4.

[^{3}H]bafilomycin loaded onto a glycerol density gradient in the absence of protein but in the presence of 1.5% $C_{12}E_9$ did not migrate into the gradient (result not shown). As can be seen in Fig. 3, fractions in the glycerol gradient that held the peak of [^{3}H]bafilomycin radioactivity appeared relatively enriched in polypeptides of apparent molecular masses of 39 kDa and 16 kDa which were also found in fractions containing the intact H^+-ATPase. As we have previously suggested [4], these fractions might contain V_B, the transmembranous proton channel subcomplex, of the osteoclast proton pump. The distribution of V-ATPase polypeptides in the gradient was therefore investigated more closely by Western analysis using antibodies against subunits of the V-ATPase. Antibodies against V_C, the catalytic subcomplex, of the ATPase (72, 56 and 42 kDa) reacted only in fractions containing the peak V-ATPase (Fig. 4A–C). In contrast, an antibody against a subunit of V_B, the 39 kDa subunit, reacted both with the peak of V-ATPase activity and more strongly in fractions containing the peak of [^{3}H]bafilomycin radioactivity (Fig. 4D). To test whether the fractions corresponding to the peak [^{3}H]bafilomycin binding contained a functional proton channel we used the method recently described for reconstitution of the transmembranous sector of the clathrin-coated vesicle H^+-ATPase [8]. Fractions from the control gradient and the [^{3}H]bafilomycin gradient corresponding to the peak of [^{3}H]bafilomycin radioactivity were reconstituted into potassium-loaded vesicles, diluted into potassium-free medium and the acidification of the vesicles in response to a valinomycin-induced membrane potential (inside negative) was measured by acridine orange fluorescence quenching. As shown in Fig. 5, the fraction from the control gradient (fraction 9, Fig. 2) catalyzed valinomycin-dependent proton transport that was not seen in the absence of reconstituted protein, indicating the presence of a functional proton channel. Bafilomycin A_1 (5 nM) inhibited the proton transport (Fig. 5), suggesting that it derives from a vacuolar ATPase and most probably represents the free V_B subcomplex of the osteoclast V-ATPase. In contrast to the fraction from the control gradient, the corresponding fraction from the [^{3}H]bafilomycin gradient showed only a small valinomycin-induced acidifi-

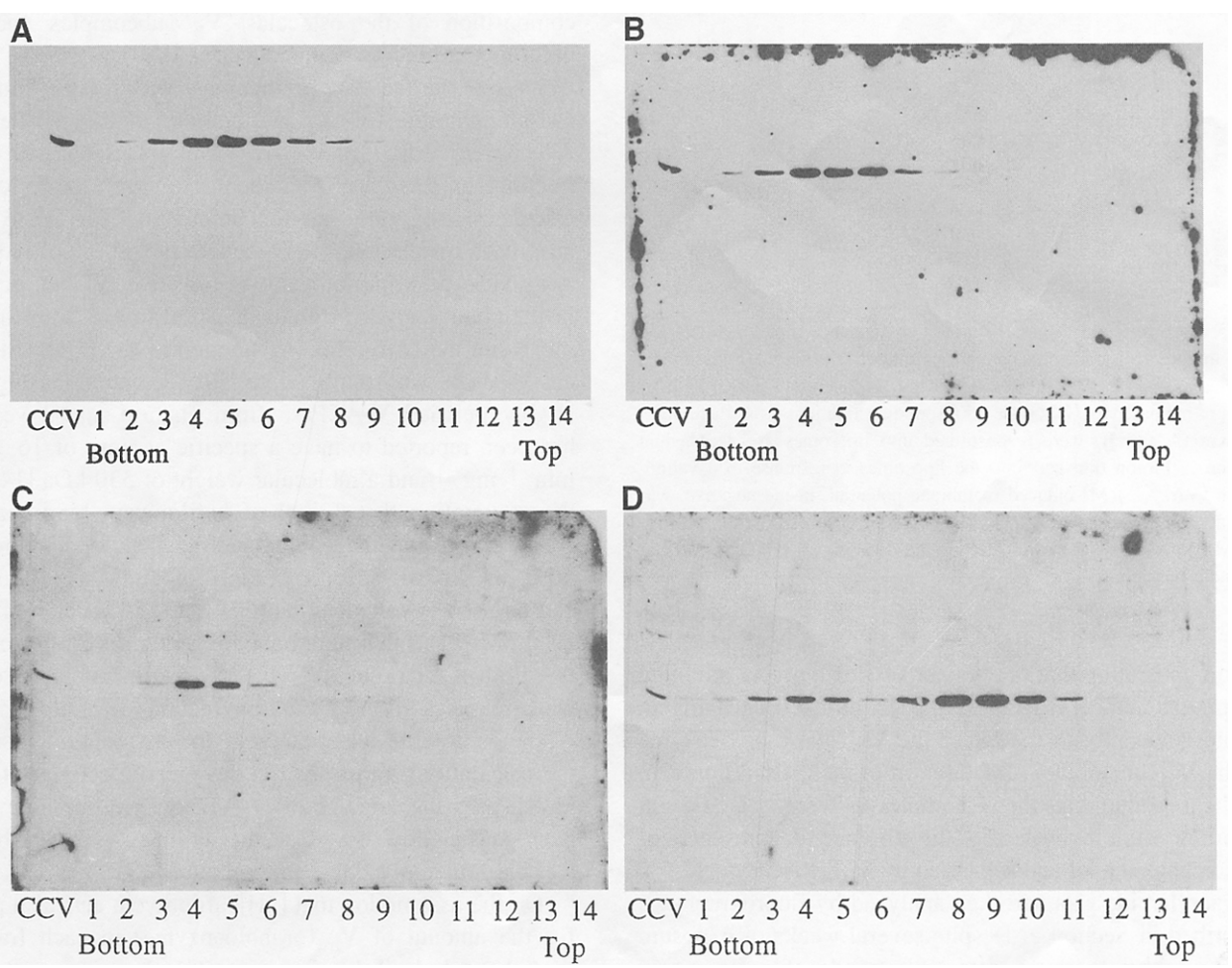

Fig. 4. Western blot of fractions from glycerol density gradient separation of solubilized osteoclast membrane vesicles using antibodies against V-ATPase subunits. Fractions (1–14) from the experiment described in the legend to Fig. 2 and the clathrin-coated vesicle H^+-ATPase (CCV) were separated by SDS-PAGE using 12.5% polyacrylamide gels. Proteins were transferred electrophoretically to nitrocellulose and Western blot analysis was performed as described in Section 2 using antibodies against the 70 kDa (A), 56 kDa (B), 40 kDa (C) and 39 kDa (D) subunits of the V-ATPase.

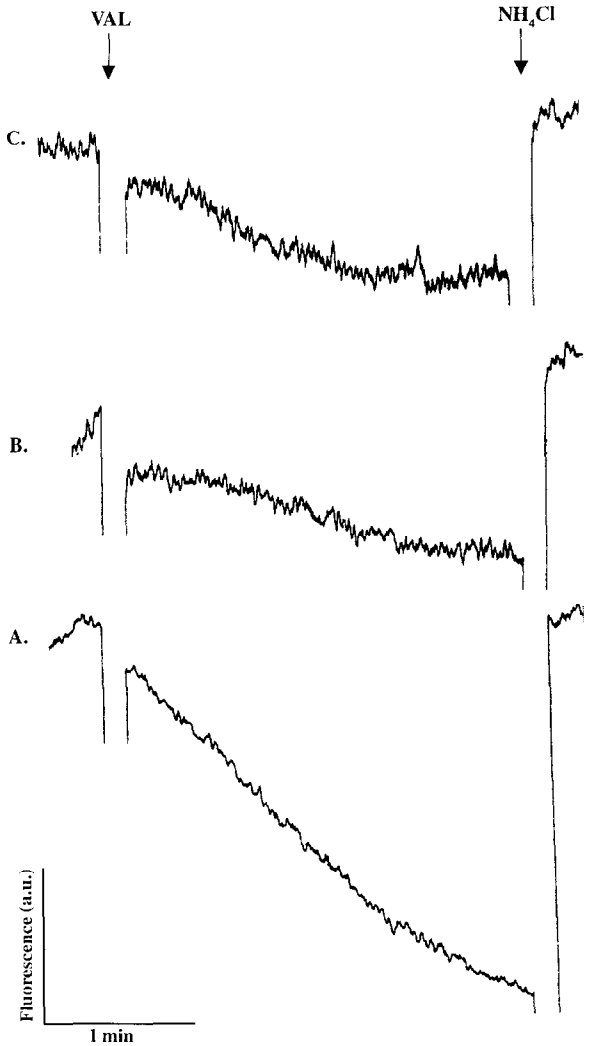

Fig. 5. Reconstitution of passive proton conductance. Fractions obtained from the glycerol gradient separation of solubilized osteoclast membrane vesicles as described in the legend to Fig. 2 containing peak [^{3}H]bafilomycin radioactivity (C) or the corresponding fractions from the control gradient (A and B) were reconstituted into liposomes as described in Section 2. Proton transport into the liposomes in response to a valinomycin (VAL, 1 μM) induced membrane potential, inside negative, was measured in the absence (A and C) or presence (B) or of bafilomycin A_1 (5 nM). Reactions were terminated by the addition of NH_4Cl (6 mM) at the indicated points.

cation, indicating that the bound [^{3}H]bafilomycin inhibited the proton channel (Fig. 5C). In order to test if bafilomycin bound covalently to the either the V-ATPase holoenzyme or the V_B subcomplex, fractions from the [^{3}H]bafilomycin gradient containing these complexes were mixed with Laemmli sample buffer in the absence or presence of β-mercaptoethanol and separated by SDS-PAGE on 12.5% polyacrylamide gels and then analyzed by fluorography as described in Section 2. Despite several weeks of exposure to film, there were no detectable bands of radioactivity either in the separated holoenzyme or the proton channel, indicating that [^{3}H]bafilomycin does not bind covalently to peptides of molecular mass greater than 14 kDa. Taken together, the results provide strong evidence that bafilomycin inhibits the osteoclast H^+-ATPase by binding tightly but non-covalently to V_B, the proton channel subcomplex, of the osteoclast V-ATPase. However, the exact subunit(s) to which bafilomycin binds remains to be elucidated. On the basis of competition studies using V-ATPase isolated from bovine brain clathrin coated vesicles it has been suggested that the 100 kDa subunit (which in some preparations is referred to as the 116 kDa subunit) is involved in bafilomycin A_1 binding [26], however direct binding to this subunit has yet to be shown. The presence or absence of this subunit in our preparations is presently unclear. As can be seen in Fig. 3, fractions holding the V_B subcomplex contained several Coomassie stained bands in the 100–120 kDa region. Furthermore, an antibody to the 116 kDa clathrin-coated vesicle H^+-ATPase reacted, albeit weakly, with two peptides in this region (data not shown). However, this antibody also reacted with a number of other peptides on Western blots so a definite conclusion about the presence of this peptide could not be drawn. Difficulties also exist detecting the presence of the 116 kDa subunit in the holoenzyme (see above and Ref. [4]). Further work is necessary to determine the exact subunit composition of the osteoclast V_B subcomplex and the bafilomycin-binding components.

Despite the fact that pretreatment with [^{3}H]bafilomycin caused inhibition of ATPase activity in the purified V-ATPase, no peak of radioactivity was associated with these fractions on the glycerol gradient. However, the following calculation suggests that the amount of V-ATPase may have been insufficient to give a detectable peak of radioactivity. The peak bafilomycin A_1-sensitive ATPase activity in the control glycerol gradient was 126 ± 8 nmol min^{-1} ml^{-1} and the corresponding fraction in the [^{3}H]bafilomycin gradient was inhibited to 50% (fraction 5, Fig. 2). Highly purified V-ATPase from clathrin-coated vesicles has been reported to have a specific activity of 16 μmol min^{-1} mg^{-1} and a molecular weight of 530 kDa [14]. On the assumption that 1 mol of bafilomycin binds per V-ATPase and that the counting efficiency for tritium was 50%, the known specific activity of the [^{3}H]bafilomycin can be used to calculate that 601 cpm/25 μl should have been present in this fraction. This amount of radioactivity is within a factor of two of the experimentally observed background radioactivity in this fraction (327 cpm/25 μl). Allowing for the uncertainty of the assumptions used in the calculations, most notably the specific activity of pure V-ATPase, the amount of V-ATPase present may have been sufficiently low that the failure to detect bound [^{3}H]bafilomycin was not unreasonable.

On the assumption that [^{3}H]bafilomycin acts as a probe for the amount of V_B (or holoenzyme) in each fraction then the data in Table 2 suggest that V_B is present in at least 10-fold molar excess over the holoenzyme. In order to quantify this difference independently, fractions containing the bafilomycin-sensitive ATPase activity and the peak

[^{3}H]bafilomycin, respectively, were pooled, blotted at serial dilutions to nitrocellulose and probed with the 39 kDa antibody. Densitometry revealed approx. 3-fold excess of the 39 kDa peptide in the pooled fractions containing the peak radioactivity over the holoenzyme (results not shown). Thus, the results indicate an excess of proton channel in the gradient of at least 3-fold and perhaps greater than 10-fold.

The presence of free V_B in the gradient might be due to the dissociation of the corresponding holoenzyme on the glycerol gradient. However, as shown in Fig. 4A–D, peptides comprising V_C (72, 56 and 42 kDa) were not found in fractions other than those containing the holoenzyme. To rule out the possibility that free V_C might co-migrate with the holoenzyme in the glycerol gradient, we dissociated the holoenzyme using cold inactivation of membrane vesicles [19], as described in Section 2. After removal of the membranes by centrifugation, the supernatant, containing V_C, was treated with $C_{12}E_9$ and run on a 15–30% glycerol gradient under the same conditions as in the experiment described in the legend to Fig. 2. Western analysis using antibodies against subunits of the V_C subcomplex (70 and 56 kDa) kDa indicated the presence of this peptide in low density fractions (fractions 10–12), indicating that free V_C, if present, should have been detectable on the original gradient.

In order to investigate further the stability of the proton pump on the gradient, fractions obtained from one glycerol gradient containing bafilomycin A_1-sensitive ATPase activity (holoenzyme) were pooled, diluted to decrease the glycerol concentration, concentrated in Centrikon-10 concentrators and then incubated in the presence or absence of a concentration of [^{3}H]bafilomycin giving 100% inhibition of reconstitutable proton transport. The control or the [^{3}H]bafilomycin-inhibited V-ATPase was then separated on a second 15–30% glycerol gradient. As shown in Fig. 6, V-ATPase activity could be recovered in high density fractions of the control gradient. After reconstitution into liposomes these fractions catalyzed ATP-dependent H^+-transport (result not shown). Two approximately equal peaks of radioactivity could be found in the gradient, one at high density corresponding to the fractions containing the H^+-ATPase and one at lower density fractions corresponding to the proton channel (Fig. 6). Thus, although there was evidence for some dissociation of the V-ATPase on the gradient, this was insufficient to explain the large excess of the V_B subcomplex seen after glycerol gradient centrifugation of solubilized membrane vesicles in the experiment shown in Fig. 2.

Another possibly explanation for the excess of the proton channel could have been that the V_C subcomplex dissociated from the holoenzyme during the preparation of the osteoclast membrane vesicles. The presence of free V_C in other sub-cellular fractions was investigated by Western analysis using the antibody against the 56 kDa subunit (Fig. 7). The final supernatant (S3) in the preparation

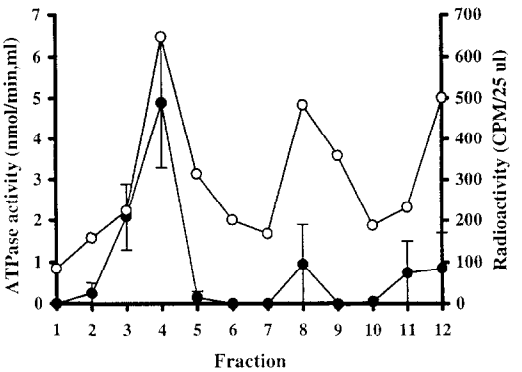

Fig. 6. Glycerol density gradient separation of partially purified osteoclast V-ATPase. Osteoclast membrane vesicles were solubilized and separated by glycerol gradient centrifugation as described in the legend to Fig. 2. Fractions containing peak bafilomycin A_1-sensitive ATPase activity were pooled, diluted in glycerol free buffer, concentrated in Centrikon-10 concentrators and incubated in the presence or absence of [^{3}H]bafilomycin followed by centrifugation on 15–30% glycerol gradients. Fractions were collected from the bottom (fraction 1 ≈ 30% glycerol) to the top (fraction 12) and bafilomycin A_1-sensitive ATPase activity in the control gradient (filled circles) and radioactivity (closed circles) in fractions from the gradient containing [^{3}H]bafilomycin pre-treated proteins were measured as described under Section 2. The ATPase activity is the mean ± range of two determinations.

where one might expect to find the free hydrophilic V_C subcomplex if it had been dissociated, did not show any cross-reaction with the 56 kDa antibody. Neither was there evidence for any proteolytic fragments of this subunit. The second pellet (P2) contained the 56 kDa peptide but this fraction also contained ATP-dependent proton transport activity, indicating that the holoenzyme was present in this fraction (result not shown).

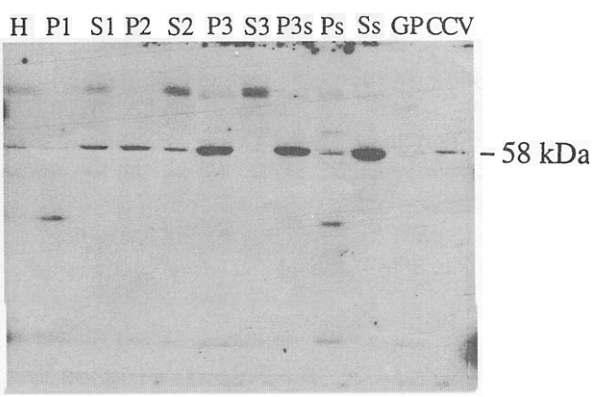

Fig. 7. Western blot analysis using a 56 kDa antibody of subcellular fractions obtained by differential centrifugation of disrupted osteoclasts. Fractions (7 μg protein) obtained during the preparation and solubilization of osteoclast membrane vesicles as detailed under Section 2 were separated by SDS-PAGE using 12.5% polyacrylamide gels. Proteins were transferred to nitrocellulose and probed with a 56 kDa subunit antibody. H, osteoclast homogenate; P1, 1000× *g* pellet; S1, 1000× *g* supernatant; P2, 6000× *g* pellet; S2, 6000× *g* supernatant; P3, 40000× *g* pellet; S3, 40000× *g* supernatant; P3s, P3 used for solubilization; Ps, 90000× *g* pellet after solubilization; Ss, 90000× *g* supernatant after solubilization; GP, small pellet after glycerol density gradient separation of Ss; CCV, partially purified clathrin-coated vesicle H^+-ATPase (0.2 μg protein).

Taken together, the results in this report suggest that the osteoclasts used for the H^+-ATPase preparation contained an excess of the transmembranous proton channel subcomplex, V_B. An excess of V_B over the holoenzyme has been found after solubilization and glycerol gradient separation of V-ATPase containing organelles from other sources [8,27,28], but if this was due to an excess of the proton channel in the original tissue or to dissociation of the holoenzyme during isolation was not investigated. The physiological relevance of the presence of free V_B in the osteoclast is not clear. However, the assembly and disassembly of the holoenzyme might play a regulatory role. In vitro, V-ATPases can be inactivated by treatment with chaotropic agents which induce dissociation of the holoenzyme [19,20]. The dissociated free V_C is unable to hydrolyze ATP and the free membrane bound V_B does not conduct protons, if not activated as in the present study [20,27]. Re-association of the subcomplexes restores ATP-driven proton transport [20]. It was recently suggested that dissociation of the catalytic complex of the V-ATPase of *Manduca sexta* larval midgut might play a role in the regulation of acid transport activity during moulting [29]. The source of the osteoclasts in the present study was egg-laying hens. In the 24 h egg-laying cycle of the hen, the osteoclasts remain attached to the medullary bone during the complete cycle but their activity is regulated by a cyclic appearance and disappearance of the ruffled membrane [30]. It is possible that during inactivation of the osteoclasts in the egg-laying cycle, the V_C subcomplex is dissociated from the holoenzyme and subsequently broken down whereas the V_B subcomplex remains in membrane structures in the cell.

An alternative possibility is that the proton channel might play a distinct role in the osteoclast separate from its function in the proton pump. There is evidence that V-ATPase subunits have other functions in the cell [31]. Ductin, a component of gap junctions, has been reported to be the same protein as the 16 kDa proteolipid of the V-ATPase proton channel [32]. Further studies on the relative distributions of the sub-complexes of the V-ATPase within the osteoclast as a function of the resorption cycle are needed to extend our understanding of the role and regulation of the acid pump in the process of bone resorption.

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