Concanamycin A, the Specific Inhibitor of V-ATPases, Binds to the V₀ Subunit c*

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Vacular-type ATPase (V-ATPase) purified from the midgut of the tobacco hornworm Manduca sexta is inhibited 50% by 10 nM of the plecomacrolide concanamycin A, the specific inhibitor of V-ATPases. To determine the binding site(s) of that antibiotic in the enzyme complex, labeling with the semisynthetic 9-O-[p-(trifluoroethyl)diazirinyl]-benzoyl]-21,23-dideoxy-23-[¹²⁵I]iodoconcanamide A (J-concanamide A) was performed, which still inhibits the V-ATPase 50% at a concentration of 15–20 μM. Upon treatment with UV light, a highly reactive carbene is generated from this concanamycin derivative, resulting in the formation of a covalent bond to the enzyme. In addition, the radioactive tracer¹²⁵I makes the detection of the labeled subunit(s) feasible. Treatment of the V₁/V₀ holoenzyme, the V₀ complex, and the V-ATPase containing goblet cell apical membranes with concanamide resulted in the labeling of only the proteolipid, subunit c, of the proton translocating V₀ complex. Binding of J-concanamide A to subunit c was prevented in a concentration-dependent manner by concanamycin A, indicating that labeling was specific. Binding was also prevented by the plecomacrolides bafilomycin A₁ and B₁, respectively, but not by the benzolactone enamide salicylihalamide, a member of a novel class of V-ATPase inhibitors.

Vacular-type ATPases (V-ATPases)³ are complex, heteromultimeric proteins consisting of a peripheral, catalytic V₁ complex and a membrane bound, ion translocating V₀ complex.

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** The abbreviations used are: V-ATPase, vacuolar-type ATPase; J-concanamide A, 9-O-[p-(trifluoroethyl)diazirinyl]-benzoyl]-21,23-dideoxy-23-[¹²⁵I]iodoconcanamide A; DCCD, bicyclohexylcarbodiimide; C₉-E₁₀ polyoxyethylene 10-lauryl ether, MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry.
id A (J-concanolide A), binds only to subunit c of the V-ATPase from tobacco hornworm (M. sexta) midgut.

EXPERIMENTAL PROCEDURES

Purification of the V1/Vo ATPase and the Vc Complex—As many as 20 whole M. sexta midguts were dissected from feeding fifth instar larvae and homogenized in ice-cold buffer A (5 mM Tris-HCl, 250 mM sucrose, 5 mM Pefabloc SC (Biomol), 5 mM Na-EDTA, pH 8.1). After centrifugation of the crude homogenate in a fixed-angle rotor at 12,000 × gmax for 5 min at 4 °C, the pellet was resuspended in buffer A and centrifuged again. The resulting pellet was resuspended in buffer A and centrifuged in a fixed-angle rotor at 233,000 × gmax for 30 min at 4 °C to yield a final pellet that was solubilized in buffer B (16 mM Tris-HCl, 0.32 mM EDTA, 9.6 mM 2-mercaptoethanol and 0.1% C12E10, pH 8.1) at 4 °C with a detergent to protein ratio of 2:1. After centrifugation in a fixed-angle rotor at 233,000 × gmax for 1 h at 4 °C, the supernatant was layered on a discontinuous sucrose density gradient (3 ml of 40%, 2 ml of 30%, 2 ml of 20%, and 1.6 ml of 10% sucrose (w/v) dissolved in buffer B with 200 mM KCl and a lower detergent concentration of 0.01% C12E10) and centrifuged in a vertical rotor at 339,000 × gmax for 90 min at 4 °C. The 30% sucrose fraction was diluted 4-fold with buffer C (20 mM Tris-HCl, 9.6 mM 2-mercaptoethanol, 0.01% C12E10, pH 8.1) containing 50 mM NaCl and subjected to anion exchange chromatography using a Mono Q (Amersham Biosciences) column. The V1/Vo ATPase was eluted in a linear salt gradient (50–400 mM NaCl dissolved in buffer C) at NaCl concentrations between 250 and 280 mM. The last step in the purification protocol was gel permeation chromatography on a Superdex 200 column (Amersham Biosciences), which was performed in buffer C containing 150 mM NaCl. The final yield was ~1 mg of V1/Vo ATPase per 20 midguts.

For purification of the Vc complex, midguts of larvae starved for 16–20 h were prepared, homogenized, and centrifuged twice as described above. The second pellet was resuspended in a buffer consisting of 5 mM Tris-HCl, 0.8 M KI, 5 mM Na-EDTA, and 5 mM Pefabloc SC (pH 8.1) and incubated for 30 min on ice. Then the sample was diluted with 16 mM Tris-HCl, 0.32 mM EDTA, 9.6 mM 2-mercaptoethanol (pH 8.1) to a KI concentration of 40 mM and centrifuged at 233,000 × gmax for 30 min at 4 °C. The pellet was resuspended in the dilution buffer and centrifuged again under the same conditions. The final pellet was solubilized and purified as described above for the V1/Vo ATPase, except for the use of 20% instead of the 30% sucrose fraction after rate zonal centrifugation. The final yield of the preparation was ~0.5 mg of Vc complex per 20 midguts.

Antibodies—J-concanolide A as well as its 125I-labeled form were synthesized as described elsewhere (11). Concanamycin A, bafilomycin A1, and bafilomycin B1 were isolated according to published procedures (12). Salicylihalamide was a generous gift from M. R. Boyd (National Cancer Institute, MD, USA). To avoid freeze thaw cycles, which have a significant influence on the stability of the substances, aliquots of stock solutions were stored in dimethyl sulfoxide and thawed only once immediately before use. The actual concentrations of the stock solutions were determined spectrophotometrically.

ATPase Assays—ATPase assays with a final volume of 160 μl and a pH of 8.1 contained 3–4 μg of protein, 50 mM Tris-Mops, 3 mM 2-mercaptoethanol, 1 mM MgCl2, 0.1 mM sodium orthovanadate, 0.05 mM sodium azide, 20 mM KCl, 0.003% C12E10, 20 mM NaCl, and 3 mM Tris-HCl. After 5 min of preincubation at 30 °C with or without inhibitors, 1 mM Tris-ATP was added, and after an additional 2 min the reaction was stopped by placing the reaction tubes in liquid nitrogen. Determination of the produced inorganic phosphate followed the protocol of Wieczorek et al. (13).

Labeling—20 μg of the samples were incubated with varying concentrations of J-concanolide A in a volume of 30–40 μl for 1 h on ice or 3 min at room temperature and irradiated for 3 min with UV light (366 nm) on ice. After irradiation, 7.5–10 μl of 5-fold sample buffer (14) was added. The mixture was heated for 45 s at 95 °C or 30 min at 40 °C, cooled on ice, and subjected to SDS-PAGE (10–15% T, 3.3% C; (15)) or to Tricine-SDS-PAGE (16.5% T, 3% C separating gel and 10% T, 3% C spacer gel (16)), followed by Coomassie staining. The gels were either stained after Western blotting (lanes 6–8) or 5 μg of protein (lanes 1–4) were loaded on each lane. Lane 1, standard proteins with molecular masses as indicated. Lane 2, purified cytosolic V1 complex. Lane 3, purified V1/Vo holoezyme. Lanes 4–8, purified Vc complex. Lane 5, [14C]DCDC labeling of the proteolipid, subunit c (34). Lane 6, monoclonal antibodies to subunit e (26). Lane 7, polyclonal antibodies to subunit d (35). Lane 8, polyclonal antibodies against the bovine chromaffin granule 116-kDa subunit (29).

RESULTS AND DISCUSSION

Revised Purification Protocol Reveals Subunit a in the Insect V-ATPase—The unequivocal assignment of an inhibitor such as concanamycin or bafilomycin to a special V-ATPase subunit requires experimental evidence for the existence of all constitutive subunits in the V-ATPase preparation used. Although nearly all subunits of the insect V-ATPase have been cloned and sequenced (22, 23), we had so far not been able to identify the 100-kDa subunit a of the insect V-ATPase unequivocally (24–26). Because this subunit on the one hand appeared to be very sensitive to proteolysis (27), and on the other hand was proposed to be a candidate for bafilomycin binding (7), we repeatedly modified purification protocols to recover a putative subunit a, also in the insect V-ATPase. Degradation during the process of purification was evidently not responsible for our lack of evidence because even with complex cocktails of protease inhibitors we were not able to resolve subunit a (28).

Purification of the M. sexta V-ATPase according to a new protocol as compared with previous procedures (Ref. 24, see under “Experimental Procedures”) resulted, mainly because of the presence of 200 mM KCl during zonal centrifugation of the detergent-solubilized protein in a sucrose gradient, for the first time in the detection of a 100-kDa subunit in an insect V-ATPase preparation (Fig. 1, lane 3). The 100-kDa band reappeared in immunoblots with polyclonal antibodies to the 116-kDa subunit a of the V-ATPase from bovine chromaffin granules (Ref. 29, not shown, but see below the results for the Vc complex), suggesting the existence of a 100-kDa subunit a, also in the M. sexta midgut V-ATPase. Respective evidence had already been provided recently by the demonstration of at least two genes encoding a V-ATPase subunit a in the midgut (23). Definitive proof that the 100-kDa band represented the V-ATPase subunit a was finally obtained from MALDI-MS. A number of tryptic peptides were found in the map of the protein.
Figure 2. Sequences of subunits a and c, indicating tryptic peptides found by MALDI-MS. A, subunit a (GenBank™ accession no. AJ249390). B, subunit c (GenBank™ accession no. X65051).

Tryptic (subunit a, subunit c) and chymotryptic (subunit c) peptides detected after enzymatic in-gel digestion of the 100-kDa band and the 17-kDa band, respectively, by MALDI-MS are marked in bold and underlined.
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**Fig. 5.** Photoaffinity labeling of the purified V-ATPase with J-concanolide A. Samples containing 20 μg each of V-ATPase were incubated for 1 h on ice with the indicated concentrations of J-concanolide A and separated afterward by Tricine-SDS-PAGE. A, 1st lane left, staining with Coomassie Blue; 2nd-9th lanes, autoradiography of the gel after exposition to a phosphoscreen. 2nd lane, without UV-illumination. Evidently during electrophoresis the hydrophobic J-concanolide A entered the gel together with the SDS of the running buffer. 3rd-9th lane, with UV-illumination. Mainly the J-concanolide A bound to protein entered the gel. Most of the unbound but photolyzed J-concanolide A, perhaps because of formation of cross-linked products with, for example, the non-ionic detergent C12E10 had no electrophoretic mobility and therefore remained in the stacking gel (data not shown). B, autoradiography of the gel after exposition to a phosphoscreen. Samples were preincubated for 1 h on ice without concanamycin A (control) or with the indicated concentrations of concanamycin A. J-concanolide A was then added to give a final concentration of 10 μM. The mixture was incubated for 1 h on ice and treated afterward with UV light. C, autoradiography of the gel after exposition to a phosphoscreen. Samples were preincubated for 1 h on ice with the indicated concentrations of bafilomycin A1 (Baf A1), bafilomycin B1 (Baf B1), or salicylhalamide (Salicyl), respectively. J-concanolide A was then added to give a final concentration of 10 μM. The mixture was incubated for 1 h on ice and treated afterward with UV light. Control with preincubation, but without effectors.

**Fig. 6.** Correlation between inhibition of V-ATPase activity by J-concanolide A and bound radioactivity. Bands of subunit c from the gel in Fig. 5A were excised. The bound radioactivities were measured in a γ-counter and then compared with the enzyme activities. The background radioactivity was subtracted by using an equivalent-sized gel slice right to the lane with the lowest concentration of J-concanolide A (0.1 μM). The radioactivity of the 100 μM band was set to 100%.

**Fig. 7.** Plecomacrolide antibiotics bind to the proteolipid, subunit c of the purified V1/Vo-ATPase, the purified V1 complex, and the membrane bound V1/V0-ATPase. SDS-PAGE gel (10–15% T, 3.3% C) stained with Coomassie Blue (lanes 1, 3, and 5). Autoradiographies of gels after exposition to a phosphoscreen (lanes 2, 4, and 6). Lanes 1–2, 20 μg of purified V1/V0 holoenzyme. Lanes 3–4, 20 μg of purified V1 complex. Lanes 5–6, 20 μg of highly purified goblet cell apical membranes. All samples contained 0.1 μM J-concanolide A; after incubation for 3 min at room temperature they were irradiated with UV light.
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explain the discrepancy between this result and the results obtained with N. crassa mutants, which exhibited a higher tolerance to baflomycin but not to concanamycin (10). Because labeling was not impaired by salicylihalamide, we conclude that the site(s) and mechanism of inhibition for benzolactone enamides may be different from that for plecomacrolidides.

In another approach we used, in addition to the purified holoenzyme, the purified V\textsubscript{o} complex and highly purified goblet cell apical membranes in which the V-ATPase is the predominant protein and, moreover, resides in its native lipid surrounding (Fig. 7). Clear labeling of subunit c was again obtained for the V\textsubscript{o} complex as well as for the goblet cell apical membrane.

Taken together, we provided for the first time direct proof that subunit c, which forms the major part of the proton translocating V\textsubscript{o} complex of V-ATPases, carries the binding site for plecomacrolidides. Experiments more precisely defining the site of covalent modification are in progress.

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