Complex Formed between Intramembrane Metalloprotease SpoIVFB and Its Substrate, Pro-αK

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Intramembrane metalloproteases (IMMPs) are conserved from bacteria to humans and control many important signaling pathways, but little is known about how IMMPs interact with their substrates. SpoIVFB is an IMMP that cleaves Pro-αK during Bacillus subtilis endospore formation. When catalytically inactive SpoIVFB was coexpressed with C-terminally truncated Pro-αK(1–126) (which can be cleaved by active SpoIVFB) in Escherichia coli, the substrate dramatically improved solubilization of the enzyme from membranes with mild detergents. Both the Pro(1–20) and αK(21–126) parts contributed to improving SpoIVFB solubilization from membranes, but only the αK part was needed to form a stable complex with SpoIVFB in a pull-down assay. The last 10 residues of SpoIVFB were required for improved solubilization from membranes by Pro-αK(1–126) and for normal interaction with the substrate. The inactive SpoIVFB-Pros(1–126)-His6 complex was stable during affinity purification and gel filtration chromatography. Disulfide cross-linking of the purified complex indicated that it resembled the complex formed in vivo. Ion mobility-mass spectrometry analysis resulted in an observed mass consistent with a 4:2 SpoIVFB-Pros(1–126)-His6 complex. Stepwise photobleaching of SpoIVFB fused to a fluorescent protein supported the notion that the enzyme is tetrameric during B. subtilis sporulation. The results provide the first evidence that an IMMP acts as a tetramer, give new insights into how SpoIVFB interacts with its substrate, and lay its foundation for further biochemical analysis of the enzyme-substrate complex and future structural studies.

Many critical cellular processes are regulated by intramembrane proteolysis (1). Intramembrane proteases (IPs) cleave their substrates within a transmembrane segment (TMS) or near the membrane surface. There are three classes of IPs: rhomboids, aspartyl IPs, and IMMPs (often called site-2 proteases or S2Ps). Rhomboids are serine IPs that promote animal cellular signaling, coordinate bacterial quorum sensing, regulate mitochondrial homeostasis, and control protozoan infection (2–5). Presenilin, an aspartyl IP, is the catalytic component of γ-secretase, which is involved in the processing of the amyloid precursor protein, Notch, and many other substrates (6, 7). Dysfunction of γ-secretase contributes to the pathogenesis of Alzheimer disease (8) and many other diseases (7). Aspartyl IPs also include preflagellin and prepilin peptidases involved in bacterial pathogenesis (9), and signal peptide peptidases, which facilitate the clearance of signal peptides from membranes, participate in viral infection, and generate small peptides as signal molecules for immune systems (10, 11). IMMPs also play critical roles in a wide variety of biological functions. In eukaryotes, cholesterol metabolism, the unfolded protein response, and the acute-phase response are regulated by IMMPs (1, 12, 13). In bacteria, IMMPs control sporulation, envelope stress responses, mating signal production, polar localization (21, 22). A serine protease, SpoIVB, expressed in the forespore of B. subtilis (15). During endospore formation, an asymmetrically positioned septum divides the cell into forespore and mother cell compartments, and then the mother cell membrane of the septum engulfs the forespore, surrounding it with a second membrane and pinching it off within the mother cell (Fig. 1, top). SpoIVFB is expressed in the mother cell and localizes to the outermost membrane surrounding the forespore, with its active site facing toward the mother cell cytoplasm (Fig. 1, bottom). SpoIVFB is kept inactive by forming a complex with BoFA and SpoIVFA (21, 22). A serine protease, SpoIVB, expressed in the forespore and secreted into the space between the membranes surrounding...
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![Diagram of morphological changes during B. subtilis sporulation and proteolytic cascade leading to cleavage of Pro-α^K in B. subtilis.](image)

**FIGURE 1.** Morphological changes during B. subtilis sporulation and proteolytic cascade leading to cleavage of Pro-α^K in B. subtilis. Top, morphological changes during sporulation of B. subtilis. Upon starvation, a polar septum forms, dividing the cell into the mother cell and forespore compartments. The mother cell membrane migrates around the forespore during the process of engulfment, and channels form connecting the two compartments. Completion of engulfment results in two membranes surrounding the forespore, triggering a proteolytic cascade detailed in the expanded view below. α^K RNA polymerase activity in the mother cell results in expression of genes with products that form the spore coat and cause the mother cell to lyse. Bottom, proteolytic cascade that leads to cleavage of Pro-α^K in the B. subtilis mother cell. This expanded view of the two membranes surrounding the forespore shows the signal transduction pathway that begins with SpoIVB in the forespore. SpoIVB crosses the first membrane and cleaves SpoVFA. CtpB from both compartments cleaves BofA. Finally, SpoIVB cleaves Pro-α^K, releasing α^K into the mother cell. See the text for references.

...ing the forespore, initiates a proteolytic cascade that causes degradation of the SpoIVFB inhibitors (23–25) (Fig. 1, bottom). The activated SpoIVFB cleaves Pro-α^K after residue 20 and releases α^K into the mother cell (26–28). Genes transcribed by α^K RNA polymerase are responsible for completing the sporulation process (29), including formation of the spore coat and lysis of the mother cell to release the spore (30) (Fig. 1, top).

SpoIVB has been studied as a model for a large subfamily of IMMPs that contain a CBS domain (31). Named after a domain found in the enzyme cystathionine β-synthase (32), CBS domains are found in thousands of proteins in all kingdoms of life (33). CBS domains undergo a conformational change upon binding a ligand and regulate protein activity in response to cellular energy levels or ion availability (34, 35). The C-terminal CBS domain of SpoIVFB is in the mother cell (Fig. 1, bottom) and has been shown to bind ATP and Pro-α^K (28). ATP is required for purified SpoIVFB to cleave Pro-α^K, so it has been proposed that CBS domains in IMMPs regulate substrate access to the active site in response to the cellular ATP level (28). Interestingly, channels form between the mother cell and the forespore during sporulation (36–38) (Fig. 1, top), and the channels have been proposed to be feeding tubes through which small molecules like ATP might pass from the mother cell to the forespore to allow late-stage gene expression in the forespore (39) and prevent its collapse (40). The SpoIVFB-BofA:SpoIVFA complex co-localizes with channel components (41, 42), and both undergo proteolytic modification upon completion of engulfment (23–25, 38, 42, 43), perhaps resulting in a rise in ATP in the mother cell that is sensed by the CBS domain of SpoIVFB as a signal that the channels have been closed and it is time to cleave Pro-α^K (15, 44). In any case, it remains to be understood how the CBS domain of SpoIVFB regulates its activity in response to ATP and how this is coupled to physiological and morphological changes during sporulation.

Recently, considerable progress has been made toward understanding how SpoIVFB interacts with Pro-α^K using mutational and cross-linking approaches. Mutational analysis revealed features of Pro-α^K important for cleavage by SpoIVFB, including preferences and tolerances somewhat different from those of other IMMPs (45). Disulfide cross-linking between single-Cys versions of Pro-α^K and catalytically inactive SpoIVFB have shown that residues in two conserved loops of SpoIVFB interact with residues near the cleavage site in Pro-α^K (46). Both studies rely on coexpression of proteins in Escherichia coli, where it had been shown that active SpoIVFB can accurately and abundantly cleave C-terminally truncated Pro-α^K(1–126) (22, 47) (Fig. 2A).

Here, we report that catalytically inactive SpoIVFB forms a complex with Pro-α^K(1–126)-His6 upon coexpression in E. coli. The complex can be solubilized from membranes with mild detergents, and it is stable during affinity purification and gel filtration chromatography. Parts of Pro-α^K(1–126)-His6 and SpoIVFB that are necessary for solubilization and affinity purification were identified. Complexes appeared to contain four SpoIVFB and at least two Pro-α^K(1–126)-His6, and evidence for tetrameric SpoIVFB in vivo was obtained. Our work deepens the knowledge of the SpoIVFB quaternary structure and interaction with its substrate and has important implications for further biochemical and structural studies of IMMP substrate recognition for the purpose of therapeutic design.

**Experimental Procedures**

**Plasmids**—Descriptions of the plasmids and primers used in this study are available upon request. DNA sequencing was used to confirm the desired sequences in cloned PCR products and in cloned genes that had been subjected to site-specific mutagenesis (QuickChange kit, Stratagene).

**Cotransformation and Induction**—Two plasmids with different antibiotic resistance genes and different B. subtilis genes...
fused to the T7 RNA polymerase promoter were used for cotransformation of *E. coli* BL21(DE3) and induction of gene expression as described (22), except 0.3 mM isoprpyl-β-D-thio-galactopyranoside (IPTG) was used for 2 h unless stated otherwise. Cells were grown in a 5-liter fermentor with vigorous aeration and pH control or in a flask with shaking, in both cases at 37 °C in Luria-Bertani medium (48) supplemented with antibiotics.

**Cell Fractionation and Detergent Solubilization of Proteins**—Cells (5 g if grown in a fermentor or about 2.5 g if harvested from 1 liter grown in a flask) were resuspended in 15 ml of lysis buffer (PBS containing 0.1 mg/ml lysozyme, 1 mg/ml RNase A, 2 mg/ml DNase I, 1 mM PMSF, 10 mM 2-mercaptoethanol, and 10% glycerol), incubated at 37 °C for 10 min, and passed three times through a French pressure cell (SLM Aminco) at 14,000 p.s.i. (96 megapascals). The cell lysate was centrifuged at low speed (15,000 × g for 15 min at 4 °C) to sediment cell debris and protein inclusion bodies. The supernatant was designated the low-speed supernatant and contains both cytoplasmic and membrane proteins. The low-speed supernatant was centrifuged at high speed (150,000 × g for 90 min at 4 °C) to sediment membrane vesicles. The supernatant was designated the high-speed supernatant and contains cytoplasmic proteins. The pellet was resuspended in 10 ml of resuspension buffer (PBS containing 1 mM Pefabloc SC, 5 mM 2-mercaptoethanol, and 10% glycerol) using a motorized Dounce homogenizer, resulting in the membrane fraction. To solubilize proteins, the membrane fraction was treated with the indicated detergent (1%; all detergents were from Anatrace, Maumee, OH) by rotating the mixture for 1 h at 4 °C. The mixture was then centrifuged at 150,000 × g for 75 min at 12 °C to sediment insoluble material. The supernatant contained detergent-solubilized proteins.

**Immunoblot Analysis**—To analyze cell fractions and detergent-solubilized proteins from the procedure described above or fractions from the cobalt affinity purification described below, samples were mixed with an equal volume of 2 × sample buffer (50 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, and 0.03% bromphenol blue) and boiled for 3 min, and then samples from an equivalent amount of cells were subjected to SDS-PAGE (12% ProSieve polyacrylamide gel) (Lanza) and immunoblotting as described previously (49). After gel filtration chromatography, samples were treated in the same way, except equal volumes of samples were analyzed. To measure expression and cleavage of proteins, an equivalent amount of cells (based on the optical density of the culture at 600 nm) were collected from 0.5 to 1.0 ml of culture by centrifugation (12,000 × g), and cell extracts were prepared as described previously (22), mixed with an equal volume of 2 × sample buffer, and boiled for 3 min; then equal volumes were subjected to SDS-PAGE and immunoblotting as described above. SeeBlue Plus2 prestained protein standard (Invitrogen) was used to judge the migration of protein species. Antibodies that recognize His6 (penta-His, Qiagen, catalogue No. 34460) or FLAG (M2 monoclonal antibody-peroxidase conjugate, Sigma, catalogue No. A8592) were used at 1:5000 or 1:10000 dilution, respectively. These antibodies specifically detected the proteins induced and exhibited very little cross-reaction with other proteins in extracts of *E. coli*. Signals were detected using a LAS-3000 imager (Fujifilm) with exposure times short enough to ensure the signals were not saturated. Signal intensities were quantified using MultiGauge (Fujifilm) software.

**Cobalt Affinity Purification (Pulldown Assays)**—Cells (about 2.5 g harvested from 1 liter grown in a flask) were fractionated as described above. The membrane fraction was treated with 1% n-decyl-β-D-maltoside (DM) to solubilize the proteins and then subjected to high-speed centrifugation as described above. The supernatant (10 ml) was mixed with 0.5 ml of Talon Superflow metal affinity resin (Clontech) that had been equilibrated with buffer (PBS containing 1% DM, 5 mM 2-mercaptoethanol, and 10% glycerol). The mixture was rotated for 1 h at 23 °C. The cobalt resin was sedimented by centrifugation at 708 × g for 2 min at 4 °C. The supernatant was the unbound sample. The cobalt resin was washed three times by rotating briefly and then sedimenting the resin as described above. The wash buffer base (WBB) was PBS containing 150 mM NaCl and 10% glycerol. The three washes were with 5 ml of WBB with 0.5% DM, 5 ml of WBB with 0.1% DM, and 3.5 ml of WBB with 0.1% DM and 80 mM imidazole. Finally, the resin was mixed with 9.5 ml of WBB with 0.1% DM, and then a 500-μl sample was added to 2× sample buffer and boiled for 3 min, resulting in the bound sample.

**Disulfide Cross-linking in Vivo**—A method described previously (50) was used with slight modifications as described recently (46). Based on the measurement of the optical density at 600 nm after induction with IPTG for 30 min, an equivalent amount of cells from 0.5 to 1.0 ml of culture was used to prepare the samples, and equal volumes were subjected to SDS-PAGE and immunoblotting as described above.

**Purification of the SpoIYFB-TEV-FLAG2 E44Q-Pro-αK(1–126)-His6 Complex**—Cells (10 g, grown in a fermentor) were fractionated as described above, except 40 ml of lysis buffer and 20 ml of resuspension buffer was used. The membrane fraction was treated with 1% DM to solubilize the proteins and then subjected to high-speed centrifugation as described above. The supernatant (20 ml) was cobalt affinity-purified as described above, except 1 ml of cobalt resin was used, and after removal of the unbound sample the resin was washed five times by rotating briefly and then sedimenting the resin as described above. The five washes were with 5 ml of WBB with 0.5% DM, 5 ml of WBB with 0.1% DM, 1 ml of WBB with 0.1% DM and 30 mM imidazole, 1 ml of WBB with 0.1% DM and 60 mM imidazole, and 1 ml of WBB with 0.1% DM and 100 mM imidazole. Finally, the resin was eluted with 2 ml of WBB with 0.1% DM and 400 mM imidazole. The eluted material was concentrated to 0.5 ml using an Amicon Ultra centrifugal device with a 10-kDa cut-off (Millipore), and the sample was loaded onto a 1.0 × 30 cm Superdex 200 gel filtration column equilibrated with PBS containing 150 mM NaCl, 5% glycerol, and 0.1% DM. The column was eluted with the same buffer at 0.4 ml/min, and 0.5-ml fractions were collected.

To purify the complex for disulfide cross-linking, cells (about 5 g harvested from 2 liters grown in a flask) were fractionated as described above, except 20 ml of lysis buffer, 10 ml of resuspension buffer, and 0.5 ml of cobalt resin was used. After removal of the unbound sample, the resin was washed three times by rotating briefly and then sedimenting the resin as described above.
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The three washes were with 5 ml of WBB with 0.5% DM, 5 ml of WBB with 0.1% DM, and 1 ml of WBB with 0.1% DM and 30 mm imidazole. Finally the resin was eluted with 1 ml of WBB with 0.1% DM and 400 mm imidazole. The eluted material was loaded directly onto the gel filtration column as described above. Equilibration and elution of the column were as described above.

Disulfide Cross-linking of Purified Proteins—Samples were treated with 1 mm Cu\(^{2+}\) (phenanthroline)\(_3\) or 3 mm 2-phenanthroline for 10 min at 37 °C followed by incubation with 12.5 mm neocuproine for 5 min at 37 °C to terminate the oxidation reaction. Samples were mixed with an equal volume of 2 x sample buffer with or without DTT and incubated for 5 min at 70 °C; then equal volumes were subjected to SDS-PAGE and immunoblotting as described above.

Ion Mobility-Mass Spectrometry (IM-MS)—To purify the SpoIVFB-TEV-FLAG\(_2\), E44Q-Pro-\(\alpha^K\)(1–126)-His\(_6\) complex for IM-MS analysis, cells (7.5 g, grown in a fermentor) were fractionated as described above, except 40 ml of lysis buffer and 40 ml of resuspension buffer was used. The membrane fraction was treated with 1% n-dodecyl-\(\beta\)-d-maltoside (DDM) by rotating the mixture for 1 h at room temperature and then subjected to high-speed centrifugation as described above. The supernatant (40 ml) was cobalt affinity-purified as described above, except 1 ml of cobalt resin was used and 0.1% DDM was substituted for 1% DM in the resin equilibration buffer. After removal of the unbound sample, the resin was washed twice with 5 ml of equilibration buffer and then with 5 ml of WBB with 0.1% DDM and 40 mm imidazole by rotating briefly and then sedimenting the resin as described above. Finally, the resin was eluted with 3 ml of WBB with 0.1% DDM and 400 mm imidazole. The eluted material was concentrated to 0.5 ml and loaded onto the gel filtration column as described above, except the column was equilibrated with buffer containing 150 mm ammonium acetate, pH 7.5, and 0.02% DDM. The column was eluted with the same buffer at 0.5 ml/min, and 0.5-ml fractions were collected. Samples were subjected to SDS-PAGE, the gel was stained with Coomassie Blue, and 3 or 4 fractions containing the most SpoIVFB-TEV-FLAG\(_2\) E44Q-Pro-\(\alpha^K\)(1–126)-His\(_6\) complex were selected, and for each a 325/550-nm band pass filter. Images were collected continuously for 30 s (to allow photobleaching) using 40-ms exposures on a 16-bit Andor DU-897 X-9795 camera set at the 10 MHz readout mode. The Nikon Perfect focus system was used to keep cells in focus during imaging. The objective was moved to a new field of view before excitation and the start of data collection to avoid missed photobleaching events.

Small foci believed to represent SpoIVFB-eGFP molecules were selected, and for each a 3 x 3 pixel region of interest (ROI) was drawn centered over the most intense pixel in the first image of the time lapse series. The total intensity of each ROI during each exposure was recorded and exported from ImageJ (53). A noise reduction algorithm (NoRSE) was used to extract an average intensity profile that aided in counting photobleaching steps (54). This algorithm was used with MATLAB (MathWorks, version R2014a 8.3.0.532). To count steps, the raw intensity and the noise-reduced profiles were plotted together. A step was defined as a decrease in intensity that was greater than noise and for which the decrease was approximately equal to other steps for that ROI. Steps were counted until the end of the time lapse series or until steps stopped occurring (steps were followed by continuous bleaching of autofluorescence). ROIs were discarded if noise exceeded steps in a major portion of the profile, if photobleaching appeared continuous rather than stepwise, or if there were inconsistent step sizes. Confidence and statistical tests (\(\gamma\) and \(\theta\)) were computed using R, version 3.2.2, to perform a Bayesian analysis designed to test support for the inferred subunit stoichiometry (55).

The dye FM 4-64 was used to stain the membranes of sporeulating cells as described previously (56). Briefly, samples collected as described above were treated with FM 4-64 (1 \(\mu g/ml\)) on ice for 30 min prior to imaging. FM 4-64 fluorescence was imaged using TIRF microscopy as described above with the following changes. Excitation used a 514-nm laser diode at 6% intensity, and emission was captured using a 600–650-nm band pass filter. Fluorescence from eGFP and step counting was carried out as described above after imaging FM 4-64. To merge the fluorescence from FM 4-64 and eGFP, duplicate FM 4-64 images of each field of view were first subjected to a
12-pixel-size Gaussian blur, which was subtracted from each original image to remove background fluorescence due to the poly-L-lysine-coated coverslips. The ImageJ “find edges” feature was used to reduce the diffuse edges of the membrane to more defined edges. The duplicate images were then averaged to reduce noise. For eGFP the first 5 images of the time series were averaged to reduce noise, and the average was convolved using the default settings of ImageJ. The resulting images from these processing strategies were then merged to show the localization of SpoIVFB-eGFP with respect to membranes of sporulating cells.

**Results**

**Effect of Pro-αK(1–126)-His6 Coexpression on Solubilization of SpoIVFB from Membranes**—We discovered that when Pro-αK(1–126)-His6 was coexpressed with catalytically inactive SpoIVFB-TEV-FLAG2, E44Q in *E. coli*, both proteins were readily solubilized by a variety of detergents (all detergents were used at 1% for solubilization of proteins from membranes). The nonionic detergent sodium dodecanoyl sarcosine (sarkosyl), the lipophilic detergents DM and DDM, the lipophilic detergents n-dodecylphosphocholine and 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine, and the zwitterionic detergents n-dodecyl-N,N-dimethylamine-N-oxide and 3-dodecylamido-N,N-dimethylpropyl amine oxide appeared to be equally effective at solubilization, whereas the nonionic detergent octaethylene glycol monododecyl ether (C12E8) appeared to be less effective (Fig. 2B). These results were surprising, because a previous study had shown that when cytTM-SpoIVFB-FLAG2-His6 alone was expressed in *E. coli*, sarkosyl was much more effective at solubilization than DM or DDM (28). The difference was not due to the tobacco etch virus (TEV) protease cleavage site in SpoIVFB-TEV-FLAG2, E44Q, because when cytTM-SpoIVFB-TEV-FLAG2-His6 alone was expressed in *E. coli*, it was readily solubilized by sarkosyl but poorly solubilized by nonionic detergents (Fig. 2C), like TM-SpoIVFB (28).

Also, cytTM-SpoIVFB-FLAG2, E44Q lacking the TEV site but having an extra TMS from rabbit cytochrome P450 2B4 (cytTM) (57), shown previously to enhance SpoIVFB accumulation in *E. coli* (28), was readily solubilized by DM only if Pro-αK(1–126)-His6 was coexpressed (Fig. 3D, lanes 2 and 10). We concluded that coexpression of Pro-αK(1–126)-His6 with SpoIVFB E44Q variants dramatically improves solubilization of the catalytically inactive enzyme from membranes with mild, nonionic detergents and also allows it to be solubilized with lipid-like or zwitterionic detergents.

It is worth noting that when both Pro-αK(1–126)-His6 and SpoIVFB-TEV-FLAG2, E44Q were coexpressed from the same plasmid (pYZ42), cytTM was not necessary for the SpoIVFB E44Q variant to accumulate abundantly. This did not depend on the TEV site or the E44Q substitution, because SpoIVFB-FLAG2, His6 accumulated abundantly and cleaved Pro-αK(1–126)-His6 when both proteins were coexpressed from the same plasmid (pYZ6) (data not shown). In both plasmids, Pro-αK(1–126)-His6 is expressed from a T7 RNA polymerase promoter, and downstream a second such promoter drives expression of SpoIVFB. We did not investigate whether this arrangement of promoters or some other aspect of coexpression from the same plasmid would explain the abundant accumulation of SpoIVFB lacking cytTM. Also worth noting is that in the figures we labeled all SpoIVFB variants as “SpoIVFB” and all Pro-αK(1–126)-His6 variants as “Pro-αK” for simplicity and to conserve space, but in the figure legends and elsewhere in the text we have fully specified the variants used in the experiments for clarity.

**Parts of Pro-αK(1–126)-His6 Required to Improve Solubilization of cytTM-SpoIVFB-FLAG2, E44Q from Membranes and to Form a Stable Complex**—To determine whether parts of Pro-αK(1–126)-His6 would be sufficient to improve the solubilization of cytTM-SpoIVFB-FLAG2, E44Q from membranes with DM, we engineered chimeras containing part of Pro-αK(1–126)-His6 and either green fluorescent protein (GFP) or cytTM (Fig. 3A). We engineered Pro-αK(1–31)-GFP, His6, because previous studies indicated that the Pro part leads to cellular localization of SpoIVFB-eGFP with respect to membranes of sporulating cells.

**FIGURE 2. Coexpression of Pro-αK(1–126)-His6 and SpoIVFB in *E. coli* and effect on solubilization of SpoIVFB from membranes.** A, *E. coli* cell and an expanded view of the inner (IM) and outer membrane (OM). Pro-αK(1–126)-His6 associates peripherally with the inner membrane (45). The topology of SpoIVFB was determined previously (77). SpoIVFB has a C-terminal CBS domain, and in some experiments an extra TMS (cytTM) was added to its N-terminal end. B, solubilization of coexpressed proteins. *E. coli* bearing pYZ42 was grown in a fermentor and induced with IPTG to coexpress Pro-αK(1–126)-His6 and SpoIVFB-TEV-FLAG2, E44Q. The membrane (M) fraction from 5 g of cells was untreated or treated with the indicated detergent to solubilize proteins. After high-speed centrifugation, the supernatant was subjected to immunoblot analysis with anti-FLAG (top) or anti-His (bottom) antibodies. PC, n-dodecylphosphocholine; LMPC, 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine; DDAO, n-dodecyl-N,N-dimethylamine-N-oxide; LAPAO, 3-dodecylamido-N,N-dimethylpropyl amine oxide. C, solubilization of enzyme alone. This is the same as in A, except pYZ250 was used to express cytTM-SpoIVFB-TEV-FLAG2, His6, alone, and immunoblot analysis was done with only anti-FLAG antibodies. A dashed white line was added to help visualize the demarcation between lanes 1 and 2. In both panels, representative results from at least two biological replicates are shown.
Pro-\(\sigma^K\) is necessary for association with membranes (28, 47, 58) and that residues 1–27 fused to GFP allow membrane association upon expression in \(E. coli\) (47). Hence, this chimera was designed to test whether the Pro part plus a few residues of \(\sigma^K\) would be sufficient to improve the solubilization of SpoIVFB.

Conversely, we engineered cytTM-\(\sigma^K(21–126)-\text{His}_6\) to test whether the \(\sigma^K\) part, associated with membrane via a generic TMS, would be sufficient to improve the solubilization of SpoIVFB. The TMS we used, cytTM, enhances the accumulation of a protein, as seen with TM-SpoIVFB (28), and/or pro-

![FIGURE 3. Effect of Pro-\(\sigma^K(1–126)-\text{His}_6\) derivatives on solubilization of cytTM-SpoIVFB-FLAG2 E44Q and interaction with it. A, diagram of chimeras and deletion derivatives. Pro-\(\sigma^K(1–126)-\text{His}_6\) associates peripherally with the \(E. coli\) inner membrane (IM) (45). cytTM (thick black segment) is expected to insert into the inner membrane like a typical TMS. GFP is depicted as a gray segment. All proteins have a C-terminal His tag that is not shown. Chimeras and deletions are described in the text and are expressed from the listed plasmids. B, cleavage of Pro-\(\sigma^K(1–126)-\text{His}_6\), derivatives by TM-SpoIVFB. \(E. coli\) bearing pZR209 to express TM-SpoIVFB as indicated at the top and/or the plasmids listed in A to express Pro-\(\sigma^K(1–126)-\text{His}_6\), derivatives, indicated at the bottom, were cultured (10 ml) and induced with IPTG. Extracts were subjected to immunoblot analysis with anti-His antibodies. The cleavage product is indicated (lanes 2 and 4). Representative results from at least two biological replicates are shown. C, protein expression and initial fractionation. \(E. coli\) bearing pYZ68 to express cytTM-SpoIVFB-FLAG2 E44Q alone or in combination with the plasmids listed in A to coexpress Pro-\(\sigma^K(1–126)-\text{His}_6\), derivatives, indicated at the bottom, were cultured (1 liter) and induced with IPTG. Cell lysates were fractionated by centrifugation to produce low-speed supernatant (LSS), high-speed supernatant (HSS), and membrane fractions (M) containing cytoplasmic and membrane proteins, cytoplasmic proteins, and membrane proteins, respectively. Samples were subjected to immunoblot analysis with anti-FLAG (top) or anti-His (bottom) antibodies. This is the same as in C, except the membrane fraction was treated with DM to solubilize cytTM-SpoIVFB-FLAG2 E44Q and coexpressed protein followed by high-speed centrifugation and collection of a sample of the supernatant (DM). The rest of the supernatant was subjected to cobalt affinity purification of coexpressed protein and interacting cytTM-SpoIVFB-FLAG2 E44Q, resulting in unbound (U) and bound (B) samples, which were subjected to immunoblot analysis with anti-FLAG (top) or anti-His (bottom) antibodies. D, quantification of cytTM-SpoIVFB-FLAG2 E44Q solubilization by DM in the absence or presence of coexpressed proteins. The immunoblots shown in D plus two biological replicates were quantified, and the signal of the DM-solubilized sample (DM) was expressed as a percentage of the signal of the membrane fraction (M). Error bars show 1 S.D. The average with coexpressed \(\Delta 2–8\) was significantly higher than with \(\Delta 2–6\) (p < 0.05, Student’s t test).

F, quantification of cytTM-SpoIVFB-FLAG2 E44Q after cobalt affinity purification in the absence or presence of coexpressed proteins. The immunoblots shown in D plus two biological replicates were quantified, and the signals of the bound and unbound samples were expressed as a ratio. Error bars show 1 S.D. The indicated averages were compared using a Student’s t test.
motes membrane association of a protein. As a negative control, we engineered cytTM-GFP-His<sub>6</sub>. We also tested two deletion derivatives of Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> reported recently to be membrane-associated when expressed in E. coli; one lacking residues 2–6 (Δ2–6-His<sub>6</sub>), which was cleaved by coexpressed TM-SpoIVFB in E. coli, and the other lacking residues 2–8 (Δ2–8-His<sub>6</sub>), which was not cleaved (45). As noted above, Δ2–6-His<sub>6</sub> but not Δ2–8-His<sub>6</sub> was reported to be cleaved by coexpressed TM-SpoIVFB in E. coli (45). We verified this finding and also found that none of the other His-tagged proteins were cleaved by coexpressed TM-SpoIVFB (Fig. 3B).

To measure solubilization from membranes and stable complex formation, each C-terminally His<sub>6</sub>-tagged protein described above was coexpressed in E. coli with cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q, as expected, cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q was present in the membrane fraction whether expressed alone or coexpressed with any of the His-tagged proteins, and each of the His-tagged proteins was predominantly in the membrane fraction, although small portions of Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> and its deletion derivatives remained in the cytosolic fraction after high-speed centrifugation (Fig. 3C). As noted above, cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q expressed alone was poorly solubilized from the membrane fraction by DM, and coexpressed Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> greatly improved its solubilization (Fig. 3D, lanes 2 and 10). In contrast, coexpressed cytTM-GFP-His<sub>6</sub> did not improve the solubilization of cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q (Fig. 3D, lane 6), as expected, because cytTM-GFP-His<sub>6</sub> does not contain any part of Pro-α<sup>K</sup>(1–126). Coexpressed cytTM-α<sup>K</sup>(21–126)-His<sub>6</sub> (Fig. 3D, lane 14), Pro-α<sup>K</sup>(1–31)-GFP-His<sub>6</sub> (lane 18), or Δ2–8-His<sub>6</sub> (lane 22) did not appear to improve the solubilization of cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q as much as Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> (lane 10), but Δ2–6-His<sub>6</sub> appeared to improve solubilization nearly as well (lane 26). Quantification from three experiments revealed that Δ2–6-His<sub>6</sub> was significantly better than Δ2–8-His<sub>6</sub> at improving the solubilization of cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q (Fig. 3E). Therefore, for all of the His-tagged proteins, including two proteins differing by just two residues, the ability to serve as substrate for the active enzyme (Fig. 3B) was positively correlated with the ability to improve the solubilization of the catalytically inactive enzyme (Fig. 3, D and E). We concluded that both the Pro(1–20) and α<sup>K</sup>(21–126) parts of Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> contribute to its ability to both serve as a substrate of TM-SpoIVFB and improve the solubilization of cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q from membranes.

It is worth noting that Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> itself was poorly solubilized by DM, and the other His-tagged proteins behaved similarly, except for cytTM-α<sup>K</sup>(21–126)-His<sub>6</sub>, which was readily solubilized (Fig. 3D, lane 14). Partial solubilization of Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> overproduced in E. coli was observed previously with the nonionic detergent Triton X-100 (1%), and this was attributed to interaction with itself and/or other cellular components resulting in detergent-insoluble aggregates too small to sediment upon low-speed centrifugation but large enough to sediment upon high-speed centrifugation (45).

Our experimental design allowed cobalt affinity purification of the His-tagged proteins to examine their interaction with cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q (i.e., pulldown assays). Proteins solubilized by DM were incubated with cobalt beads, and the unbound fraction was compared with the bound fraction using immunoblots. As expected, the major portion of each His-tagged protein was in the bound fraction (Fig. 3D), and cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q remained unbound when expressed alone (lanes 3 and 4) or in combination with cytTM-GFP-His<sub>6</sub> (lanes 7 and 8). In contrast, the major portion of cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q was in the bound fraction upon coexpression with Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> (Fig. 3D, lanes 11 and 12), indicative of stable complex formation. Interestingly, cytTM-α<sup>K</sup>(21–126)-His<sub>6</sub> appeared to interact with cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q, because a considerable portion was in the bound fraction (Fig. 3D, lanes 15 and 16), but this was not the case for Pro-α<sup>K</sup>(1–31)-GFP-His<sub>6</sub> (lanes 19 and 20). Δ2–8-His<sub>6</sub> (Fig. 3D, lanes 23 and 24) and especially Δ2–6-His<sub>6</sub> (lanes 27 and 28) also appeared to interact with cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q. Quantification from three experiments (Fig. 3F) revealed that Δ2–6-His<sub>6</sub> may be significantly better than Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> at forming a stable complex with cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q (p ≈ 0.05, Student’s t test), suggesting that residues 2–6 inhibit the interaction of Pro-α<sup>K</sup> with SpoIVFB. Loss of just two more residues in Δ2–8-His<sub>6</sub> or replacement of the Pro part with cytTM in cytTM-α<sup>K</sup>(21–126)-His<sub>6</sub> resulted in similar levels of interaction, as observed for Pro-α<sup>K</sup>(1–126)-His<sub>6</sub>. As all the His-tagged proteins containing the α<sup>K</sup> part of Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> interacted with cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q better than did Pro-α<sup>K</sup>(1–31)-GFP-His<sub>6</sub>, we concluded that the α<sup>K</sup> part of Pro-α<sup>K</sup>(1–126)-His<sub>6</sub> is required to form a stable complex with cytTM-SpoIVFB-FLAG<sub>2</sub> E44Q upon solubilization from membranes with DM and that the Pro part is insufficient for stable complex formation under these conditions.
To ensure that single-Cys Pro-\(\sigma^K(1–126)\)-His\(_6\), N24C (or Y21C or a Cys-less version that served as a negative control) was not limiting for complex formation with single-Cys TM-SpoIVFB E44C or its C\(\Delta\)10 derivative, and in each case Pro-\(\sigma^K(1–126)\)-His\(_6\) accumulated in excess of TM-SpoIVFB E44C or its C\(\Delta\)10 derivative (Fig. 4B). Cells coexpressing the same combinations of enzyme and substrate were treated with the oxidant Cu\(^{2+}\) (phenanthroline)\(_3\) to promote disulfide bond formation between Cys residues in proximity; then cells were lysed and proteins were precipitated by the addition of TCA, and finally samples were subjected to immunoblot analysis without or with DTT treatment to reverse disulfide cross-links. As shown previously (46), antibodies against the FLAG tags present in full-length TM-SpoIVFB E44C detected two species that migrated more slowly than the monomer in some of the samples (Fig. 4C), corresponding to a cross-linked complex with the N24C (lane 3) or Y21C (lane 5) versions of Pro-\(\sigma^K(1–126)\)-His\(_6\) and to a cross-linked dimer of TM-SpoIVFB E44C (lanes 1, 3, and 5), all of which were not present upon treatment with DTT, as expected (lanes 2, 4, and 6). Cys-less Pro-\(\sigma^K(1–126)\)-His\(_6\) cannot form a cross-linked complex, so the faint species migrating at a similar position as the cross-linked complex represents the background of the assay (Fig. 4C, lane 1). The C\(\Delta\)10 derivative of TM-SpoIVFB E44C failed to form a cross-linked complex with the N24C (Fig. 4C, lane 7) or Y21C (lane 9) versions of Pro-\(\sigma^K(1–126)\)-His\(_6\), showing only the faint background species. We concluded that the C\(\Delta\)10 derivative fails to interact with Pro-\(\sigma^K(1–126)\)-His\(_6\) in a way that brings the SpoIVFB active site into proximity with residues near the substrate cleavage site. The results do not distinguish between a lack of interaction and an altered interaction. In either case, the abnormal interaction presumably explains why TM-SpoIVFB C\(\Delta\)10 fails to cleave Pro-\(\sigma^K(1–126)\)-FLAG\(_2\) (28) and why neither Pro-\(\sigma^K(1–126)\)-FLAG\(_2\) nor single-Cys Pro-\(\sigma^K(1–126)\)-His\(_6\) N24C coexpression facilitated solubilization of catalytically inactive TM-SpoIVFB from membranes with DM (Fig. 4A).

**Purification and Characterization of SpoIVFB-FLAG\(_2\), E44Q-**

Pro-\(\sigma^K(1–126)\)-Histidine Complexes—As noted above, Pro-\(\sigma^K(1–126)\)-His\(_6\) and SpoIVFB-TEV-FLAG\(_2\), E44Q both accumulate abundantly when coexpressed from pYZ42. We took advantage of this and used the cobalt affinity (pulldown) strategy described above for initial purification of a complex between the two proteins (Fig. 5A). After concentrating the bound fraction, it was subjected to gel filtration chromatography, monitoring the effluent absorbance at 280 nm and characterizing the fractions by immunoblot. The absorbance of the effluent reached a maximum in fractions 21–23, and this was followed by a smaller peak in fractions 29–31 (Fig. 5B). Immunoblotting showed that the larger peak contained both Pro-\(\sigma^K(1–126)\)-His\(_6\) and SpoIVFB-TEV-FLAG\(_2\), E44Q, whereas the smaller peak and subsequent fractions contained only Pro-\(\sigma^K(1–126)\)-His\(_6\) (Fig. 5C). Because Pro-\(\sigma^K(1–126)\)-His\(_6\) alone was retained for a longer time on the column, it may be monomeric and/or in relatively small multimeric complexes. Pro-\(\sigma^K(1–126)\)-His\(_6\) in complex with SpoIVFB-TEV-FLAG\(_2\), E44Q eluted earlier from the column, indicative of a relatively large complex. As a control, cells expressing only Pro-\(\sigma^K(1–126)\)-His\(_6\) were subjected to the same purification protocol, and Pro-\(\sigma^K(1–126)\)-His\(_6\) was not found in the early-eluting fractions from the gel filtration column (Fig. 5D). Taken together, the results suggest that Pro-
Complex of Bacillus subtilis SpoIVFB with Pro-\(\sigma^K\)

We repeated the purification several times and found the absorbance pattern in the effluent of the gel filtration column to be reproducible (data not shown). Fractions from the column were subjected to SDS-PAGE as described for immunoblot analysis, but in this case gel electrophoresis was followed by Coomassie Blue staining of proteins. The early-eluting peak fractions 22–24 were subjected to SDS-PAGE followed by Coomassie Blue staining of proteins. The early-eluting peak fractions 22–24 were pooled, and samples of the pooled fractions were treated with 1 mM DTT and 1 mM 2-phenanthroline for 10 min (\(\text{DTT} + \text{2-phenanthroline}\)) as a control and then mixed with sample buffer with or without DTT as indicated and subjected to immunoblot analysis with antibodies against the FLAG tag on single-Cys cytTM-SpoIVFB-FLAG2 E44C (lanes 1–3) or against the His\(_6\) tag on single-Cys Pro-\(\sigma^K\)(1–126)-His\(_6\) K23C (lane 4). The immunoblot was cut between lanes 3 and 4 and realigned based on the migration of prestained marker proteins, for which sizes in kDa are indicated along the right side. Representative results from two biological replicates are shown.

\(\sigma^K\)(1–126)-His\(_6\) forms a complex with SpoIVFB-TEV-FLAG2 E44Q that is stable during gel filtration chromatography.

To estimate the size of the protein complex in the early-eluting peak fraction, we quantified the two species in peak fraction 23 in this and another experiment. The upper species was 3-fold more intense than the lower species. If the upper species is exclusively SpoIVFB-TEV-FLAG2 E44Q and the lower species is exclusively Pro-\(\sigma^K\)(1–126)-His\(_6\), and if the two proteins stain equally well, the upper species should be 2.5-fold more intense than the lower species if the complex has 1:1 stoichiometry. Therefore, Pro-\(\sigma^K\)(1–126)-His\(_6\) appeared to be slightly substoichiometric compared with SpoIVFB-TEV-FLAG2 E44Q in the early-eluting peak fraction.
the $K_{on}$ of the protein complex (0.22) was used to estimate its molecular mass, yielding 360 kDa (Fig. 5f). This estimate is slightly larger than the estimate of 320 kDa for TM-SpoIVFB solubilized from membranes with sarkosyl after expression in *E. coli* as found previously (28); in that study, it was assumed that detergent associated with protein doubles the apparent molecular weight, so it was inferred that TM-SpoIVFB is tetrameric. If the same assumption is made for the complex of Pro-\(\sigma^B\)(1–126)-His6 with SpoIVFB-TEV-FLAG2 E44Q, our data suggest that the protein complex is about 180 kDa, which is close to the size expected (208 kDa) for a complex containing four monomers of each protein. Some complexes may contain less than four monomers of Pro-\(\sigma^B\)(1–126)-His6, as it appeared to be slightly substoichiometric compared with SpoIVFB-TEV-FLAG2 E44Q in the early-eluting peak fraction described above.

To test whether a purified SpoIVFB-FLAG2 E44Q-Pro-\(\sigma^B\)(1–126)-His6 complex resembles the complex formed when the two proteins are coexpressed in *E. coli*, we employed single-Cys versions of the proteins so that purified complexes could be subjected to disulfide cross-linking to probe the proximity between the enzyme active site and the substrate cleavage site. We constructed single-Cys cytTM-SpoIVFB-FLAG2 E44C for this experiment and coexpressed it in *E. coli* with single-Cys Pro-\(\sigma^B\)(1–126)-His6 K23C, because E44C of the enzyme had been shown previously to cross-link abundantly with K23C of the substrate upon coexpression in *vivo* (46). Cobalt affinity purification followed by gel filtration chromatography and immunoblotting yielded results similar to those presented in Fig. 5G, A–C (data not shown). Both single-Cys Pro-\(\sigma^B\)(1–126)-His6 K23C and single-Cys cytTM-SpoIVFB-FLAG2 E44C reached a maximum in early-eluting fractions 22–24; late-eluting fractions 29–31 contained predominantly single-Cys Pro-\(\sigma^B\)(1–126)-His6 K23C. Fractions 22–24, pooled and treated with Cu(II) (phenanthroline)3+ to promote disulfide bond formation, were then subjected to immunoblot analysis without or with DTT treatment to reverse cross-links. Single-Cys Pro-\(\sigma^B\)(1–126)-His6 K23C could be cross-linked to single-Cys cytTM-SpoIVFB-FLAG2 E44C, forming a complex that migrates more slowly than the monomer of single-Cys cytTM-SpoIVFB-FLAG2 E44C (Fig. 5G, lane 2). This species, as well as one that migrated at the position expected for a dimer of single-Cys cytTM-SpoIVFB-FLAG2 E44C, was much less abundant upon treatment with DTT, as expected for reversal of disulfide cross-links (Fig. 5G, lane 3). An immunoblot of the cross-linked sample was probed with anti-His antibodies to detect single-Cys Pro-\(\sigma^B\)(1–126)-His6 K23C, confirming its presence in the species migrating at the position expected for a cross-linked complex (Fig. 5G, lane 4). A species migrating at the position expected for cross-linked single-Cys Pro-\(\sigma^B\)(1–126)-His6 K23C dimer was also observed (Fig. 5G, lane 4). We concluded that in the purified complex, the active site of cytTM-SpoIVFB-FLAG2 (represented by E44C) is in close proximity to the cleavage site in Pro-\(\sigma^B\)(1–126)-His6 (represented by K23C), suggesting that the purified complex resembles the complex formed *in vivo*.

**IM-MS Analysis of the SpoIVFB-TEV-FLAG2 E44Q-Pro-\(\sigma^B\)(1–126)-His6 Complex**—To purify the complex for IM-MS analysis, we modified the purification procedure to produce samples containing low levels of DDM or C8E4 detergent in ammonium acetate buffer (see “Experimental Procedures” for details). Briefly, we substituted DDM for DM during cobalt affinity purification, and we used ammonium acetate buffer containing a low concentration of DDM (0.02%) during gel filtration chromatography. The changes resulted in a higher yield of the complex with a slightly different elution profile in which the larger peak was preceded by a shoulder and the smaller peak was more prominent (compare Fig. 6, A and B). This appears to reflect more dissociation of Pro-\(\sigma^B\)(1–126)-His6 from SpoIVFB-TEV-FLAG2 E44Q during gel filtration chromatography (Fig. 6B). Fractions 25–27 containing the most SpoIVFB-TEV-FLAG2 E44Q-Pro-\(\sigma^B\)(1–126)-His6 complex were pooled, concentrated, and buffer-exchanged into ammonium acetate buffer containing low levels of DDM or C8E4 for IM-MS analysis.

To identify the mass of the complex, we used gentle collision-induced dissociation (CID) conditions to remove lipid and detergent molecules bound to the surface of the protein complex. Analysis resolved an observed mass of 183.2 kDa (Fig. 6C), agreeing well with the expected mass of a 4:2 SpoIVFB-TEV-FLAG2 E44Q-Pro-\(\sigma^B\)(1–126)-His6 complex (176.4 kDa). The difference between the observed and expected masses (6.8 kDa) is likely due to lipid or detergent molecules that remained bound to the complex (51).

Samples prepared in DDM were further heated collisionally upon post-ion mobility (post-IM) separation to confirm our stoichiometry assignment. This approach exploited the fact that product ions produced by post-IM CID share the same arrival time as their antecedent precursor ions, enabling the mass assignment of precursor stoichiometry through the accurate correlation of precursor and product ions (59–62). This method was used because lipid and detergent adducts inhibited the quadrupole isolation of the 4:2 complex. Post-IM CID analysis of the data presented in Fig. 6C (transfer voltage 240 V, drift time dimension (Fig. 6D)) enabled the identification of three dissociation products from the 4:2 complex. Mass analysis of these dissociation productssupported the identification of monomeric Pro-\(\sigma^B\)(1–126)-His6, along with two different masses in agreement with a 1:1 SpoIVFB-TEV-FLAG2 E44Q-Pro-\(\sigma^B\)(1–126)-His6 complex (55.3 and 56.6 kDa, respectively). These two closely related masses support the conclusion that some lipid or detergent molecules are retained within the complex (51).

Additional analyses using 0.5% C8E4 supported the identification of further masses corresponding to monomeric SpoIVFB-TEV-FLAG2 E44Q and 1:2 and 4:2 SpoIVFB-TEV-FLAG2 E44Q-Pro-\(\sigma^B\)(1–126)-His6 complexes (data not shown). A list of the observed masses and charge state distributions for these complexes, as well as those from Fig. 6, C and D, is available upon request. Although we do not understand why post-IM CID analysis detected different dissociation products when the complex was buffer-exchanged into two different detergents, in both cases the native IM-MS analysis resolved an observed mass that agreed well with the expected mass of a 4:2 SpoIVFB-TEV-FLAG2 E44Q-Pro-\(\sigma^B\)(1–126)-His6 complex.

**Stepwise Photobleaching of SpoIVFB-eGFP in Vivo**—The *in vitro* analyses described above predicted that SpoIVFB is tetrameric. To test this prediction *in vivo*, stepwise photobleaching
Complex of Bacillus subtilis SpoIVFB with Pro-σK

Our results include several important advances: 1) the discovery that substrate improves the solubilization of inactive SpoIVFB from membranes with mild detergent and that the complex is stable during purification; 2) the identification of parts of SpoIVFB and Pro-σK important for solubilization and complex formation; 3) the use of IM-MS to determine the mass and composition of the complex, which agreed well with a 4:2 enzyme-substrate complex; and 4) the use of stepwise photobleaching to obtain evidence that SpoIVFB is tetrameric during sporulation. The implications of these advances are discussed below.

Discussion

was performed on SpoIVFB fused at its C terminus to eGFP and expressed from the native spoIVF promoter during sporulation. Photobleaching of a fluorescent protein can decrease its fluorescence intensity in a stepwise fashion over time, revealing the number of subunits in a multisubunit protein (63–67). We used TIRF microscopy to limit excitation to the evanescent field and only excite SpoIVFB-eGFP near the coverslip. Small, fluorescent foci believed to represent individual molecules were analyzed for the number of photobleaching steps observed before continuous photobleaching of autofluorescence ensued. Most fluorescent foci were larger and likely represented multiple molecules (data not shown); these were not analyzed. The inset in Fig. 7A shows representative large and small foci in a single sporulating cell. For the small focus, the raw fluorescence intensity as a function of time during excitation and the noise-reduced profile were plotted together to illustrate the type of data used to count photobleaching steps (Fig. 7A). The small foci with countable photobleaching steps appeared to be localized to the mother cell membrane engulfing the forespore (Fig. 7B), as expected for sporulating cells at 3 h poststarvation based on standard fluorescence microscopy of SpoIVFB-GFP and membranes stained with FM 4-64 reported previously (42). The number of steps counted for many such foci is summarized in Fig. 7C. The majority of foci exhibited 4 steps, consistent with SpoIVFB-eGFP being tetrameric. Statistical analysis of the data supported this conclusion; using Bayesian inference, confidence was only slightly higher for 4 steps than 5 steps, but analysis suggested that a count of 5 steps was erroneous (Table 1). Also, the value of θ, representing the likelihood of observing every photobleaching event given the data, was highest for 4 steps and decreased with increasing step numbers (note that although 6-step profiles were not observed, the θ value for 6 steps was calculated to show that unobserved steps beyond step 5 would be unlikely). We concluded that stepwise photobleaching provides evidence that SpoIVFB is tetrameric in sporulating cells, in agreement with predictions based on in vitro analyses of the SpoIVFB-TEV-FLAG2 E44Q-Pro-σK(1–126)-His6 complex (Figs. 5 and 6) and of TM-SpoIVFB solubilized from membranes with the harsh, ionic detergent sarkosyl (28).

Our results include several important advances: 1) the discovery that substrate improves the solubilization of inactive SpoIVFB from membranes with mild detergent and that the complex is stable during purification; 2) the identification of parts of SpoIVFB and Pro-σK important for solubilization and complex formation; 3) the use of IM-MS to determine the mass and composition of the complex, which agreed well with a 4:2 enzyme-substrate complex; and 4) the use of stepwise photobleaching to obtain evidence that SpoIVFB is tetrameric during sporulation. The implications of these advances are discussed below.
Complex of Bacillus subtilis SpoIVFB with Pro-αK

Statistical analysis of stepwise photobleaching data

<table>
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<tr>
<th>No. of steps</th>
<th>Confidence</th>
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<td>5</td>
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<tr>
<td>6a</td>
<td>NAa</td>
<td>NAa</td>
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* No foci with 6 photobleaching steps were observed, but θ could nevertheless be calculated, showing that the probability of having missed any 6-step profiles was relatively small.
  a Not applicable.

Solubilization of an IMMP-Substrate Complex from Membranes—Our discoveries that Pro-αK(1–126)-His₆ improves the solubilization of catalytically inactive SpoIVFB from membranes with mild detergents and that the complex is stable during purification are important advances for several reasons. First, proteins solubilized with mild, nonionic detergents are more likely to retain their native structure and be amenable to crystallization efforts (68). The process of membrane protein solubilization by detergents is complex and not well understood. It appears typically to involve replacement of protein-lipid interactions with protein-detergent interactions in micelles, although some lipids often remain associated with the protein (68). Ionic detergents have charged head groups that in many cases facilitate membrane protein solubilization but may also alter protein structure and diminish activity. The ionic detergent sarkosyl was used previously for initial solubilization of overexpressed TM-SpoIVFB from E. coli membranes followed by a transition to nonionic detergent in an effort to preserve activity, which proved successful (28). However, partial loss of activity and alteration of structure could not be ruled out. It seemed possible that the interaction of the substrate with the enzyme might change its oligomeric state and/or its interactions with lipids in a way that facilitated solubilization from membranes. In recent studies of other types of IPs, small substrate mimics or inhibitors induced conformational changes ranging from modest shifts of TM-Ss and loops seen in rod-shaped GlpG crystal structures (69–71) to widespread domain movements observed in presenilin-containing γ-secretase complexes using cryoelectron microscopy methods (72). Our results show that the SpoIVFB-TEV-FLAG₂ E₄₄Q-Pro-αK(1–126)-His₆ complex is efficiently solubilized from membranes with nonionic detergents, as well as with lipid-like zwitterionic detergents (Fig. 2A). After affinity purification, the complex was fairly stable during gel filtration chromatography, although the large peak of absorbance at 280 nm was broad (Fig. 5B), suggesting some degree of heterogeneity in the composition of the complex. If the oligomeric complex dissociates slowly after affinity purification, a variety of smaller complexes could be produced and result in peak broadening. In some experiments, a very small peak preceded the large peak, and the small peak contained SpoIVFB-TEV-FLAG₂ E₄₄Q, based on immunoblotting, and eluted near the void volume of the column (data not shown), suggesting that protein aggregates had formed. In other experiments, the small peak was not observed, but the large peak was preceded by a shoulder (Fig. 5B) containing SpoIVFB-TEV-FLAG₂ E₄₄Q (Fig. 5C). SpoIVFB may have a tendency to aggregate after dissociation from Pro-αK(1–126)-His₆. It might be necessary to optimize detergent and salt in the buffer to stabilize the SpoIVFB-TEV-FLAG₂ E₄₄Q-Pro-αK(1–126)-His₆ complex to facilitate further biochemical analyses and future structural studies (e.g. x-ray crystallography and cryoelectron microscopy).

A second reason that our discoveries are important is that other IMMP-substrate complexes might exhibit similar solubilization and stability characteristics as the SpoIVFB-TEV-
FLAG, E44Q-Pro–αK(1–126)-His6 complex. To our knowledge, the only other IMMPs reported to be successfully solubilized from membranes are a fragment of a Methanocaldovoraceae jacobsii enzyme (referred to as mjS2P) lacking its CBS domain (17) and E. coli RseP (73). mjS2P produced in E. coli was solubilized from membranes with 1% DM, and it crystallized as a dimer; but the relevance of these observations to the full-length enzyme is unknown, as CBS domains typically promote oligomerization (34). RseP does not belong to the SpoIVFB subfamily, as it does not contain a CBS domain (31). Rather, RseP belongs to a large subfamily of IMMPs that contain one or more PDZ domains. RseP was readily solubilized from membranes with 1% DDM in the absence of its substrate (73). Also, catalytically inactive RseP could be solubilized from membranes with 1% DDM and co-immunoprecipitated with its substrate, RseA, suggesting that the two proteins form a fairly stable complex (50). This result, together with our discovery that the SpoIVFB–TEV-FLAG, E44Q-Pro–αK(1–126)-His6 complex is fairly stable during purification, suggests that catalytically inactive IMMPs may form stable complexes with their substrates, allowing the substrates to be identified. For example, using a His-tagged IMMP with an inactivating substitution in place of the catalytic Glu residue of its easily recognizable HEXXH motif, it may be possible to solubilize complexes from membranes, affinity-purify them, and identify putative substrates by mass spectrometry after protease digestion. The conditions under which the substrate is associated with its cognate IMMP must be known for this approach to work. At least one IMMP in the RseP subfamily, Rip1 of Mycobacterium tuberculosis, requires a substrate-specific adapter protein, Ppr1, in order to interact with its substrate, RsmA (74). Such adapter proteins might also be identified by the approach just described.

A third reason that our discoveries are important is that they suggest a strategy to facilitate further biochemical analyses of active IMMPs that are difficult to solubilize from membranes, such as SpoIVFB. For example, coexpression of active SpoIVFB with uncleavable substrate in E. coli should result in a complex that can be solubilized from membranes with nonionic detergent and purified. If the uncleavable substrate can be exchanged for a cleavable one (e.g. upon the addition of ATP), this strategy might result in a more active enzyme than a previous purification strategy using the harsh ionic detergent sarkosyl for initial solubilization of TM-SpoIVFB from membranes (28). Recent progress toward identifying the features of Pro–αK important for cleavage by SpoIVFB revealed several single-residue substitutions in Pro–αK(1–126)-His6 (e.g. S20W, V22P, V22N, and V22G) that virtually eliminate cleavage by coexpressed TM-SpoIVFB (45). These uncleavable substrates are candidates for use in the purification strategy just described.

**Parts of SpoIVFB and Pro–αK Important for Solubilization and Complex Formation.—**Both the Pro(1–20) and αK(21–126) parts contribute to improving the solubilization of cytTM-SpoIVFB–FLAG2, E44Q from membranes with 1% DM (Fig. 3, D and E). Analysis of two deletion derivatives of Pro–αK(1–126)-His6 provided further insight into the requirement for the Pro part. Δ2–6-His6 and Δ2–8-His6, differ by just two residues, yet Δ2–6-His6 improved enzyme solubilization significantly more than Δ2–8-His6. This implies that Ala17 and Leu16 of Pro–αK(1–126)-His6 change its interaction with the membrane and/or with SpoIVFB. In agreement, Δ2–6-His6, but not Δ2–8-His6, was observed previously to be cleaved by coexpressed TM-SpoIVFB in E. coli (45), as confirmed here (Fig. 3B, lanes 4 and 6). Slightly more Δ2–8-His6 than Δ2–6-His6, was in the supernatant after high-speed centrifugation (Fig. 3C, lanes 17 and 20), suggesting that slightly less Δ2–8-His6 was membrane-associated; but neither was wild-type Pro–αK(1–126) found exclusively in the membrane fraction (Fig. 3C, lane 8), so it seems unlikely that the slight difference in fractionation accounts for the observed differences in the solubilization of cytTM-SpoIVFB–FLAG, E44Q and in cleavage by active TM-SpoIVFB. Rather, it seems likely that Δ2–6-His6 and Δ2–8-His6 differ subtly in their interaction with the membrane and/or with SpoIVFB. The Pro sequence, MTGVFAALGFV–VKELVFLVS, has two hydrophobic regions separated by two adjacent residues (Lys13 and Glu14). It was proposed that the charged residues interact with the membrane surface and the hydrophobic regions loop into the membrane, because Pro–αK(1–126)-His6, appeared to interact peripherally with the E. coli inner membrane (45). If so, the absence of Ala17 and Leu16 in the Pro part of Δ2–8-His6 might change the way its first hydrophobic region interacts with the membrane, perturbing its interaction with SpoIVFB.

The last 10 residues of SpoIVFB in catalytically inactive TM-SpoIVFB are also necessary for Pro–αK(1–126)-His6, to improve its solubilization from membranes with mild detergents (Fig. 4A). The single-Cys version of TM-SpoIVFB E44C lacking the 10 residues failed to interact normally with single-Cys versions of Pro–αK(1–126)-His6, in in vivo disulfide cross-linking assays (Fig. 4C). Although it is tempting to speculate that the C-terminal 10 residues of SpoIVFB interact directly with Pro–αK(1–126)-His6, we cannot rule out the possibility that the 10 residues are important for folding of the CBS domain and that other parts of the SpoIVFB CBS domain interact directly with the substrate.

The αK(21–126) part, but not the Pro(1–20) part, of Pro–αK(1–126) forms a stable complex with cytTM-SpoIVFB–FLAG2, E44Q when the parts of Pro–αK(1–126) are fused to heterologous domains. This conclusion is based on pulldown assays in which very little cytTM-SpoIVFB–FLAG2, E44Q bound to Pro–αK(1–31)-GFP-His6, and considerably more of the enzyme bound to cytTM-αK(21–126)-His6 (Fig. 3, D, lanes 16 and 20, and F). The inability of Pro–αK(1–31)-GFP-His6, to form a stable complex with the enzyme may reflect an inability to interact at all, which would explain the lack of cleavage (Fig. 3B, lane 12). On the other hand, despite the ability of cytTM-αK(21–126)-His6, to form a stable complex with the enzyme, it too is not cleaved (Fig. 3B, lane 8). cytTM-αK(21–126)-His6 may be unsuitable as a substrate for two reasons. First, cytTM likely interacts with membranes differently than the Pro part of Pro–αK(1–126)-His6, cytTM is expected to insert into membranes like a typical TMS (57), whereas the Pro sequence appears to interact peripherally with membranes (45), as discussed above. Second, certain residues near the cleavage site in Pro–αK(1–126)-His6, are important for cleavage to occur (45). cytTM-αK(21–126)-His6, may not contain a sequence of residues suitable for cleavage.
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How does the \(\alpha^K\) part of Pro-\(\alpha^K\) (1–126)-His\(_6\) interact with SpoIVFB? Previously, it was shown that Pro-\(\alpha^K\) (1–126)-FLAG\(_2\) interacts with the CBS domain of SpoIVFB (28). It will be important to determine whether Pro-\(\alpha^K\) (21–126) interacts with the CBS domain and if so, how. Our purification of the SpoIVFB-TEV-FLAG\(_2\), E44Q-Pro-\(\alpha^K\) (1–126)-His\(_6\) complex lays the foundation for approaches like chemical cross-linking followed by protease digestion and mass spectrometry to identify residues of the two proteins that are in proximity. At least with respect to proximity between the active site of SpoIVFB (represented by E44C) and the cleavage site in Pro-\(\alpha^K\) (1–126)-His\(_6\) (represented by Y21C, K23C, or N24C), a purified complex resembled the complex formed in vivo, based on disulide cross-linking results (Figs. 4C and 5G) (46).

Interestingly, five residues near the N terminus of Pro-\(\alpha^K\) (1–126)-His\(_6\) appear to inhibit its interaction with SpoIVFB slightly. \(\Delta2–6\)-His\(_6\), lacking the five residues, appeared to pull down cytTM-SpoIVFB-FLAG\(_2\), E44Q more efficiently than did Pro-\(\alpha^K\) (1–126)-His\(_6\) (Fig. 3, D and F). The loss of just two more residues in \(\Delta2–8\)-His\(_6\) restored the pulldown efficiency to that observed for Pro-\(\alpha^K\) (1–126)-His\(_6\) and cytTM-\(\alpha^K\) (21–126)-His\(_6\). Although \(\Delta2–6\)-His\(_6\) appeared to interact more strongly than Pro-\(\alpha^K\) (1–126)-His\(_6\) with the enzyme in the pulldown assay, Pro-\(\alpha^K\) (1–126)-His\(_6\) appeared to be cleaved more efficiently (Fig. 3B, lanes 2 and 4). This suggests that a slightly weaker interaction benefits cleavage, perhaps by allowing better positioning of the substrate in the enzyme active site.

**IM-MS Analysis of an IP-Substrate Complex**—We used IM-MS to determine the composition of the SpoIVFB-TEV-FLAG\(_2\), E44Q-Pro-\(\alpha^K\) (1–126)-His\(_6\) complex. To our knowledge, this is the first time the composition of an IP substrate complex has been determined. The detergent and buffer conditions used to prepare samples of the complex for IM-MS analysis appeared to cause more dissociation of Pro-\(\alpha^K\) (1–126)-His\(_6\) from SpoIVFB-TEV-FLAG\(_2\), E44Q during gel filtration chromatography, but analysis of pooled fractions containing the complex, after buffer exchange into two different detergents, resulted in an observed mass that agreed well with the expected mass of a 4:2 SpoIVFB-TEV-FLAG\(_2\), E44Q-Pro-\(\alpha^K\) (1–126)-His\(_6\) complex in both cases. Post-IM CID analysis of the samples detected some of the expected dissociation products, although the products were difficult depending on the detergent, as observed in other studies (75, 76). Notably, dissociation products in agreement with a 1:1 SpoIVFB-TEV-FLAG\(_2\), E44Q-Pro-\(\alpha^K\) (1–126)-His\(_6\) complex were observed in the 0.02% DDM sample (Fig. 6D), indicative of stable interaction between monomers of the two proteins. Interestingly, a dissociation product in agreement with a 1:2 SpoIVFB-TEV-FLAG\(_2\), E44Q-Pro-\(\alpha^K\) (1–126)-His\(_6\) complex was observed in the 0.5% C8E4 sample (data not shown). We speculated that this reflected SpoIVFB interacting with a dimer of Pro-\(\alpha^K\) (1–126)-His\(_6\), because a disulfide cross-linked dimer of Pro-\(\alpha^K\) (1–126)-His\(_6\) was detected upon treatment with Cu\(^{2+}\) (phenanthroline)\(_3\) (Fig. 5G, lane 4). In any case, the IM-MS data strongly support the previous inference (28) that SpoIVFB is tetrameric and, together with our finding that Pro-\(\alpha^K\) (1–126)-His\(_6\) may be slightly substoichiometric compared with SpoIVFB-TEV-FLAG\(_2\), E44Q in the peak fraction during gel filtration in buffer containing 0.1% DM (Fig. 5E), at least two and perhaps as many as four Pro-\(\alpha^K\) (1–126)-His\(_6\) proteins may associate with the SpoIVFB tetramer.

**Evidence That SpoIVFB Is Tetrameric in Vivo**—Because previous analysis of purified TM-SpoIVFB (28) and our analyses of the purified SpoIVFB-TEV-FLAG\(_2\), E44Q-Pro-\(\alpha^K\) (1–126)-His\(_6\) complex (Figs. 5 and 6) suggested that SpoIVFB is tetrameric, we used TIRF microscopy and stepwise photobleaching to determine the number of subunits in SpoIVFB in sporulating *B. subtilis*. To our knowledge, this is the first time this methodology has been applied to an IP. The analysis was challenging because even though we used TIRF microscopy to only excite SpoIVFB-eGFP near the coverslip, we observed mostly large fluorescent foci likely representing multiple molecules and were unusable for counting photobleaching steps. We found that the small fluorescent foci had countable steps and such foci were localized to the mother cell membrane engulfing the forespore (Fig. 7, A and B). Most foci with countable steps exhibited 4 steps, but some had other numbers of steps (Fig. 7C). Potential sources of error in step counting are misfolded eGFP, quenching, partial overlap of foci, malformed complexes, and the subjective nature of counting steps. Hence, a distribution of the number of steps counted is expected, and our data are consistent with prior studies in this respect (63–67). The highest number of steps we counted was 5. Typically in stepwise photobleaching analysis, the highest number of steps is hypothesized to be the number of subunits in the protein. However, we rarely counted 5 steps, and statistical analysis of the \(\gamma\) function (55), which allows one to ask if a number of steps was counted erroneously, or more formally, tests the null hypothesis that the highest number of steps is not the number of subunits, yielded a very low \(\gamma\) value for 5 steps (Table 1), strongly suggesting that SpoIVFB is not pentameric. Moreover, analysis of the \(\theta\) function yielded the highest value for 4 steps, indicating the lowest probability of missed photobleaching events. We concluded that the data provided strong evidence that SpoIVFB is tetrameric in vivo. Because purified TM-SpoIVFB appears to be tetrameric (28) and SpoIVFB in the purified SpoIVFB-TEV-FLAG\(_2\), E44Q-Pro-\(\alpha^K\) (1–126)-His\(_6\) complex (Figs. 5 and 6) appears to be tetrameric, we further conclude that the purified proteins resemble native SpoIVFB in terms of the number of subunits.

The C-terminal CBS domain of SpoIVFB may account for its formation of a tetramer. Four CBS domains form a conserved disk-like structure in a variety of proteins (34). The four CBS domains most commonly occur as pairs within two identical polypeptides of a homodimeric protein but may occur within a single polypeptide. In this respect, SpoIVFB, with its single CBS domain, is somewhat unusual among the CBS domain-containing proteins studied thus far. A pair of CBS domains forms a characteristic structure called a Bateman module, to which an adenine nucleotide typically binds in a cleft, although each CBS domain contains a potential nucleotide binding cavity (34). If the SpoIVFB tetramer follows this trend, it may bind two molecules of ATP. However, previously the recombinant CBS domain from SpoIVFB was shown to migrate during gel electrophoresis under mildly denaturing conditions as a mixture of predominantly dimers and some monomers, and radioactive ATP bound primarily to the monomers (28). ATP was required. 
for the activity of purified TM-SpoIVFB, and it was speculated that ATP binding changes the conformation and/or oligomeric state of the enzyme. In most cases, ligand binding changes the conformation to relieve an autoinhibitory effect of the CBS domain (34). The tetrameric quaternary structure of SpoIVFB might allow cooperative binding of ATP and/or other adenine nucleotides, making the enzyme very sensitive to the energy level of the mother cell. Cooperative binding of the substrate is also possible. These questions and many others about the large subfamily of CBS domain-containing IMMPs represented by SpoIVFB can now be tackled by building on the foundation of insights and approaches reported here.

**Author Contributions**—Y. Z. and L. K. designed most of the study and wrote most of the paper, and Y. Z. performed most of the experiments. S. H. designed, performed, and analyzed the experiments shown in Fig. 6, A and B. R. A. K. and B. R. designed and analyzed the experiments shown in Fig. 6, C and D, and R. A. K. performed the experiments and wrote the corresponding parts of the paper. D. P. designed and performed the experiments shown in Fig. 7 and together with L. K. analyzed the results and wrote the corresponding parts of the paper. All authors reviewed the results and approved the final version of the manuscript.

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**References**


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