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PURIFICATION OF VACUOLAR ATPase WITH BAFILOMYCIN C_1 AFFINITY CHROMATOGRAPHY

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SUMMARY: We have developed a fast and simple affinity purification method for vacuolar ATPases (V-ATPases) using bafilomycin C_1 -coupled cellulose column. The purified protein from solubilized chicken medullary bone microsomal vesicles contains 8 subunits at sizes of 72, 65, 55, 41, 34, 20, 17 and 15 kDa. This subunit structure is typical to V-ATPases. Although the enzyme obtained is inactive, this method is useful in search of various isoforms of known V-ATPase subunits. We also present further evidence that the binding site of bafilomycin is located in the 17 kDa proteolipid subunit of V-ATPase.

Vacuolar ATPases consist a group of proton translocating ATPases, which have been purified or characterised from several different sources including vacuoles of yeast, vacuoles of plants, cromaffin granules, clathrin coated vesicles, kidney microsomes, lysosomes, endosomes and Golgi apparatus (1-10).

All characterized enzymes have quite a similar subunit structure containing about 70, 60 and 17 kDa polypeptides and two to six other subunits at the approximate sizes of 115, 41, 39, 34, 33 and 20 kDa. The 115 and 39 kDa subunits are found only from mammalian cells and they are not necessary for the function of the ATPase although they may have some regulatory roles. The subunits are divided into two sectors: hydrophobic membrane sector and hydrophilic catalytic sector. It has been reported that these two sectors can be separated apart by cold treatment thus inactivating the enzyme (11). The vacuolar ATPases are

The abbreviations used are: DCCD, dicyclohexylcarbodi-imide; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride.

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electrogenic proton pumps, which require charge neutralisation for continuous proton transport. Usually this is achieved via chloride ion flux through membrane Cl-channels (12). The vacuolar ATPases are insensitive to vanadate and ouabain, inhibitors of E_1E_2 ATPases and to azide and oligomycin, inhibitors of F_0F_1 ATPases. However, they can be inhibited by N-ethylmaleimide, N,N'-dicyclohexylcarbodi-imide (DCCD) and most of all by bafilomycin (13), which is so far the only known specific and potent inhibitor of these enzymes.

There are several members in bafilomycin family, but bafilomycin A_1 and C_1 are the most potent forms known at the moment. In this work we describe a fast and simple method for coupling bafilomycin C_1 to cellulose matrix and for using it in affinity chromatography purification of vacuolar ATPases.

MATERIALS & METHODS

Reagents - Bafilomycin C_1 was obtained from Astra, Sweden and it was stored as 15 mM stock solution in dimethylsulfoxide. N-octyl β-D-glucopyranoside was purchased from Sigma.

Preparing the microsomal vesicle fraction from chicken medullary bone - For the source of vacuolar H+-ATPase we used osteoclast enriched medullary bone (14, 15). The medullary bone was removed from tibia and femur of regularly laying hens and it was homogenised with Ultra-Turrax homogenizator in homogenizing buffer containing 20 mM Tris-HCl pH 7.4, 250 mM sucrose, 1 mM PMSF, 1 mM EGTA and 1 mM DTT. The homogenate was centrifuged at 1000 x g for 15 minutes and the pellet was rehomogenized and recentrifuged. Combined supernatants were centrifuged at 10 000 x g for 15 minutes and the resulting supernatant finally at 100 000 x g for 50 minutes. The final pellet was suspended in the homogenizing buffer with glass-Teflon homogenizator and frozen with liquid nitrogen for later use. The pellet from second centrifugation (10 000 x g) was also collected and it contained most of the lysosomal vesicles (16).

Solubilizing the protein samples - All samples were solubilized with 50 mM n-octyl β-D-glucopyranoside at +4°C during 30 minutes with light rotation . The protein concentration was about 10 mg / ml. The samples were then ultracentrifuged at 100 000 x g for 50 minutes and the pellet was discarded.

Proton transport experiment - Proton transport by isolated membrane vesicles was measured as described in the paper of Sundquist et al. 1990 (17).

ATPase experiment - The assay conditions for the Mg^+-ATPase activity are described by Sallman et al. 1986 (18).

Coupling bafilomycin C_1 to cellulose matrix - Bafilomycin C_1 was diluted to 10 ml of dimethylsulfoxide so that the final concentration was 3 mM. All incubations were done at room temperature with light mixing. Bafilomycin was incubated with 3 mM succinic acid anhydride during five minutes. 3 mM dicyclohexylcarbodi-imide and 3 mM hydroxybenzotriazol was added to solution and after two minutes 1.0 g cellulose powder was added and the suspension was incubated for one hour. Succinic acid anhydride binds to bafilomycin's hydroxide group creating an arm from which the complex binds to free hydroxide group in the cellulose, thus creating covalent binding between these two molecules. Half of the resulting matrix, volume 2 ml, was packed to a column and was washed with 200 ml of buffer A (20 mM Tris-HCl pH 7.4, 1 mM N-octyl β-D-glucopyranoside).

Affinity chromatography - The bafilomycin bound protein was eluated with 1 M KCl buffer containing 1 mM N-octyl β-D-glucopyranoside and 2 mM HCl. Usually it was enough to collect three 2 ml fractions. Eluated samples were dialysed overnight against buffer A and concentrated with Speed Vac - concentrator before SDS-PAGE.

RESULTS & DISCUSSION

Both the solid and solution state structures of bafilomycin A_1 are known (19, 20, 21) and these studies indicate that the hydroxyl groups at C19 and C17 are involved in a tight hydrogen bonding network with the carbonyl group at C1 (shown as broken bond lines in the X-ray structure in Figure 1). As the only remaining free hydroxyl group of bafilomycin C_1 is the one at C7, we suggest that this is the site for attachment of the bafilomycin core to linker and cellulose matrix. The tight hydrogen bonding network notwithstanding, the hydroxyl group at C7 is also sterically the least encumbered one, thus giving further evidence for its reactivity.

The column binds less than 0.1% of the total protein from the solubilized chicken medullary bone derived microsomal vesicles. The protein yield decreases to 30 % from original if the microsomal vesicle fraction is saturated with 10 μM bafilomycin C_1 before loading to column. Also saturation with 1 mM DCCD decreases the yield indicating that the binding site of bafilomycin is near the binding site of DCCD. This explains why we did not get any peak of radioactivity when we labelled our sample with [^{14}C] DCCD. The purified protein was characterized with SDS-PAGE and the results are shown in figure 2. From chicken medullary bone microsomal vesicles we obtained with bafilomycin affinity purification 8 subunits at the sizes of 72, 65, 55, 41, 34, 20, 17 and 15 kDa (figure 2, lane A), when eluated with 1 M KCl, 2 mM HCl, 1 mM N-octyl β-D-glucopyranoside.

The subunit structure from lysosome enriched fraction (figure 2, lane B) is similar to the one from microsomal fraction. However, the 55 kDa subunit is missing and there is a double band at the size of 33-34 kDa. There is also a 44 kDa and 22 kDa polypeptides present in the purified protein from lysosome enriched fraction. The differences between purified lysomal and microsomal fractions (figure 2, lanes B and A) show that there are different isoforms of some subunits present in membrane vesicles derived from medullary bone. This is in accordance with recent results of Chatterjee et al. (22). If no acid is used in eluation buffer, most of the subunits dissipate from the column (figure 2, lane C) but not all. After 1 M KCl eluation we eluated the same column with 1 M KCl containing 2 mM HCl and we obtained only two subunits: 17 and 41 kDa (figure 2. lane D). This indicates that the bafilomycin binds either to the 17 kDa or 41 kDa subunit. Most of the 41 kDa subunit is obtained already in KCl eluation but some of it only with

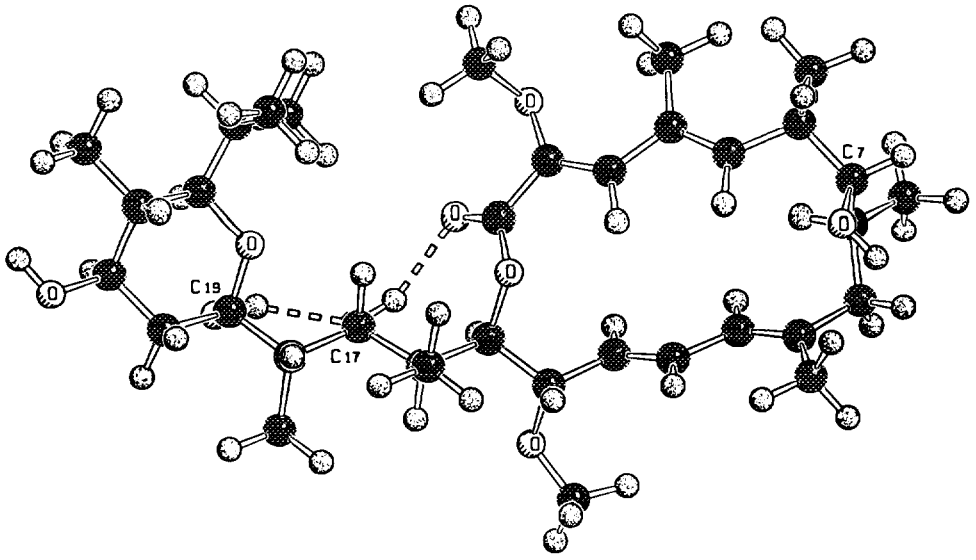

Figure 1. **The X-ray structure of bafilomycin A_1.**

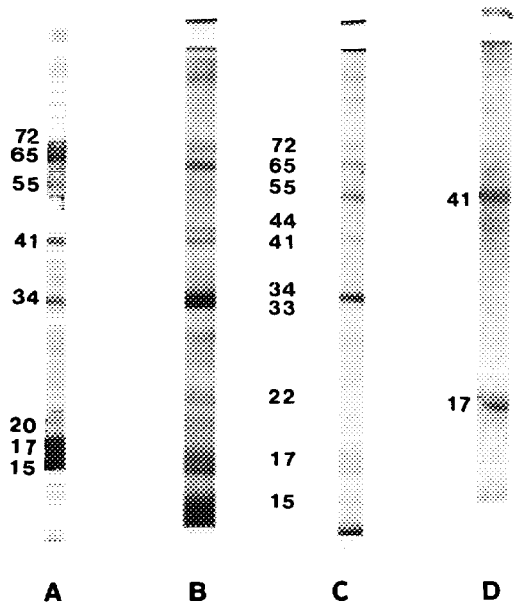

Figure 2. **Subunit structure of affinity purified protein.** Lanes A, C and D represent the purified protein from the chicken medullary bone derived microsomal vesicles and lane B the purified protein from lysosomal enriched vesicles. Lane C is the protein from eluation with 1 M KCl and lane D is the purified protein when eluated with 1 M KCl containing 2 mM HCl after 1 M KCl eluation. The polyacrylamide gel had 5% concentration gel and 15% separation gel and it was stained with Coomassie Brilliant Blue.

Table I. **Proton transport experiments.** Effluent contains vesicles which do not bind to the column and eluent contains column-bound vesicles which were eluated with 1 M KCl. Washing buffer contains 20 mM Tris-HCl, pH 7.4, and effluent buffer is the same buffer except that it was run through the column. The control column was prepared like the bafilomycin C_1 column, except that instead of bafilomycin we used ouabain.

	H^+-transport activity %
microsomal vesicles	100
effluent	2
eluent	0
microsomal vesicles + washing buffer	91
microsomal vesicles + effluent buffer	92
effluent of control column	99

combined acid-salt eluation, which suggests the existence of a strong binding between 41 kDa subunit and proteolipid. These results support the earlier suggestion of Hanada et al. (23) that the binding site for bafilomycin locates in the 17 kDa subunit.

All subunits in our purified protein complex correspond with the sizes of known subunits of vacuolar ATPases. The ATP hydrolysing 72 kDa subunit A, 34 kDa subunit D and 41 - 44 kDa subunit C are present in all purified preparations. The size of subunit B is in our samples 55 or 65 kDa. Also the 26 kDa subunit E and 20 kDa subunit a are present. The proteolipid, subunit c, has the size of 14 - 18 kDa depending from the source. In our preparation we obtained 15 and 17 kDa bands which may be different isoforms from the same enzyme or the proteolipids from two different vacuolar ATPases.

There is no ATPase activity in purified protein, which can be explained by the fact that the subunits are separated from each other during eluation.

Table I presents the results from the proton transport measurements of nonsolubilized microsomal vesicle fraction enriched with bafilomycin cellulose affinity chromatography. Loading of vesicles to the column was done in the same way as the purification of solubilized material except no N-octyl β-D-glucopyranoside was used in solutions. Although the bafilomycin C_1 inhibits only about 1 % of total ATPase activity in this fraction, it inhibits all proton transport at 20 nM concentration. The column binds almost totally the proton transport activity, but there is no activity left in eluent. The loss of activity is not due to the bafilomycin leak because the effluent buffer coming from column does not inhibit the proton transport more than buffer itself. The loss of activity is not the consequence of vesicles binding to the DCCD which is used in coupling reaction, because the control column, containing ouabain instead of bafilomycin, does not bind the protein responsible for proton transport activity in these vesicles.

On the basis of SDS-gels in purified protein complex the amount of membrane sector subunits is much higher than the amount of catalytic sector subunits indicating that the main part of the enzyme is dissociated or dissociates during the purification and that the bafilomycin C_1 binds to the membrane sector. In proton transport measurement we can only detect the accumulation of acridine orange to inside-outside vesicles in which the catalytic sector of the vacuolar ATPase is located outside. Because we can bind these vesicles to the column, the binding site for bafilomycin C_1 must be in the catalytic side of the membrane sector and probably in 17 kDa proteolipid thus supporting the results obtained by solubilized protein (see above).

Vacuolar proton ATPases have been isolated from many sources with various methods which are specific for each source material and they can not be used with other vacuolar ATPases. The bafilomycin affinity purification can be used to purify enzymes from any material as long as one keeps in mind that several different vacuolar ATPases may be purified at the same time. This method also allows purification from considerably smaller amount of starting material than other published procedures. Although the resulting yield is in inactive form, it can be used in search of different isoforms of known subunits.

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