Recombinant SFD Isoforms Activate Vacuolar Proton Pumps*

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The vacuolar proton pump of clathrin-coated vesicles is composed of two general sectors, a cytosolic, ATP hydrolytic domain (V\(_1\)) and an intramembranous proton channel, V\(_0\). V\(_1\) is comprised of 8–9 subunits including polypeptides of 50 and 57 kDa, termed SFD (Sub Fifty-eight-kDa Doublet). Although SFD is essential to the activation of ATPase and proton pumping activities catalyzed by holoenzyme, its constituent polypeptides have not been separated to determine their respective roles in ATPase functions. Recent molecular characterization of these subunits revealed that they are isoforms that arise through an alternative splicing mechanism (Zhou, Z., Peng, S.-B., Crider, B.P., Slaughter, C., Xie, X.S., and Stone, D.K. (1998) J. Biol. Chem. 273, 5878–5884).

To determine the functional characteristics of the 57-kDa (SFD\(_a\)) and 50-kDa (SFD\(_b\)) isoforms, we expressed these proteins in *Escherichia coli*. We determined that purified recombinant proteins, rSFD\(_a\) and rSFD\(_b\), when reassembled with SFD-depleted holoenzyme, are functionally interchangeable in restoration of ATPase and proton pumping activities. In addition, we determined that the V-pump of chromaffin granules has only the SFD\(_a\) isoform in its native state and that rSFD\(_a\) and rSFD\(_b\) are equally effective in restoring ATPase and proton pumping activities to SFD-depleted enzyme. Finally, we found that SFD\(_a\) and SFD\(_b\) structurally interact not only with V\(_1\), but also with V\(_0\), indicating that these activator subunits may play both structural and functional roles in coupling ATP hydrolysis to proton flow.

Vacular, or V-type proton pumps acidify a wide array of intracellular compartments and are essential to functions of constitutive endocytotic and regulated secretory pathways. These H\(^+\) pumps are also found in the plasma membrane of polarized cells such as osteoclasts and renal tubular epithelial cells, where they function to acidify discrete extracellular compartments. In fact, V-pumps have been localized to virtually all intracellular compartments, except the nucleus and mitochondria, and their functions are equally diverse, ranging from promoting receptor-ligand dissociation in clathrin-coated vesicles and endosomes, to energizing neurotransmitter and catecholamine storage in synaptic vesicles and adrenal chromaffin granules (1–3).

Key questions thus arise regarding the mechanisms by which these pumps are targeted to their cellular sites and how differential regulation of the enzymes is achieved in these disparate locales. Several regulatory elements of V-pumps have been described, including activator proteins (4, 5) and chloride channels that operate in parallel with V-pump to dissipate the charge generated by these electrogenic pumps and thereby facilitate pH gradient formation (6, 7).

Recently, we provided biochemical (8) and molecular (9) evidence for the roles of a 50- and 57-kDa polypeptide doublet, termed SFD (Sub Fifty-eight-kDa Dimer, or Doublet), in the activation of the V-pump of clathrin-coated vesicles (CCV) of bovine brain. These proteins were discovered in the course of our attempts to achieve biochemical resolution of components of the V-pump of CCV. This enzyme, like all V-pumps, is comprised of two general sectors, a multisubunit, cytosolic ATP hydrolytic domain (V\(_1\)), and a multisubunit proton channel (V\(_0\)). Biochemical and genetic studies have revealed that the V\(_1\) domain in eukaryotic organisms is composed of 7 core subunits (A-G), some of which are present in multiple copies (10). Although less well characterized, the V\(_0\) domain of the V-pump of CCV contains between 3–6 different subunits, ranging from a 116-kDa polypeptide (subunit a) to a small protein (subunit c) (11).

Attempts at defining the components of V\(_1\) (subunits A-G) and their functions revealed that an additional factor(s) was required for pump function, namely the polypeptides of SFD. When selectively depleted of these proteins, the V-pump of CCV, though assembled as a V\(_1\)V\(_0\) complex, cannot support ATP hydrolysis or proton pumping. Purified SFD, when added to SFD-depleted pump, was shown to restore these functions (8).

From a molecular standpoint, we recently determined the 57- and 50-kDa polypeptides of SFD, termed SFD\(_a\) and SFD\(_b\), respectively, arise from a single gene by an alternative splicing mechanism and that the SFD\(_b\) isoform has a smaller molecular mass because of an 18-amino acid deletion (9). Further characterization revealed that the SFD proteins have sequence homology to the VMA13 product of *Saccharomyces cerevisiae*. As is the case with SFD, loss of the VMA13 gene product yielded a yeast vacuolar proton pump that was assembled but inactive (12).

Of note, the activation properties of SFD had been previously ascribed by others (13, 14) to AP50, a component of the AP2 complex which is responsible for the assembly of the clathrin...
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coats of coated pits and vesicles (15–17). Our more recent work demonstrated that SFD, and in particular its 50-kDa component (SFDβ), is molecularly distinct from AP50 and that removal of AP50 from impure pump preparations had no effect on enzyme activity, whereas removal of SFDα and SFDβ accounted for the deactivation we had previously observed (9).

The current studies add final proof to the molecular identity of the SFD isoforms. Purified, recombinant SFDα and SFDβ are shown to restore ATPase and proton pumping activity to V-pump depleted of SFD. Moreover, we have identified a source of V-pump (chromaffin granules) that in its native form has only SFDα. When depleted of SFD, the chromaffin granule pump loses ATPase and proton pumping activities, and both are restored by addition of either SFDα or SFDβ. Finally, we have determined that SFD binds to both isolated V1 and V0, thus providing evidence that it may function in a structural role of coupling ATP hydrolysis to proton pumping.

**EXPERIMENTAL PROCEDURES**

**Preparations and Materials**—Isolation of clathrin-coated vesicles from bovine brains (18) and preparations of liposomes from purified lipid rafts was reported as previously described (19). As a translocating ATPase of clathrin-coated vesicles was performed by sequential solubilization with ChE, hydroxylapatite chromatography, (NH4)2SO4 fractionation, and glycerol gradient centrifugations (20). Purified H2-ATPase had a specific activity of 14–16 μmol of P1, mg of protein −1 min−1. V0 was isolated from purified V-pump (21), and recombinant subunit B (22) was prepared as described. Purified proton pump was depleted of SFD by treatment with Zwittergent 3–16, followed by glycerol gradient centrifugation, as reported (8). Partial purification of the vacuolar proton pump of chromaffin granules was achieved by modification of the protocol used for preparation of the vacuolar proton pump of clathrin-coated vesicles of bovine brain. Briefly, 500 mg of bovine chromaffin granule membranes were incubated at 4°C for 1 h with 20 ml of solubilization buffer, consisting of 10 mM Tris-HCl (pH 7.5), 1% CH3CN, 0.5 mM EDTA, and 5 mM dithiothreitol. The mixture was centrifuged at 150,000 × g for 1 h, and the supernatant was removed and mixed with (NH4)2SO4 to achieve 45% final saturation. After a 30-min incubation at 4°C, the mixture was centrifuged at 150,000 × g for 30 min, and the supernatant was discarded. The pellet was dissolved in 1 ml of 20 mM Tris-HCl (pH 7.5), 0.05% CH3CN, 5 mM dithiothreitol, and 0.5 mM EDTA and was layered over a 13-ml continuous 15–30% glycerol gradient prepared in the same buffer. The gradients were centrifuged at 38,000 rpm for 2 h at 4°C in a Beckman SW40 rotor. Proton pump was harvested from the bottom one-third of the gradient in 1-mL fractions.

Restriction enzymes, T4 DNA sequencing ligase, and a nick translation kit were obtained from Roche Molecular Biochemicals; DNA sequencing enzymes and PCR were designed to express fusion proteins containing 10 neighboring histidine residues and a Factor Xa recognition site at the NH2 terminus. For expression of SFDα and SFDβ, E. coli strain BL21 (DE3) pLys S was transformed with expression vectors pET16b-SFDα or pET16b-SFDβ, respectively, and grown and induced with IPTG at 37°C, as described (23).

**Northern blot Analysis**—An 856-bp PCR product, originally used to isolate the cDNAs encoding SFDα (9), was used to generate a 23P-labeled RNA probe with a MAXI-Script Sp6/T7 kit from Ambion. Designated amounts of bovine poly(A)−enriched mRNA were subjected to agarose (1.2%) gel electrophoresis and were subsequently transferred to nylon filter. After baking at 80°C for 1 h, the filters were prehybridized with 5× Denhardt’s solution, 0.1 mg/ml sheared, single-stranded salmon DNA, and 0.1% SDS at 65°C for 3 h. Subsequently the filter was hybridized with RNA probe (0.3 × 106 cpm/ml) for 12 h at 65°C. Filters were sequentially washed with 2× SSC, 0.1% SDS at 65°C for 15 min, 0.5× SSC, 0.1% SDS at 65°C for 15 min, and 0.1× SSC, 0.1% SDS at 65°C for 30 min. Washed filters were exposed overnight using Amersham Pharmacia Biotech Hyperfilm. RT-PCR—RT-PCR was performed using a GeneAmp RNA PCR kit from Perkin-Elmer. Bovine poly(A)+ RNA (0.1 μg) was reverse-transcribed using poly(dt)16 as primer, and PCR amplification was subsequently performed using Primer 1 (5′-CTGGAGGAACGGAGGATG-3′) and Primer II (5′-GACATCTGTCAGACATCGACCG-3′). These primers were designed to yield a PCR product that included the 54-bp insertion distinguishing SFDα and SFDβ. Using primers as a template, these primers are predicted to yield a 450-bp PCR product, and with SFDβ transcript template, a 396-bp PCR product. PCR products were separated electrophoretically on 1% agarose gels and were visualized by etidium bromide staining.

**Purification of Recombinant SFDα and SFDβ**—Bacterial cells were harvested by centrifugation at 3,840 × g for 20 min. The pellets were resuspended in 100 ml of lysis buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl), and the cells were broken by sonication. The lysate was centrifuged at 186,000 × g for 1 h, and the supernatants, which contained most of the recombinant proteins, were collected and loaded on 1 ml of Ni2+–NTA columns. After loading, the columns were washed with 20 ml of lysis buffer and then eluted with lysis buffer containing 20 mM imidazole (pH 7.5). The eluents were diluted 1:10 with 5 mM Tris-Cl (pH 7.5) and loaded on a Mono-Q (5 mm × 5 cm) column. The column was washed with 10 ml of binding buffer (20 mM Tris-Cl (pH 7.5), and 10% glycerol). Proteins were eluted with linear (0–400 mM) NaCl gradients prepared in 15 ml of binding buffer. The fractions (1 ml) contained as much as 10–20 mg of the recombinant proteins, with >95% purity, as assessed by SDS-PAGE (24) and by the Bradford protein assay (25).

**Assembly of SFD-depleted Proton Pump with Recombinant SFDα and SFDβ**—For each assay, 0.5 pmol of SFD-depleted proton pump was mixed with designated amounts (0–2.5 pmol) of recombinant SFDα and/or SFDβ, and the final volume was brought to 10 μl with buffer consisting of 20 mM Tris-Cl (pH 7.5), 0.05% CH3CN, 0.5 mM EDTA, and 2 mM dithiothreitol. After incubation at room temperature for 10 min, recombinant pump was assessed for ATPase and/or proton pumping activities.

**Measurement of ATPase and Proton Pumping**—Measurement of ATPase activity was assessed by the liberation of 32P from [γ-32P]ATP. Reassembled pump (0.5 pmol/assay) was incubated with 5 μl of phosphatidylserine (0.5 mg/ml) at room temperature for 5 min. Reactions were initiated with addition of 200 μl of assay buffer, consisting of 50 mM Tris-MES (pH 7.0), 30 mM NaCl, 3 mM MgCl2, 0.15 mM NaVO4, and 3 mM [γ-32P]ATP (200–400 cpm/nmol). After incubation at 37°C for 30 min, reactions were terminated by the addition of 1 ml of 1.25 N perchloric acid, and liberated 32P was extracted and counted as described (26).

For assessment of ATP-driven proton pumping, samples of reassembled proton pump (0.5 pmol/assay) were reconstituted into liposomes (300 μmol/assay) prepared from purified lipids by the cholate-dilution, freeze-thaw method, as described (19). Protein liposomes were diluted into 1.6 ml of pumping assay buffer consisting of 150 mM KCl, 10 mM Tricine (pH 7.5), 2.5 mM MgCl2, 0.15 mM NaVO4, and 7 μM atride orange. Proton pumping was assessed by ATP-dependent quenching of the absorbance of acridine orange and was measured in Amino DW2C detector at 510 nm. Samples were excited at 492 nm, and luminescence was monitored at 540 nm. Reactions were initiated by addition of 1.3 mM Na-ATP and 1 μM valinomycin and were terminated by addition of 1 mM 17β.
RESULTS

Our previous attempts to resolve the 50- and 57-kDa polypeptides of SFD by conventional biochemical methods failed (8). While this may have owed to the fact that SFD existed as a heterodimer, it was also possible that their copurification owed to the fact that the two proteins differed by only 18 amino acids (9). Moreover, results of molecular mass determination by gel filtration chromatography were equivocal, perhaps because of the presence of residual amounts of detergent used for release of the polypeptide pair from holoenzyme. Key issues regarding the 50- and 57-kDa components of SFD are thus whether both components are required for pump functions, and if not, do differences exist in the functional properties of the two proteins.

To explore these points, we expressed and purified recombinant forms of the two polypeptides, as described under "Experimental Procedures." Shown in panel A of Fig. 1 are the purified recombinant α (lane 3) and β (lane 5) components of SFD. Panels B and C are Western blot analysis of the two components. In panel B, antibody Q48, directed against an epitope common to SFDα and SFDβ, reacts with both species in lysates of E. coli transformed with expression vectors for the α polypeptide and SFDβ, respectively. Western blot analysis (panel C) using antibody Q50, directed against the unique 18-amino acid insert of SFDα demonstrates that the antibody recognizes rSFDα before (lane 2) and after purification (lane 3) from E. coli lysate; in contrast, the antibody does not recognize rSFDβ (lanes 4 and 5).

Of note was the finding that both rSFDα and rSFDβ were present as soluble proteins in E. coli lysates. This differed from our experience with production of recombinant forms of all other V1 subunits, each of which required detergents for solubilization, and many of which required denaturation with 8 M urea to achieve dissolution of inclusion bodies that contained the recombinant proteins.

To address whether SFDα or SFDβ had independent activities, or synergistic effects when used in combination, reconstitution experiments were performed using the recombinant SFD proteins and biochemically prepared proton pump that had been depleted of SFD. As shown in Fig. 2, proton pump depleted of SFD lacked significant Mg2+-activated ATPase activity. Reassembly of recombinant forms of SFD with SFD-depleted pump resulted in a saturable activation of MgATPase activity. No differences were detected with the use of rSFDα alone, rSFDβ alone, or equimolar mixtures of rSFDα and rSFDβ. Maximum restoration of MgATPase activity was achieved at a molar ratio of rSFD to SFD-depleted proton pump of 2. The final specific activity of reassembled enzyme was about 1.5 μmol of Pi/mg of protein 1.2

To further characterize the functional properties of rSFDα and rSFDβ, we performed reconstitution experiments in which the rSFD proteins were first reassembled with SFD-depleted pump, and the resulting holoenzyme was then reconstituted into liposomes and assayed for proton pumping activity. Shown in Fig. 3 are the proton pumping activities of these preparations, as assessed by ATP-generated acridine orange quenching. SFD-depleted pump lacked activity (trace 1), whereas rSFDα (trace 2), rSFDβ (trace 3), and equimolar mixtures of rSFDα and rSFDβ (trace 4) activated protein pumping in SFD-depleted pump. In this particular experiment, proton pumping, as assessed by the initial rate of ATP-generated acridine orange quenching, was about 1.3-fold higher when a mixture of rSFDα and rSFDβ were used (trace 4), as compared with rates attained with a single isoform of SFD (traces 2 and 3). This, however, was not a consistent observation, and coupled with the results obtained in the experiments of Fig. 2, we find no compelling evidence at present that there exist functional differences between the two isoforms.

Tissue distributions of the SFD isoforms were assessed by Northern blot analysis, as shown in Fig. 4. The highest copy number of transcripts was found in brain (lane 1) followed by kidney (lane 3) and lung (lane 4); much lower levels of SFD

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Fig. 1. Expression and purification of rSFDα and rSFDβ. SDS-PAGE (panel A) followed by Coomassie Blue staining and Western blot analysis using Q48 (panel B) or Q50 (panel C) IgG were performed as described under "Experimental Procedures." Lanes 1, 5 μl of cell lysate of E. coli transformed with pET16b SFDα, before IPTG induction; lanes 2, 5 μl of cell lysate of E. coli transformed with pET16b SFDα after IPTG induction; lanes 3, purified recombinant SFDα (150 ng); lanes 4, 5 μl of cell lysate of E. coli transformed with pET16b SFDβ after IPTG induction; and lanes 5, purified recombinant SFDβ (150 ng).

Fig. 2. Stimulation of MgATPase activity by rSFDα and rSFDβ. Reassembly of rSFDα and rSFDβ with SFD-depleted proton pump and measurements of ATPase activity were performed as described under "Experimental Procedures." SFD-depleted proton pump (0.5 pmol) and 0.5–2.5 pmol(s) of either SFDα (○), SFDβ (■), or an equal molar mixture of SFDα and SFDβ (●) were used for these experiments.

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This is about one-tenth the specific activity of native enzyme (20) and is close to the specific activity obtained by reassembling biochemically prepared SFD with SFD-depleted proton pump. This reduced activity likely owes to treatment of the holoenzyme with Zwittergent 3–16 in preparation of SFD-depleted pump. We have found that this detergent is relatively toxic to enzyme function and is not easily removed because of its low critical micellar concentration.
transcripts were detected in heart (lane 2). Because of the minor difference in molecular mass in the transcripts for SFDα and SFDβ, we performed RT-PCR to determine whether tissue differences in the transcript level of SFDα and SFDβ existed. As described in “Experimental Procedures,” primers were utilized that would predictably yield products of 450 bp for SFDα and 396 bp for SFDβ. Although transcripts for SFDα and SFDβ were found in all tissues, it appears that SFDβ is in relative abundance in brain (lane 2), whereas relatively more SFDα RT-PCR product was demonstrated in all other tissues examined.

We next sought to determine whether proton pump preparations from sources other than brain contained both SFDα and SFDβ isoforms. As shown in Fig. 5, proton pump prepared from bovine brain (lane 1) contained roughly equimolar amounts of SFDα and SFDβ, whereas V-pump purified from bovine chromaffin granules (lane 2) had only the SFDα isoform, as determined by immunoblot analysis (panels B and C). This finding allowed us to examine by another approach whether any functional differences could be attributed to the isoforms, and we specifically sought to determine whether SFDβ could substitute for SFDα in the proton pump of chromaffin granules, which, in its native form, contains only SFDα.

To test this possibility, we first depleted the purified chromaffin granule V-pump of SFD (Fig. 6) by treatment of holoenzyme with Zwittergent 3–16, followed by glycerol gradient centrifugations as described under “Experimental Procedures.” In panel A, SDS-PAGE reveals that the holoenzyme was found to migrate to its usual position in the glycerol gradient, as evidenced by the characteristic Coomassie stained bands of 70-, 58-, and 33-kDa, that can be visualized in lanes 3 and 4. In contrast, an immunoblot performed with anti-SFD antibody (panel B) demonstrate that all immunoreactive protein was present near the top of the gradient (e.g. lanes 8 and 9), where released SFD is typically found (8, 9).

Next, we utilized SFD-depleted proton pump of chromaffin granules (as shown in Fig. 6, lanes 3 and 4) to compare the effects of rSFDα and rSFDβ in restoration of enzyme activity. Shown in Fig. 7 are the results of these experiments. rSFDα and rSFDβ were found to be equimolar in restoration of MgATPase (panel A) and proton pumping (panel B) activities of the SFD-depleted V-pump of chromaffin granules.

Finally, we have begun to investigate the structural basis of the effects of SFD on pump function. As described previously (8, 9), and as demonstrated in these studies, SFD activates ATPase and proton pump activities of the holoenzyme. In addition, SFD has been shown to activate MgATPase activity of SFD-depleted V₁ (8). To determine the domain(s) through which SFD regulates pump function, we performed binding experiments using rSFD, SFD-depleted proton pump, SFD-depleted V₁, and isolated V₅. For these studies (Fig. 8), recombinant, histidine-tagged SFD was incubated with SFD-depleted holoenzyme, SFD-depleted V₁, or isolated V₅. The mixtures were passed over Ni²⁺-NTA columns; and bound proteins
the coupling of V1 to V0, and thereby, the functional coupling of Ni2+ATP hydrolysis to proton flow.

Panel A (SDS-PAGE) shows that SFD-depleted pump (with dominant polypeptides of 70-, 58-, and 33-kDa) migrates near the bottom (lanes 3 and 4) of the glycerol gradient. Panel B (Western blot) using Q48 IgG shows that the released SFDα stays in fractions (lanes 9 and 10) near the top of the glycerol gradient.

were subsequently eluted with imidazole. After SDS-PAGE, the eluents were then tested by immunoblot analysis to determine whether there had been binding of the 116-kDa polypeptide (a V₀ constituent) and/or the 70-kDa subunit (a V₁ constituent). As shown in panel A, SFD-depleted holoenzyme binds to both rSFDα (lane 3) and rSFDβ (lane 4). In addition, both recombinant forms of SFD bind isolated V₀ (panel B, lanes 3 and 4) as well as SFD-depleted V₁ (panel C, lanes 3 and 4). Important controls for this study included passage of the biochemically prepared SFD-depleted pump, and V₁ or V₀, over a Ni²⁺-NTA column without preincubation with rSFD isoforms (lanes 1 of panels A, B, and C). In addition, histidine-tagged, recombinant (22) subunit B (a component of V₁) was substituted for rSFD and was demonstrated to not bind either V₁, V₀, or holoenzyme (lanes 2 of panels A, B, and C). This study adds an additional insight into SFD function with the demonstration that SFD isoforms bind not only to V₁, but also to V₀, thus raising the possibility that SFD may play a structural role in the coupling of V₁ to V₀, and thereby, the functional coupling of ATP hydrolysis to proton flow.

**DISCUSSION**

Expression of functional forms of 50- and 57-kDa components of SFD has allowed us to address several key questions regarding the role of SFD in V-pump function. First, a lingering question from our previous studies was whether both forms of SFD were essential to V-pump function. A strict biochemical approach to this issue was thwarted by our inability to separate the two isoforms (8). With the molecular cloning of SFDα and SFDβ, it became apparent that their (near) identical sequences might explain the difficulty in separating these two species by conventional biochemical methods (9). Moreover, attempts to determine the overall molecular mass of the putative SFDα/SFDβ complex by size exclusion chromatography were equivocal, perhaps because of the presence of detergent-protein micelles resulting from residual Zwittergent 3–16. Two lines of evidence now demonstrate that the α and β isoforms can function independently in activation of ATPase and proton pumping activities.

First, we have shown that recombinant forms of either SFDα or SFDβ can restore the ATPase and proton pumping activities of SFD-depleted proton pump prepared from clathrin-coated vesicles of bovine brain. Further, titration of mixtures of rSFDα and rSFDβ did not yield synergistic effects. Second, we demonstrate that V-pump of chromaffin granules contains only the SFDα isoform, indicating that a native form of the enzyme functions with only one isoform. In additional experiments, rSFDα and rSFDβ had equivalent effects on SFDα-depleted pump of chromaffin granule, further demonstrating the independent, and interchangeable functions of the two isoforms. Additional evidence indicates that the purified recombinant SFD isoforms exist in monomeric forms prior to reassembly with the pump, as determined by high performance liquid chromatography performed with size exclusion columns (data not shown). We therefore believe it is most likely that the SFD protein exist in a monomeric form if, indeed, they are present as isolated species in the cytosol.

Our finding that optimal stimulation of pump activities occurs at a molar ratio of SFD to holoenzyme of 2:1 suggests that there may be more than one SFD binding site per holoenzyme. We, however, view the issue of SFD copy number as unresolved at present, in part because recombinant SFD isoforms may not be fully active and thus spuriously increasing the apparent SMF/hoenzyme ratio. Also, as discussed below, it is possible that SFD may act as a dissociable regulatory element, and the optimal ratio required for the binding of SFD to holoenzyme may differ from the copy number of SFD needed for activation of the enzyme, once binding has occurred. The finding that reassembly of SFD-depleted V-pump of CCV with mixtures of rSFDα and rSFDβ does not yield additive, or synergistic, stimulation of activities indicates that the SFD binding sites of a given V-pump molecule are promiscuous, at least under these conditions. In addition, reassembly of SFD-depleted V-pump of chromaffin granule with either rSFDα or rSFDβ provides additional evidence that SFD isoforms can act interchangeably, even in a pump which, in its native form, has only one (SFDα) isoform. We thus favor the view that more than one isoform can be present in a single proton pump.

These studies add final clarification to a controversy regarding the nature of the 50-kDa polypeptide present in the V-pump of clathrin-coated vesicles. Previously it was reported by others (13, 14) that this polypeptide was AP-50, a component of the AP2 complex that is essential to the assembly of clathrin coats (15–17). Previous work from our laboratory demonstrated that the 50-kDa polypeptide was in fact SFDβ (9), and with the results of the current work, we have functional proof that SFD isoforms are causally related to the functional effect ascribed by others to AP-50. As we continue to note, these observations do not exclude the possibility that AP-50 may interact with V-pumps of clathrin-coated vesicles to induce changes in enzyme behavior that are at present unknown (e.g., targeting). In composite, however, our data do demonstrate that SFD components, and not AP-50, are required for V-pump function, as defined by activation of ATPase and proton pumping activities in enzyme depleted of this key component.

Given that SFDα and SFDβ have interchangeable functions in activation of ATPase and proton pumping activities, a key question remains as to the roles these isoforms might play in the overall biology of V-pumps. Although we have no direct evidence to address this issue, it is likely that an important clue to this question resides in our finding that SFD isoforms can bind to both the V₁ and V₀ sectors of V-pumps. This observation is of some surprise, as all evidence to date has indicated that SFD is a V₁ component, as demonstrated by its copurification with isolated V₁. Our current experiments now indicate
that SFD subunits may play a structural role in coordinating the activities of V₁ and V₀ and may thus function not only as activators of V-pumps but also as true coupling factors. In this respect, it is notable that V-pumps (27), like F-type proton pumps (28), appear to have two links between the ATP hydrolytic sector and the intramembranous channel. In addition to the γ subunit that plays a key role in energy transduction, the F-type pump of *E. coli* has a stator arm that links F₁ and F₀ together in such a manner that F₁ is held stationary relative to the membrane. For the F-type pump of *E. coli*, this stator arm is composed of the b subunits of F₀ and OSCP of F₁ (28). Based upon other investigations in our laboratory, it appears that the NH₂ terminus of the γ subunit of F₁ and the intramembranous channel. In addition to the γ subunit that plays a key role in energy transduction, the F-type pump of *E. coli* has a stator arm that links F₁ and F₀ together in such a manner that F₁ is held stationary relative to the membrane. For the F-type pump of *E. coli*, this stator arm is composed of the b subunits of F₀, and δ subunit of F₁, whereas in mitochondrial F₁F₀ this linkage is composed of the b subunits of F₀ and OSCP of F₁ (28). Based upon other investigations in our laboratory, it appears that the NH₂ terminus of the 116-kDa component (subunit a) of V₀ is cytosolic in orientation and may interact with SFD.³ We thus speculate that subunit a with SFD may represent the stator arm of V-pumps. As such, SFD could play a pivotal role in governing the structural and functional assembly of V-pumps.

V-pumps, unlike F-type proton pumps, have been shown to reversibly dissociate into V₁ and V₀ components as a mechanism of recruitment of V-pump function in instances requiring increased proton pumping capability (29, 30). Recently, we have demonstrated that the tendency of V₁ to dissociate from V₀ correlates with isoform diversity of subunit a (11). Thus it is possible that the isoform diversity of the SFD subunits interplays with the isoforms of subunit a to provide a multiplicative diversity in the interactions of V₁ with V₀. In this turn, may represent an important means by which proton pumps undergo differential regulation in their myriad cellular locations.

We also believe that the solubility of recombinant SFD isoforms may be highly significant. Of the 10 subunits and subunit isoforms of V₁ that we have expressed in functional, recombinant forms, only rSFDα and rSFDβ exist as soluble proteins in expression cell lysates. Although our current data speak only to the roles these proteins play in activation of enzyme activity, and possibly coupling functions, we believe that SFDα and SFDβ may exist as soluble, regulatory factors of V-pumps within the cytosol. Current studies are underway to investigate this issue.

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