Isolation and Reconstitution of the Dicyclohexylcarbodiimide-sensitive Proton Pore of the Clathrin-coated Vesicle Proton Translocating Complex*

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The clathrin-coated vesicle proton translocating complex is composed of a maximum of eight polypeptides. The function of the components of this system have not been defined. Proton pumping catalyzed by the reconstituted, 200-fold purified proton translocating complex of clathrin-coated vesicles is inhibited 50% at a dicyclohexylcarbodiimide (DCCD)/protein ratio of 0.66 μmol of DCCD/mg of protein. At an identical DCCD/protein ratio, the 17-kDa component of the proton pump is labeled by [14C]DCCD. Through toluene extraction, the 17-kDa subunit has been isolated from the holoenzyme. The 17-kDa polypeptide diminished proteoliposome acidification when coreconstituted with either bacteriorhodopsin or the intact clathrin-coated vesicle proton translocating ATPase. In both instances, treatment of the 17-kDa polypeptide with DCCD restored proteoliposome acidification. Moreover, the proton-conducting activity of the 17-kDa polypeptide is abolished by trypsin digestion. These results demonstrate that the 17-kDa polypeptide present in the isolated proton ATPase of clathrin-coated vesicles is a subunit which functions as a transmembranous proton pore.

Clathrin-coated vesicle acidification is mediated by an electronegic proton translocating ATPase which is resistant to oligomycin and efrapeptin, but is inhibited by N-ethylmaleimide, to which the mitochondrial proton pump is resistant (1-3). In addition, the coated vesicle proton pump is inhibited by dicyclohexylcarbodiimide (DCCD) at a DCCD/protein ratio over 100-fold greater than that required to inhibit the mitochondrial ATPase (1). Similar responses to inhibitors have been demonstrated for a number of evidently related intracellular organelles (4-10). The clathrin-coated vesicle proton translocating complex has recently been purified 200-fold and reconstituted into liposomes prepared from both crude (11) and defined (12) lipids. At the current stage of purification, the enzyme preparation contains eight major polypeptides, and the holoenzyme has a molecular mass of 530 kDa (11). There is a similarity in the polypeptide composition of the coated vesicle proton pump to those of several ATPases which have been isolated from acidifying organelles prepared from fungal, plant, and mammalian sources (13-17). Notable are apparent conservations of 67-80-, 57-64-, and 15-19.5-kDa polypeptides amongst these preparations. Speculations as to the functions of these putative subunits have been based exclusively on labeling experiments. Controversy exists regarding the catalytic center of these ATPases in that there is not uniformity in labeling with ATP or ATP analogues (13, 15, 16). In contrast, most investigators have identified the low molecular weight species present within these ATPase isolates as the site of DCCD binding (13-17). Based upon an analogy to the Fo component of mitochondrial ATPase (18), it has been proposed that this low molecular weight component is a DCCD-inhibitable proton pore, which serves to lower the activation energy for the movement of protons across the lipid bilayer.

Beyond such labeling experiments, there is little direct evidence for the functional properties of the putative subunits of this new class of proton pumps. Indeed, the actual subunit composition of these pumps is not known and the polypeptide components of the isolates range from three for plant (15, 16) and fungal (17) ATPases to eight for the clathrin-coated vesicle complex (11). It is possible that subunits critical for proton pumping are missing from preparations with apparently fewer components.

In order to determine the minimal polypeptide requirements for proton pumping, as well as to define subunit function, we have undertaken the dissociation of the isolated clathrin-coated vesicle proton translocating complex. In this report, we describe the isolation and reconstitution of the 17-kDa subunit, which we demonstrate to serve as a DCCD-sensitive, transmembranous proton pore.

EXPERIMENTAL PROCEDURES

Materials—Dicyclohexylcarbodiimide was obtained from Aldrich, acridine orange from Eastman, Enlighting from Du Pont, [14C] dicyclohexylcarbodiimide (60 mCi/mmol) and [γ-32P]ATP from Amersham Corp., phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine from Avanti Polar Lipids (Birmingham, AL), octylglucopyranoside from Behring Diagnostics, and toluene from Fisher. All other materials, including bacteriorhodopsin, were obtained from Sigma.

Preparations—Clathrin-coated vesicles were isolated from batches of 30 bovine brains as described (19). The proton translocating complex was solubilized with the nonionic detergent polyoxyethylene 9-lauryl ether (C12E8) and was purified as we have reported (11).
ATPase activities of these preparations range from 12–16 pmol of P_i per min.

Isolation of the Proton Pore from the Intact Proton Translocating Complex of Clathrin-coated Vesicles—Twenty ml (150 µg protein/ml) of final glycerol gradient fractions containing the purified clathrin-coated vesicle proton pump (11) were pooled, and the ATPase was competitively inhibited by MgATP (50 µM) (12) and NaPi (100 µM) to a final specific activity of 50% and centrifuging the mixture of 100,000 x g for 30 min. The pellets were dissolved in 2 ml of Buffer A, consisting of 0.5 mM dithiothreitol, 0.1% CHAPS, and 10 mM Tris brought to pH 7.0 with MES. Twenty ml of ice-cold toluene was added and the mixture was stirred for 1 h at 4 °C. (Extractions performed at room temperature did not yield a functional proton pore.) The toluene phase was collected and was dried under N_2. The residue was either dissolved in 1.5 ml of Buffer Awhile for reconstitution with the clathrin-coated vesicle proton translocating complex or with 1.5 ml of 7 M Tricine at pH 8.0 and 1.25% octylglucosanarone for reconstitution with bacteriorhodopsin. The isolated proton pore was stored at 4 °C and was stable for 2 weeks.

Reconstitution—For reconstitution of both bacteriorhodopsin and the clathrin-coated vesicle proton translocating complex, liposome composition was exactly as described (12). Briefly, lipid mixtures (125 mg protein/ml in 150 mM KCl, 1.25% octylglucosanarone, and cholesterol at a weight ratio of 40:26:5:7:5:26) were also dissolved. The mixture was sonicated for 3 10-min cycles under 20% SDS (v/v), 1%, 0.1 M, and 2.5 mM, respectively.

As shown in Fig. 2, panel I, of the eight major polypeptides present in our preparation, only the 17-kDa component is labeled with [³²P]DCCD.

Isolation of the 17-kDa polypeptide from the 200-fold purified holoenzyme was accomplished by toluene extraction. Approximately 60 µg of protein was extracted from the 3 mg of starting material. Shown in Fig. 2, panel II are the Coomassie-stained extract and the [³²P]DCCD-labeled extract. Loss of a sharply focused band on SDS-polyacrylamide gel electrophoresis was a consistent (unassignable) feature and likely was caused by the co-extraction of lipids present within our ATPase preparation. Nonetheless, the predominant constituent within the extract is a protein with an apparent molecular mass of 15–20 kDa.

To determine if the isolated 17-kDa polypeptide could function as a proton pore, reconstitution of the toluene extracted protein was performed using two different proton gradient generating systems: the light-driven proton pump,
bacteriorhodopsin (24), and the ATP-energized clathrin-coated vesicle proton pump itself.

Reproduced in Fig. 3 are the results of coreconstituting the isolated 17-kDa polypeptide with bacteriorhodopsin. As shown in panel A, illumination of reconstituted bacteriorhodopsin, in the presence of valinomycin, results in alkalinization of the extravesicular medium due to proton accumulation within the proteoliposomes. Inclusion of 0.8 μg of the 17-kDa polypeptide within the reconstitution mixture, in the absence of DCCD, results in restoration of bacteriorhodopsin catalyzed medium alkalization. Taken together, these results indicate that the uncoupling effect of the 17-kDa polypeptide on proteoliposome acidification is DCCD-sensitive and thus demonstrate that the 17-kDa subunit acts as a proton pore. Similar results were obtained when the 17-kDa subunit was coreconstituted with the clathrin-coated vesicle proton translocating complex, as shown in Fig. 5. As can be seen, addition of 0.4 μg of the isolated 17-kDa polypeptide to the reconstitution mixture, in the absence of DCCD, reduces the initial slope of ATP-generated acridine orange quenching from a control rate of 4.6 to 2.1. With pretreatment of the 17-kDa subunit with DCCD (25 μM or 0.35 μmol of DCCD/mg of protein), restoration of the initial rate of proton pumping is achieved to a rate (4.1) which is indistinguishable from that occurring with the holoenzyme reconstituted with an equal amount of DCCD, but without the 17-kDa component. At higher concentrations of DCCD (40 μM or 0.56 μmol of DCCD/mg of protein), the inhibitory effect of DCCD on the

![Fig. 2. SDS-polyacrylamide gel electrophoresis and autoradiographic determination of [14C]DCCD labeling of the holoenzyme and the toluene extract. Panel I, A: SDS-polyacrylamide gel electrophoresis (15% acrylamide) of 0.6 pg of isolated proton translocating ATPase which was preincubated with [14C]DCCD for 5 min at 25 °C with 0.69 μmol of DCCD/mg of protein. B, autoradiograph of the 0.6 μg of the extract which was prelabeled with [14C]DCCD at a DCCD/protein ratio of 0.69 μmol of DCCD/mg of protein.

![Fig. 3. Effects of DCCD on the 17-kDa polypeptide when coreconstituted with bacteriorhodopsin. For all experiments, 20 μg of bacteriorhodopsin was reconstituted with 625 μg of lipid and was assayed as described under "Experimental Procedures." Trace A is the control experiment performed with bacteriorhodopsin proteoliposomes. Trace B (−DCCD) is the effect of coreconstituting 0.8 μg of the 17-kDa polypeptide with bacteriorhodopsin. As denoted by the asterisk (*), 2 μmol of DCCD were added to the proteoliposomes, and after a 5-min incubation at 25 °C, the system was reilluminated, resulting in the final trace (+DCCD).

![Fig. 4. Effects of the 17-kDa polypeptide on proteoliposome acidification catalyzed by light-energized bacteriorhodopsin. For all experiments, bacteriorhodopsin (45 μg) was reconstituted with 625 μg of lipids and assayed as described under "Experimental Procedures." Trace A is the control experiment performed with bacteriorhodopsin proteoliposomes. Trace B is the effect of coreconstituting 0.8 μg of the 17-kDa subunit with bacteriorhodopsin. Trace C, 0.8 μg of the 17-kDa subunit was preincubated with DCCD at a DCCD/protein ratio of 0.7 μmol of DCCD/mg of protein for 20 min prior to coreconstitution with bacteriorhodopsin.

![Fig. 5. Effects of the 17-kDa polypeptide on proteoliposome acidification catalyzed by the 530-kDa clathrin-coated vesicle proton pump. For all experiments, 1.5 μg of the purified clathrin-coated vesicle proton pump was reconstituted with 300 μg of lipids, and proteoliposome acidification was assessed by ATP-generated acridine orange quenching, as described under "Experimental Procedures." Circles (−17 kDa) indicate the effect of preincubating the holoenzyme for 5 min prior to reconstitution with designated concentrations of DCCD. Squares (+17 kDa), indicate the effect of coreconstituting 0.4 μg of the 17-kDa subunit with holoenzyme. The 17-kDa subunit was preincubated for 5 min with designated concentrations of DCCD, as indicated. In all instances, (squares and circles) the amount of DCCD in the final reaction mixture was identical for each preincubation concentration.
TABLE I
Effect of trypsin on the inhibition by the 17-kDa polypeptide of bacteriorhodopsin-catalyzed proteoliposome acidification

| Addition                  | Medium alkalinization | Initial rate | Maximum extent |
|---------------------------|-----------------------|--------------|-----------------|
|                           | nmol H⁺/s             |               | Initial-final nmol H⁺ |
| 17-kDa subunit            | 4.9                   | 5.1           |                 |
| Trypsin-treated 17-kDa subunit | 2.8              | 2.8           |                 |

Proton Channel Isolation and Reconstitution

holoenzyme is predominant; i.e. the effects of DCCD on the 17-kDa component are obscured by direct inhibition of the proton gradient generating system itself, the proton ATPase. Thus, in a second proton gradient generating system, the 17-kDa polypeptide preparation can be shown to function as a DCCD-inhibitable proton pore.

The results of experiments listed in Table I demonstrate that the uncoupling effect of the 17-kDa polypeptide is trypsin-sensitive and hence due to the protein moiety of the subunit, as opposed to contaminating lipids.

DISCUSSION

Taken together, these experiments demonstrate that the 17-kDa polypeptide of the clathrin-coated vesicle proton translocating complex is a DCCD-inhibitable, transmembranous proton pore. Resemblance to the F₀ component of F₁,F₀ ATPases (18) is striking and supports the popular thesis that endomembrane pumps in general, and the clathrin-coated vesicle proton pump in particular, are F₁,F₀-type pumps and share a distant ancestry with the mitochondrial pump in the proton ATPase of anaerobic bacteria (25). Indeed, other investigators have used the coreconstitution of mitochondrial F₀ (18, 26) and chloroplast F₀ (27) with bacteriorhodopsin to demonstrate the proton-conducting activity of these proteolipids. Furthermore, the finding that the coreconstitution of the 17-kDa subunit with the clathrin-coated vesicle proton pump yields a DCCD-inhibitable proton conductance is highly reminiscent of experiments conducted with submitochondrial particles which were partially depleted of F₁ (28).

Because of the close analogy to mitochondrial F₀, it is important to note that the 17-kDa subunit differs from mitochondrial F₀ in several respects, and thus we have isolated the proton-conducting unit of the clathrin-coated vesicle proton pump. First, the 8–10-kDa mitochondrial F₀ is substantially smaller (18) than the 17-kDa proton pore of the clathrin-coated vesicle proton pump. Second, 100-fold greater DCCD/protein ratios are required to inhibit the clathrin-coated vesicle proton pump and the 17-kDa subunit, as compared to the mitochondrial F₁,F₀ (1). Third, in side-by-side comparison (11) we have shown that our final clathrin-coated vesicle ATPase preparation shares no polypeptides in common with purified mitochondrial F₁. As our final step in purification is a glycerol gradient centrifugation, it is highly unlikely that the 530-kDa clathrin-coated vesicle preparation is contaminated with dissociated mitochondrial F₀, a complex of a molecular mass of 8 kDa.

Further experiments are required to determine the compo-

sition of the clathrin-coated vesicle proton pore. By analogy to F₀, it is likely that the 17-kDa subunit of the clathrin-coated vesicle proton pump is a proteolipid. We cannot at present discern whether the proton pore of coated vesicles is a single polypeptide or is a composite of several polypeptides and proteolipids (as is F₀); loss of resolution after extraction precludes such definition and at present we claim isolation, rather than purification, of the proton pore.

A key issue in the field of vacuolar H⁺ ATPases, of which the clathrin-coated vesicle proton pump is an example, is the possibility of relationship to F₁,F₀ type pumps. Although the presence of a dissociable proton pore (and likely proteolipid) in the coated vesicle ATPase suggests a relationship, a more important differentiating feature is the mechanism of the catalytic sector. The vanadate insensitivity of the coated vesicle ATPase (1) suggests the absence of a phosphosapartyl intermediate which participates in the catalytic cycle of E₁,E₂ type ATPases such as Na⁺K-ATPase (29). However, sensitivity to vanadate can be a latent feature of known E₁,E₂-type enzymes (30), and at present, the lack of vanadate inhibition of our system can be viewed as only indirect evidence that the coated vesicle pump does not have a phosphoenzyme intermediate. It is our view that if the clathrin-coated vesicle proton pump is an F₁,F₀-type ATPase, then the divergence from the mitochondrial ATPase is extreme. In order to gain insight into this issue, resolution and functional definition of the catalytic cycle of the clathrin-coated vesicle proton translocating ATPase is necessary.

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