SHORT COMMUNICATION

DICYCLOHEXYLCARBODIIMIDE REACTS SPECIFICALLY WITH THE 16kDa SUBUNIT OF LARVAL MANDUCA SEXTA MIDGUT VACUOLAR-TYPE ATPase

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Proton-motive vacuolar-class ATPases (V-ATPases) are commonly associated with membrane-bound organelles other than mitochondria and chloroplasts. They are found in the vacuoles of Neurospora crassa and yeasts, in tonoplasts of plants and in endomembranes such as lysosomes, endosomes, clathrin-coated vesicles and secretory granules (Sze, 1985; Rudnick, 1986; Mellman et al. 1986; Rodman et al. 1991). However, they have also been found in plasma membranes of kidney tubules (Brown et al. 1987; Wang and Gluck, 1990). Recently, the pump responsible for the large lumen-directed net flux of potassium across larval Manduca sexta midgut has been shown to consist of a plasma membrane proton-motive V-ATPase functioning in conjunction with an $\text{nH}^+/\text{K}^+$ antiporter (Wieczorek et al. 1991).

Purification of V-ATPases from native membranes has shown that these enzymes consist of several polypeptides, ranging in molecular mass from approximately 116 kDa to 15 kDa (Forgac, 1989). The holoenzymes range from 300 kDa to 600 kDa and thus, like F-ATPases, V-ATPases are multipolypeptide complexes. There is considerable diversity in the subunit composition of V-ATPases. However, there is a general consensus that three polypeptides, of approximately 70 kDa, 60 kDa and 16 kDa, are always present (Stone et al. 1989). The V-ATPase of M. sexta midgut contains major subunits of approximately 67 kDa, 56 kDa, 43 kDa, 28 kDa and 16 kDa (Schweikl et al. 1989).

V-ATPases are distinguished from P-ATPases and F-ATPases by their sensitivity to various inhibitors. V-ATPases are resistant to the P-ATPase inhibitor vanadate and to classic F-ATPase inhibitors such as oligomycin and azide. In contrast, the V-ATPases are highly sensitive to bafilomycin (Bowman et al. 1988; Wieczorek et al. 1991) and inhibited by sulfhydryl-alkylating agents such as N-ethylmaleimide and 7-chloro-4-nitro-benz-2-oxa-1,3-diazole (Forgac, 1989). In general, V-ATPases are inhibited by $N,N'$-dicyclohexylcarbodiimide (DCCD) concentrations approximately an order of magnitude higher than those required to inhibit F-ATPases but an order of magnitude lower than those required to inhibit P-ATPases (Forgac, 1989).

Key words: DCCD, Manduca sexta, midgut, V-ATPase.
The functions of different V-ATPase subunits are not yet completely known. Studies performed with \[^{14}\text{C}]\text{DCCD}\) reveal that the 17kDa subunit of several V-ATPases is predominantly labeled (Sutton and Apps, 1981; Bowman, 1983; Manolson \textit{et al.} 1985; Uchida \textit{et al.} 1985; Mandala and Taiz, 1986; Arai \textit{et al.} 1987; Kaestner \textit{et al.} 1988; Jehmlich \textit{et al.} 1991). Based upon an analogy with mitochondrial F-ATPase, there has been speculation that this component is similar to \(F_{0}\), which is also labeled with \[^{14}\text{C}]\text{DCCD}\), and that it is part of the transmembrane proton-conducting sector of the mitochondrial enzyme.

The aim of the following investigation was to use DCCD to identify the putative proton translocation subunit and thereby tentatively to assign a function to one of the individual subunits of the V-ATPase in the apical membrane of the larval midgut goblet cells of the tobacco hornworm \textit{Manduca sexta}.

Unless noted otherwise, all chemicals were purchased from either Sigma (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). \textit{M. sexta} eggs and larval diet were purchased from Carolina Biological Supply (Burlington, NC). The larvae were raised to the fifth instar, 5.0±0.5 g, at 27°C with constant light. Midgut goblet cell apical membrane V-ATPase was prepared as described by Weiczorek \textit{et al.} (1990) from the fifth-instar larvae. Enzyme concentration was determined by the Amido Black method of Popov \textit{et al.} (1975) as modified and described by Weiczorek \textit{et al.} (1990).

ATPase activity was assayed by determining the amount of released inorganic phosphate (Wieczorek \textit{et al.} 1990). The incubation mixture for ATPase activity assays contained approximately 0.5 \(\mu\text{g}\) of purified ATPase protein, 1mmol\(\text{l}^{-1}\) MgCl\(_2\), 1mmol\(\text{l}^{-1}\) Tris-ATP, 20mmol\(\text{l}^{-1}\) KCl, 50mmol\(\text{l}^{-1}\) Tris-Mops, 0.1mmol\(\text{l}^{-1}\) EDTA, 3mmol\(\text{l}^{-1}\) 2-mercaptoethanol and 0.003 % C\(_{12}\)E\(_{10}\) (polyoxyethylene 10 lauryl ether). Incubation mixtures, prepared in triplicate, had a total volume of 0.16 ml and a pH of 8.1. The mixtures were pre-incubated in the absence of the substrate for 5–20 min at 30°C. Assays were started by adding ATP and were incubated for 5 min. Under these conditions, release of inorganic phosphate was a linear function of enzyme concentration and of incubation time. Reactions were stopped by placing the assay tubes in liquid nitrogen.

The reaction mixtures for measuring the inhibition of ATPase activity by DCCD were similar to those for measuring enzyme activity except for the addition of specific concentrations (0–1.5mmol\(\text{l}^{-1}\)) of DCCD. Concentrated DCCD solutions were prepared in dimethylformamide (DMF). Concentrations of DMF up to 2% had no effect on enzyme activity. All reaction mixtures to test the effects of DCCD contained less than 2% DMF.

The midgut ATPase was labeled with \[^{14}\text{C}]\text{DCCD}\) by incubating purified enzyme (15 \(\mu\text{g}\text{ml}^{-1}\)) with 10 \(\mu\text{mol}^{-1}\) or 100 \(\mu\text{mol}^{-1}\) \(N,N'\)-dicyclohexyl\[^{14}\text{C}\text{carbodiimide}\) (50–60 Ci mol\(^{-1}\); Amersham, Arlington Heights, IL) in 1mmol\(\text{l}^{-1}\) MgCl\(_2\), 20mmol\(\text{l}^{-1}\) KCl, 50mmol\(\text{l}^{-1}\) Tris–Mops (pH 8.1), 0.1mmol\(\text{l}^{-1}\) EDTA, 1mmol\(\text{l}^{-1}\) 2-mercaptoethanol and 0.003 % C\(_{12}\)E\(_{10}\) for 10 min at 30°C. Reactions were stopped by placing the assay tubes in liquid nitrogen.

Radioactive DCCD-treated ATPase was precipitated with trichloroacetic acid
Reaction of DCCD with midgut V-ATPase

Fig. 1. Activity of solubilized midgut V-ATPase versus \(N,N'-\text{dicyclohexylcarbodiimide}\) concentration. Assay conditions are described in the text. Mean values with s.e.m. error bars from three independent assays are shown.

A dose–response curve for the effect of DCCD on ATPase activity is shown in Fig. 1. When the DCCD concentration is 0.15 mmol l\(^{-1}\), enzyme activity is inhibited by 50%. At a DCCD concentration of 1.5 mmol l\(^{-1}\), enzyme activity is less than 20% of that in the absence of DCCD.

The 16 kDa subunit of \(M.\ sexta\) midgut V-ATPase is labeled heavily in the presence of 10 \(\mu\text{mol l}^{-1}\) [\(^{14}\text{C}\)]DCCD (approximately 37 \(\mu\text{Ci mg}^{-1}\) enzyme). When the labeling mixture contains 100 \(\mu\text{mol l}^{-1}\) [\(^{14}\text{C}\)]DCCD (approximately 367 \(\mu\text{Ci mg}^{-1}\) enzyme), labeling of the 16 kDa subunit is similar to that obtained with 10 \(\mu\text{mol l}^{-1}\) DCCD, but most other ATPase subunits are also labeled (Fig. 2).

In this study, solubilized \(M.\ sexta\) midgut V-ATPase was labeled specifically in the presence of 10 \(\mu\text{mol l}^{-1}\) DCCD and its catalytic activity was inhibited significantly in the presence of 15 \(\mu\text{mol l}^{-1}\) DCCD. However, 150 \(\mu\text{mol l}^{-1}\) DCCD was required for 50% inhibition and even 1.5 mmol l\(^{-1}\) DCCD did not consistently
inhibit enzyme activity completely. Two previous studies have determined the effects of 1.5 μmol l⁻¹ and 150 μmol l⁻¹ DCCD on midgut goblet cell apical membrane ATPase. In the study of Wieczorek et al. (1986), 1.5 μmol l⁻¹ and 150 μmol l⁻¹ DCCD were found to inhibit the activity of highly purified membrane-bound enzyme by 6% and 94%, respectively. In the study of Schweikl et al. (1989), no inhibition of membrane-bound ATPase activity and 81% inhibition of solubilized ATPase activity were found in the presence of 1.5 μmol l⁻¹ DCCD, whereas 93% inhibition of membrane-bound ATPase activity and 95% inhibition of solubilized ATPase activity were found in the presence of 150 μmol l⁻¹ DCCD. Differences between the inhibitor sensitivities of membrane-bound and detergent-solubilized forms of the same enzyme are not unusual (Uchida et al. 1985; Arai et al. 1987). However, the large difference between the DCCD sensitivity of the solubilized midgut V-ATPase found in the present study and that reported by Schweikl et al. (1989) is unusual. The larvae used in the two studies came from different colonies but the methods of enzyme purification were the same. The assays of enzyme activity were also the same in both studies except that in the present studies concentrated DCCD stock solutions were prepared in DMF and

Fig. 2. Labeling of midgut V-ATPase subunits by N,N'-dicyclohexylcarbodiimide (DCCD). Lane A, positions of standard proteins. Lane B, 10 μmol l⁻¹ [¹⁴C]DCCD. Lane C, 100 μmol l⁻¹ [¹⁴C]DCCD. Lane D, blot of ATPase stained for protein.
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Assay mixtures were incubated for 5 min, whereas Schweikl et al. (1989) prepared concentrated DCCD stock solutions in ethanol and incubated their assay mixtures for 12 min. The amount of ATP hydrolysed did not increase linearly with time in the assays of Schweikl et al. (1989). Furthermore, both sodium azide and sodium orthovanadate were present in the assay mixtures used by Schweikl et al. (1989) but not in those used in the present study.

Specific binding of DCCD to the 16 kDa subunit of midgut V-ATPase is shown clearly in Fig. 2. Although DCCD can interact with carboxyl groups, sulphydryl groups and tyrosine residues, it usually forms covalent bonds with hydrophobically located carboxyl groups, such as those on glutamate and aspartate residues in transmembrane domains (Nalecz et al. 1986). DCCD blocks proton translocation through Fo by reacting with the c-subunit at a single glutamic acid or aspartic acid carboxyl group in F-type ATPases (Sebald et al. 1979, 1980; Schneider and Altendorf, 1987). Reaction of DCCD with midgut V-ATPase is quite specific for the 16 kDa subunit. The similarities between the 16 kDa subunit of several V-ATPases and the 8 kDa subunit of mitochondrial, chloroplast and bacterial F-ATPases are striking (Wang and Sze, 1985). The findings reported in this communication suggest that the 16 kDa subunit of midgut V-ATPase may be involved in forming the transmembrane proton-conducting pore of this proton-motive ATPase.

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References


