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Initial Binding Process of the Membrane Insertase YidC with Its Substrate Pf3 Coat Protein Is Reversible

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ABSTRACT: The binding of the inner membrane insertase YidC from *Escherichia coli* to its substrate, the Pf3 coat protein, was examined *in vitro* by fluorescence spectroscopy. Purified YidC protein was solubilized with the lipid-like detergent *n*-dodecylphosphocholine and noncovalently labeled with 1-anilino-naphthalene-8-sulfonate (ANS), whereas the Pf3 coat protein was kept in solution by the addition of 10% (v/v) isopropanol to the buffer. The binding of Pf3 coat protein was analyzed by fluorescence quenching of ANS bound to YidC. All binding curves showed a strict hyperbolic form at pH values between 9.0 and 5.0, indicating a reversible and noncooperative binding between YidC and its substrate. Analysis of the data revealed a dissociation constant K_D for the binding process in the range of 1 μM. The pH profile of the K_D values suggests that the binding of the Pf3 coat protein is dominated by hydrophobic interactions. The titration experiments provide strong evidence for a conformational change of the insertase upon binding a Pf3 coat protein molecule.

Membrane proteins use insertases and translocases to achieve their correctly folded conformation within the membrane bilayer. Whereas the more complex proteins interact with the multimeric Sec-translocase complex (*1*), small single- or double-spanning proteins, such as the major coat proteins of filamentous phage Pf3 or M13, use the membrane insertase YidC. YidC is an integral membrane protein (molecular mass of 61 kDa) of the bacterial inner membrane, which spans the bilayer 6 times. It is ubiquitously present among both Gram-negative and Gram-positive bacteria and also in chloroplasts and mitochondria (*2*). In *Escherichia coli*, YidC is essential for growth (*3*), most likely because YidC is required for the membrane insertion of subunit c of the F_oF_1 ATP synthase and for subunit II of the ubiquinol oxidase (*4, 5*). In the absence of YidC, a number of membrane proteins cannot achieve their integral conformation and remain bound to the cytoplasmic surface of the inner membrane (*6*). Likewise, mitochondrial mutants that are deficient in the YidC homologue Oxa-1 are inhibited in oxidative respiration (*7*).

Deletion analyses have shown that the C-terminal five hydrophobic transmembrane regions of YidC are important for function (*8*). These are also the most conserved regions among the bacterial homologues (*9*). A detailed analysis of the large periplasmic domain of YidC from *E. coli* has recently shown that it has a binding motif for SecF but is not essential for the insertase function (*10*).

An unresolved question is how YidC functions to insert membrane proteins. As an approach to elucidate the molecular mechanism, YidC has been purified and reconstituted into proteoliposomes. The reconstituted YidC-containing liposomes were able to catalyze the insertion of a purified natural substrate, the coat protein from the Pf3 phage, when the liposomes were subject to an electrochemical potential, whereas control liposomes lacking YidC were insertion-incompetent (*11*). The mechanism of YidC-catalyzed Pf3 insertion is thought to involve an initial binding of noninserted Pf3 prior to membrane insertion. However, neither the binding kinetics nor the stoichiometry of this step has yet been examined explicitly. In the reconstituted system, we expect that, in the absence of prior information, the resolution of the YidC−Pf3 binding step may be difficult to distinguish from a membrane-adsorption process. For this purpose, we have chosen to examine here the binding of Pf3 to native *E. coli* YidC solubilized in a mild lipid-like detergent, *n*-dodecyl-phosphocholine. We have previously demonstrated that this detergent is highly suitable for maintaining YidC functional over a long period of time (*11*).

To characterize the initial binding step, YidC was purified and noncovalently labeled with the dye 1-anilino-naphthalene-8-sulfonate (ANS).[1] The binding of the Pf3 coat protein to YidC was monitored by fluorescence quenching of the ANS dye. Although ANS has been extensively used for examining conformational changes of water-soluble proteins, it has rarely been employed for the study of membrane proteins

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[1] Abbreviations: ANS, 1-anilino-naphthalene-8-sulfonate; bis-Tris, 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol; cmc, critical micelle conentration; DMF, *N*,*N*-dimethylformamide; FRET, fluorescence energy transfer; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria broth; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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because of the possible interactions with the solubilizing detergent. In this study, we show that, with the appropriate controls, conformational changes of membrane proteins can also be examined reliably.

MATERIALS AND METHODS

Expression and Purification of YidC and Pf3 Coat. *E. coli* C43 cells were transformed with the wild-type *yidC* containing pET16b vector. Cells were aerobically grown in Luria broth (LB) medium at 37 °C, and the overexpression of YidC was induced at an OD_{600} of about 0.5−0.6 by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h, the cells were harvested and resuspended in 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl buffer (20 mM Tris-HCl at pH 8.0, 300 mM NaCl, and 10% glycerol). The cells were broken with a French pressure cell at 8000 psi, and the membranes were separated from the crude lysate by centrifugation for 50 min at 160000*g*. The membrane pellet was extracted with 1% (w/v) *n*-dodecylphosphocholine (FOS-CHOLINE-12, Anatrace, Maumee, OH) in Tris-HCl buffer (20 mM Tris-HCl at pH 8.0, 200 mM NaCl, and 10% glycerol) for 30 min at 4 °C and homogenized with a Dounce homogenizer. To remove all nonsolubilized components, the suspension was centrifuged for 20 min at 160000*g* and the supernatant was subsequently loaded on a Ni^{2+}-affinity column (Qiagen, Hilden). The column was washed twice with 40 mM imidazole/6 mM (w/v) *n*-dodecylphosphocholine in Tris-HCl buffer, and the protein was eluted with 300 mM imidazole/6 mM (w/v) *n*-dodecylphosphocholine in Tris-HCl buffer [20 mM Tris-HCl at pH 8.0, 200 mM NaCl, and 10% (v/v) glycerol]. Pf3 coat protein was purified as described previously (*11*) without using any detergent. The solubilizing agent for the Pf3 coat protein was 10% (v/v) isopropanol in Tris-HCl buffer (100 mM Tris-HCl at pH 8.0). The purity of both Pf3 coat and YidC were confirmed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) followed by staining with Coomassie Blue R250.

ANS Labeling of YidC. For the fluorometric titration measurements, YidC was labeled by adding 10 μL of 1.19 mM ANS (Invitrogen GmbH, Karlsruhe) dissolved in *N*,*N*-dimethylformamide (DMF) to 0.52 μM YidC in 460 μL of buffer. The ANS/YidC molar ratio was 40:1 here. After the addition of ANS, the solution was allowed to equilibrate for 5 min. Fluorescence spectra and energy-transfer measurements were performed with YidC labeled with the same protocol but only with ANS present in a 2−4-fold molar excess.

Fluorometric Titration and Data Analysis. The titration experiments were made in a quartz cuvette (Hellma AG) with a path length of 10 mm using a spectrofluorimeter (Fluorolog FL-2, Horiba Jobin-Yvon, Inc.). The temperature of the sample holder was controlled by a bath thermostat (Julabo F10) and held constant at 25 °C. The ANS was excited at 370 nm, and fluorescence spectra were measured in the range of 405−550 nm with a stepwidth of 1 nm and an integration time of 1 s. The slit widths of the monochromators were both set to a spectral width of 2 nm. To measure the binding of ANS to YidC, 470 μL of Tris-HCl buffer [20 mM Tris-HCl at pH 8.0, 50 mM NaCl, and 10% (v/v) isopropanol] containing 1.0 μM YidC was titrated by stepwise addition of small aliquots (1−5 μL) of 119 μM ANS dissolved in DMF. Similarly, the binding of YidC to Pf3 coat was performed by the stepwise addition of small aliquots (0.25−1 μL) of 0.65 mM Pf3 coat stock solution to 0.51 μM ANS-labeled YidC dissolved in 470 μL of buffer. This experiment was performed at different pH values using 100 mM sodium acetate (pH 5.0−5.5), 100 mM 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol (bis-Tris)-HCl (pH 6.0), and 100 mM Tris-HCl (pH 7.0−9.0) buffers. All buffers contained 50 mM NaCl and 10% (v/v) isopropanol. The interaction of Pf3 coat with ANS was examined by titrating 28 μM ANS in 460 μL of Tris-HCl buffer [50 mM Tris-HCl at pH 8.0, 50 mM NaCl, and 10% (v/v) isopropanol] with Pf3 coat as described above. After each addition of ANS or Pf3 coat, respectively, the solution was allowed to equilibrate for 5 min, and subsequently, a fluorescence spectrum was recorded. All spectra were background-corrected by subtraction of a buffer blank spectrum. The binding curves were constructed by taking the fluorescence values at 460 nm and plotting $\Delta F_i = |F_i - F_0|$ against the molar substrate concentration, where F_0 is the initial fluorescence value of YidC without substrate and F_i is the value at 460 nm at the *i*-th titration step. All fluorescence values were corrected for the increased volume because of the titration by multiplying each value with the factor $(1 + V_i/V_0)$, where V_i is the total added volume at at the *i*-th titration step and V_0 is the initial volume.

In this study, the Pf3 coat was used in molar excess compared to the initial YidC concentration, so that the concentration of free Pf3 coat [Pf3] is approximately equal the total Pf3 coat concentration. The dissociation constant K_D was obtained by fitting the binding curve to the hyperbolic equation

$$\Delta F = \Delta F_{max}\frac{[\mathrm{Pf3}]}{[\mathrm{Pf3}] + K_D} \quad (1)$$

where ΔF_{max} is the maximum fluorescence change at saturation. To reduce the error, each K_D value was determined in two consecutive experiments and averaged.

For the titration of YidC with ANS, the equation

$$\Delta F = \Delta F_{max}\left(\frac{[\mathrm{ANS}] + [\mathrm{YidC_0}] + K_D}{2[\mathrm{YidC_0}]} - \frac{\sqrt{([\mathrm{ANS}] + [\mathrm{YidC_0}] + K_D)^2 - 4[\mathrm{YidC_0}][\mathrm{ANS}]}}{2[\mathrm{YidC_0}]}\right) \quad (2)$$

was used for fitting the binding curves, where [ANS] and $[YidC_0]$ is the total ANS and initial YidC concentration, respectively. Scatchard plots for the binding of Pf3 coat to YidC were constructed by plotting the fractional saturation $p = \Delta F/\Delta F_{max}$ (see eq 1) against the ratio of the fractional saturation to the substrate concentration p/[Pf3]. Nonlinear and linear least-squares fitting procedures were performed using the Origin6.1 software (OriginLab Corp.).

Measurement of Fluorescence Spectra and Energy Transfer from Tryptophan (Trp) to YidC-Bound ANS. All measurements were performed in the same setup and under the same conditions as described above. Spectra of free ANS and ANS-labeled YidC were obtained by exciting the sample at 370 nm and recording the emission spectrum in the range of 405−550 nm. To measure the intrinsic Trp fluorescence

of YidC and the energy transfer from the YidC Trp residues to YidC-bound ANS, 0.49 μM of the protein was excited at 295 nm and a spectrum was recorded in the range of 305−560 nm before and after the addition of different amounts of ANS (0.44 and 0.87 μM). For this experiment, it was crucial to remove traces of imidazole (which fluoresces intensely in the same wavelength region as Trp) remaining after the Ni^{2+}-affinity step from the YidC samples. An overnight dialysis of the sample (≈500 μL against 100 mL of 50 mM Tris-HCl at pH 8.0, 50 mM NaCl, and 1.2 mM *n*-dodecylphosphocholine) at 4 °C proved sufficient for this purpose.

In all experiments with solubilized YidC, the detergent concentration was kept as low as possible to prevent interactions between Pf3 coat or ANS and the *n*-dodecylphosphocholine molecules. We observed that it was possible to keep the YidC protein in solution at a *n*-dodecylphosphocholine concentration of about 0.12 mM, which is below the critical micelle concentration (cmc) value of 1.2 mM.

RESULTS

Binding of ANS to YidC. Figure 1A shows the fluorescence spectrum of 1.1 μM free ANS and a spectrum of the same sample after the addition of 0.9 μM solubilized YidC. The addition of the YidC protein resulted in an increased fluoresecence yield and a blue shift of the ANS fluorescence peak of about 50 nm from 520 to 468 nm. This behavior is indicative for a binding of the ANS dye to hydrophobic sites of the protein (*12*). Further control experiments without protein employing detergent concentrations comparable to those used in this experiment yielded an small increase of the ANS fluorescence intensity concomitant with a blue shift of the entire spectrum. A spectrum of these control experiments is also shown in Figure 1A. However, the observed increase in intensity as well as the blue shift were not comparable to those caused by the binding of ANS to the solubilized YidC protein.

The series of fluorescence spectra that were recorded during titration of YidC with ANS shown in Figure 1B exhibit two uncommon features: (i) the intensity on the red wings of the spectra increase with each titration step, and (ii) the fluorescence maximum λ_{max} is red-shifted with increasing ANS concentration (Figure 1C). The sharp increase of λ_{max} between 0.21 and 1.7 μM ANS is caused by the superposition of the spectra of labeled YidC with the spectrum of the unlabeled sample (background spectrum at the bottom of Figure 1B), which has its maximum at 428 nm. At higher ANS concentrations, the fraction of labeled YidC exceeds the fraction of unlabeled protein; therefore, the spectral contribution of unlabeled sample becomes negligible.

An analysis of difference spectra calculated from spectra recorded at consecutive titration steps revealed the appearance of an additional band at ANS concentrations ≥ 5 μM centered around 517 nm. Three of these difference spectra are shown in Figure 1D. In comparison to the spectrum of free ANS (Figure 1A), the additional band (spectrum 3 in Figure 1D) is blue-shifted by ≈5 nm, which indicates that this band is a result of another binding process. Because of its spectral position and low intensity, it can be concluded

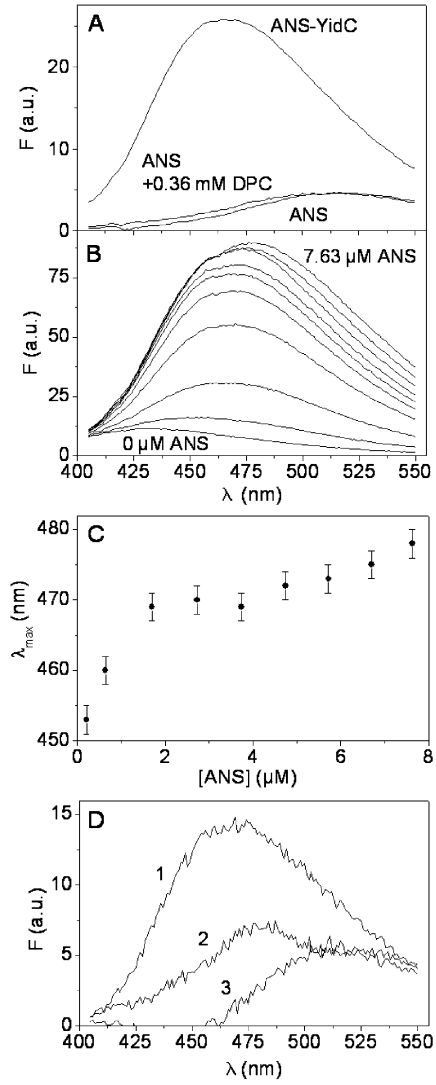

FIGURE 1: (A) Fluorescence spectra of 1.1 μM free ANS (ANS) and after addition of 0.9 μM detergent-solubilzed YidC (ANS−YidC) (0.16 mM *n*-dodecylphosphocholine) as well as a fluorescence spectrum of 1.1 μM free ANS in the presence of 0.38 mM *n*-dodecylphosphocholine (DPC) [in 20 mM Tris-HCl at pH 8.0, 50 mM NaCl, and 10% (v/v) isopropanol]. (B) Series of fluorescence spectra recorded after the titration of 1.0 μM YidC with increasing ANS concentrations [steps 0, 0.21, 0.64, 1.7, 2.73, 3.74, 4.74, 5.72, 6.7, and 7.63 μM, in 20 mM Tris-HCl at pH 8.0, 50 mM NaCl, and 10% (v/v) isopropanol]. (C) Dependence of λ_{max} of the ANS−YidC fluorescence on the ANS concentration. Because of the broad and flat maxima of the emission spectra, the error was arbitrarily set to ±2 nm. (D) Three difference spectra calculated from the spectra in B. The spectra are the differences from the spectra recorded with 2.73 and 1.7 μM ANS (1), 5.72 and 4.74 μM ANS (2), as well as 7.63 and 6.7 μM ANS (3).

the YidC protein possesses one or more low-affinity binding sites for the ANS molecule, which are strongly exposed to the polar environment.

To examine the binding process of ANS to YidC, which dominates the observed change of the emission, the spectra in Figure 1B were analyzed at 460 nm. At this spectral position, the resulting binding curve shown in Figure 2A could be fitted with good accuracy to eq 2 (see the Materials and Methods) with a best-fit value for the dissociation constant of $K_D = (1.37 \pm 0.11)$ μM. Because eq 2 is only true for homogeneous noncooperative binding, the excellent

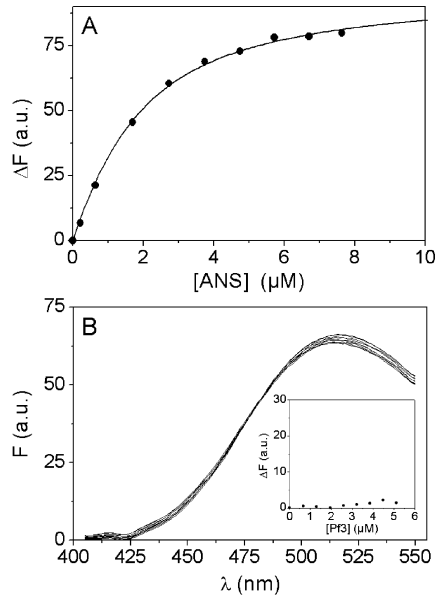

FIGURE 2: (A) Binding curve of ANS to 1.0 μM YidC constructed from the fluorescence emission at 460 nm (Figure 1B) and fitted to eq 2 with a best-fit value of $K_D = (1.37 \pm 0.11)$ μM. (B) Series of fluorescence spectra recorded after the titration of 25 μM ANS with increasing amounts of Pf3 coat protein (0−5 μM). The inset shows the bindng curve constructed from the fluorescence emission at 520 nm [in 20 mM Tris-HCl at pH 8.0, 50 mM NaCl, 10% (v/v) isopropanol, and 0.12 mM *n*-dodecylphosphocholine].

fit to the data indicates that only a single binding process is dominant here under the conditions studied.

Binding of Pf3 Coat to YidC. The sequence of the Pf3 coat protein includes an approximately 20 amino acid residue long hydrophobic helix, which might also bind ANS (*13, 14*). Titration of 24 μM ANS with Pf3 coat protein (0−5 μM) in a detergent containing buffer (0.12 mM *n*-dodecylphosphocholine) showed neither a spectral shift nor a concentration dependence of the fluorescence spectra compared to the spectrum of free ANS as shown in Figure 2B. The binding curve constructed from the fluorescence values at 520 nm (inset in Figure 2B) indicates that there is no measurable affinity of the ANS dye to the Pf3 coat protein.

To measure the binding of the Pf3 coat protein to YidC, a solution with 0.51 μM YidC pre-equlibrated with 20 μM ANS was titrated with increasing amounts of Pf3 coat protein. Under these conditions, more than 95% of the YidC was labeled with ANS (ANS−YidC). Figure 3A shows a representative series of fluorescence spectra recorded after titration of ANS−YidC with Pf3 coat at pH 8.0. These spectra reveal that the fluorescence yield of the ANS−YidC decreases together with a red shift of the spectra upon the addition of the Pf3 coat protein. With increasing substrate concentrations, the fluorescence maximum λ_{max} shifts from 480 nm (0 μM Pf3 coat) to 487 nm (10.7 μM Pf3 coat) as shown in Figure 3B. This shift indicates that the binding event influences the surrounding of the YidC-bound ANS (see the Binding of ANS to YidC section above). The binding curve in Figure 3C extracted from the spectra in Figure 3B shows a strict hyperbolic behavior and was fitted with good accuracy to eq 1 with a best-fit value for the dissociation constant of $K_D = (1.74 \pm 0.15)$ μM. In fact, all measured binding curves between pH 5.0 and 9.0 show similar hyperbolic characteristics with best-fit values for K_D in the range of 1 μM. A representative selection of the binding

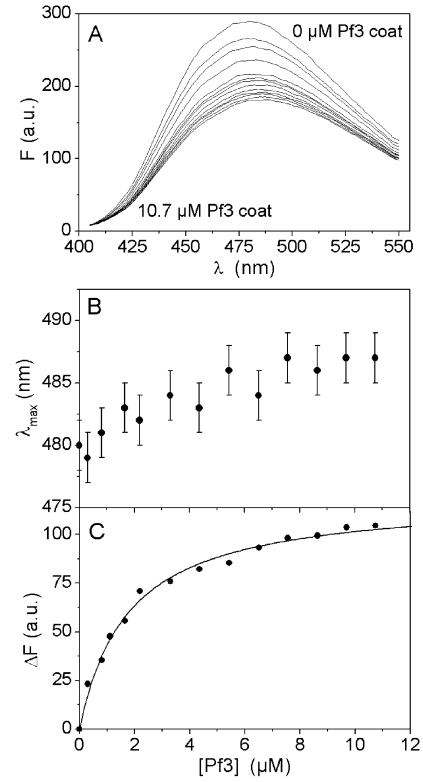

FIGURE 3: (A) Series of fluorescence spectra recorded after the titration of 0.51 μM ANS−YidC with increasing amounts of Pf3 coat protein and (B) dependence of λ_{max} of the fluorescence of ANS−YidC on the Pf3 coat protein concentration. As discussed in the caption of Figure 1, the error was arbitrarily set to ±2 nm. (C) Binding curve constructed from the fluorescence emission at 460 nm from the spectra in A. The curve was fitted to eq 1 with a best fit value of $K_D = (1.74 \pm 0.15)$ μM [in 20 mM Tris-HCl at pH 8.0, 50 mM NaCl, 10% (v/v) isopropanol, and 0.12 mM *n*-dodecylphosphocholine].

curves measured at different pH values is displayed in Figure 4A. To visualize the K_D values calculated from the curves in A, the corresponding Scatchard plots are shown in Figure 4B. The pH profile of the K_D values, shown in Figure 4C, reveals that the dissociation constants are independent of pH in the range between pH 9.0 and 5.5 and show a slight increase below pH 5.5. The low pH dependence between 5.5 and 9.0 indicates that the binding process is dominated by hydrophobic interactions.

Fluorescence Spectroscopy and Fluorescence Energy Transfer (FRET) between the YidC−Trp Residues and YidC-Bound ANS. To follow the fluorescence intensity of ANS−YidC, 0.51 μM YidC and 2 μM ANS were mixed and excited at 370 nm. We observed that the fluorescence intensity of the protein-bound ANS remained almost constant between pH 5.5 and 9.0, with a substantial increase occurring below pH 5.5 as shown in Figure 5A. The latter is probably due to a major conformational change of the YidC, resulting in a further shielding of the ANS-binding sites.

The amino acid sequence of YidC contains 11 Trp residues, which are predicted to be located either in the periplasmic portions of the protein or in the transmembrane α helices close to the interface of the periplasmic side of the membrane with its aqueous surrounding (*9*). Because we expected ANS to be bound to a hydrophobic site either at the cytoplasmic side of the protein or at a transmembrane location, we expected FRET between Trp and bound ANS

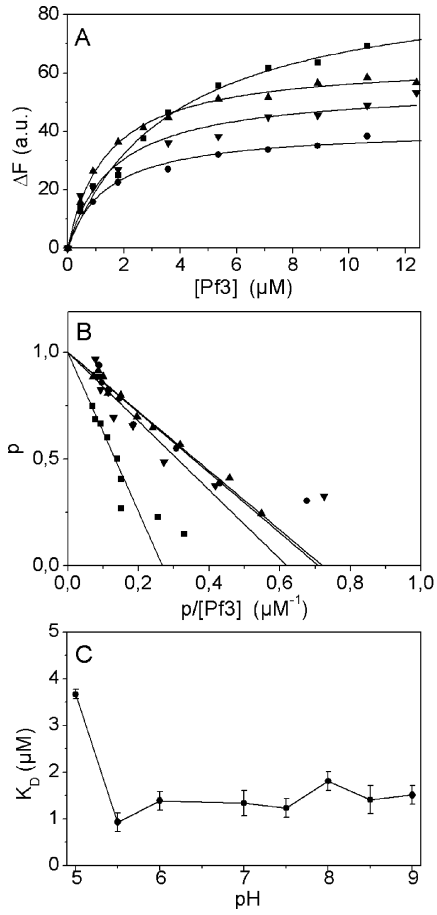

FIGURE 4: (A) Binding curves of Pf3 coat to 0.51 μM YidC and (B) corresponding Scatchard plots determined at pH 5.0 (■), pH 6.0 (▲), pH 7.0 (▼), and pH 9.0 (●). The best fit values for the dissociation constant were $K_D = (3.73 \pm 0.53)$ μM at pH 5.0, (1.39 ± 0.09) μM at pH 6.0, (1.61 ± 0.33) μM at pH 7.0, and (1.41 ± 0.20) μM at pH 9.0. (C) pH profile of the dissociation constants between pH 5.0 and 9.0. Each data point is the mean value calcuated from two consecutive experiments, and the error bars represent the standard deviations of the mean values.

to be experimentally observable. Figure 5B shows the fluorescence emission spectra of 0.45 μM YidC excited at 295 nm in the presence of different amounts of ANS. At this excitation wavelength, the fluorescence intensity obtained from ANS in the absence of protein is extremely weak, so that changes at 468 nm are due almost exclusively to Trp−ANS FRET. As expected for Trp−ANS FRET, the addition of increasing amounts of ANS (to about a 2-fold molar excess over YidC) led to the appearance of an additional emission maximum at 468 nm because of FRET with a concomitant decrease of the intrinsic fluorescence from Trp, as well as an isoemissive point at 417 nm. The emission maximum Trp at 348 nm corresponds closely to that observed for Trp dissolved in water (*15*) and is thus relatively far-red-shifted when compared to other proteins (*16*). This suggests that the Trp residues at the periplasmic positions are probably exposed to the aqueous environment. The corresponding Förster radius R_0 for the energy transfer from Trp to ANS was calculated using the absorption spectrum of free ANS (data not shown) and the intrinsic Trp emission spectrum in Figure 5 (*17*). The calculation suggests an approximate value of $R_0 = 23$ Å for the distance between the bound ANS molecule and the Trp residues.

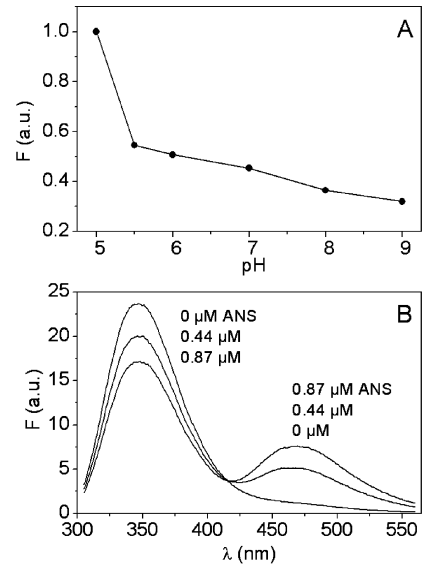

FIGURE 5: (A) pH profile of the ANS−YidC fluorescence maxima between pH 5.0 and 9.0. The values were arbitrarily normalized to the fluorescence maximum at pH 5.0. (B) Energy transfer from the Trp residues of YidC to the bound ANS molecules. The intrinsic Trp fluorescence spectra (excitation at 295 nm) of 0.49 μM YidC without ANS and after addition of different amounts of ANS are shown (in 50 mM Tris-HCl at pH 8.0, 50 mM NaCl, and 0.17 mM *n*-dodecylphosphocholine).

DISCUSSION

In this study, we have used the fluorescent properties of the reporter ANS to examine the binding kinetics of a natural substrate, the Pf3 coat protein, to detergent-solubilized YidC. The advantages of ANS as a fluorescent reporter molecule are well-known: (i) the quantum yield in water is almost negligible [$\Phi = 0.002$ (*17*)], (ii) the molecule binds readily to a wide variety of hydrophobic binding sites, (iii) the quantum yield of protein-bound ANS is usually about 200-fold higher than that in water (*17*), and (iv) the quantum yield is often exquisitely sensitive to very small conformational or local viscosity changes difficult to detect by other means. For these reasons, ANS has been widely used for measuring conformational changes and binding kinetics of water-soluble proteins. However, to our knowledge, ANS has been rarely if ever used for the same purpose for integral membrane proteins, most likely because of uncertainties concerning nonspecific interactions with the detergent environment necessary for their solubilization. Our work suggests that, for the case studied here, these effects occur but can be addressed by considering the proper controls and quantitative treatment of the fluorescent data.

The initial binding of ANS to detergent-solubilized YidC (Figure 1) illustrates the particular problems arising with membrane proteins present in an isopropanol-containing buffer. First, a significant fluorescence emission was observed in buffer in the absence of protein. Because of the low quantum yield of free ANS in water and aqueous buffers, the fluorescence spectrum is expected to be much smaller in comparison to the spectrum of ANS in Figure 1A. Presumably, the presence of 10% isopropanol lowers the polarity of the buffer, thereby enhancing the quantum yield of ANS. In the presence of YidC, there is a clear partitioning of the ANS to at least one hydrophobic site of the protein, as indicated by the large increase in the fluorescence intensity

and the blue-shift to 468 nm. The latter is fairly typical for ANS−protein binding in general. The appearance of a second weak emission maximum at 517 nm at high ANS/protein ratios (Figure 1D), showing a fluorescence intensity at lower wavelengths than that observed in the absence of protein but significantly lower than the main ANS−protein maximum at 468 nm, may indicate another class of binding sites on the protein. Although the appearance of multiple binding sites is not specific for membrane proteins, the effect may be exacerbated by the larger amount of accessible hydrophobic area within the solubilizing micelle that they exhibit. Despite these complex effects, the excellent fit (Figure 2A) of the fluorescence intensity data to eq 2, which implies only a single class of binding sites, indicates that ANS binds strongly (in the micromolar range) and preferentially to protein, even in the presence of solubilizing detergent. The interpretation of the YidC−Pf3 binding data is further facilitated by the finding that Pf3 coat alone does not bind ANS, probably because of its small size.

The first step in the membrane insertion of the Pf3 coat protein is the binding to the YidC insertase. In this study, detergent-solubilized YidC protein and the purified Pf3 coat protein were used to characterize this first step. Again, the good fit of the fluorescence intensity data obtained indicates that the binding process is reversible and that the Pf3 coat protein binds moderately tight to YidC with a K_D of approximately 1 μM. This value is in the same range of preprotein binding to the SecA protein (*18*). Although the Pf3 coat protein, as all other known substrates of YidC, is a protein of the inner membrane, it can be solubilized with the aid of 10% (v/v) isopropanol (*11*). Therefore, the purified Pf3 coat protein is not enclosed in a detergent micelle, which may disturb or inhibit the binding process. We therefore conclude that YidC interacts directly with the Pf3 coat protein. At present, however, the Scatchard analysis of the fluorescence titration data does not allow us to distinguish between a single substrate binding isotherm or multiple equivalent isotherms, corresponding to identical binding sites.

We found that the binding of the Pf3 coat protein is only slightly affected by pH. This indicates that hydrophobic interactions and not ionic interactions are the main forces for binding. When the pH was below 5.5, the binding of Pf3 coat protein was reduced (Figure 4B), as a result of a conformational change of the YidC protein, because at this pH, the fluorescence of ANS−YidC changed considerably (Figure 5A). However, circular dichroism measurements at pH 5.0 and 7.0 did not show any change in the secondary structure of YidC (data not shown).

According to current topological predictions, all 11 Trp residues are located at the periplasmic surface of YidC or in the large periplasmic domain (*9, 19, 20*). This unique situation facilitates the use of FRET to localize the approximate position of the bound ANS molecules. The observed Förster energy transfer from Trp to ANS (Figure 5A) indicates that the ANS molecules are localized within Förster radius of 23 Å from the Trp periplasmic cluster. In one interpretation, this distance would correspond closely to that expected for a transmembrane span and, if correct, would place the ANS-binding site(s) close to the cytoplasmic surface. We emphasize, however, that the Förster radius only provides an upper limit of the distance between the donor (periplasmic located Trp residues) and acceptor (ANS). A precise distance measurement would require detailed information as the molar donor/acceptor ratio, which is not available here. The observed red shift of the emission spectra (parts A and B of Figure 3) shows that the ANS molecule at this particular site is subjected to the quenching process upon binding Pf3 coat to the insertase. Thus, it appears that the Pf3 coat-binding site at the YidC is conformationally coupled to the region where the ANS molecule is bound.

We think that our results are physiologically relevant for the following reasons. First, it is now well-accepted that membrane proteins solubilized in mild detergents retain their native conformation, allowing us to study their enzymatic mechansims as well as allowing three-dimensional crystals to be obtained. For YidC, we have shown previously that *n*-dodecylphosphocholine-solubilized protein reconstituted into phospholipid bilayers yields preparations of high activity (*11*). Second, the Pf3−YidC binding curves shown here show the binding process to be reversible and to arise from a single class of binding sites, which would not be expected for nonspecific binding to a denatured or non-native conformation. Finally, preliminary FRET results indicate that the ANS molecule(s) are localized at discrete distance from the periplasmic Trp cluster, once again implying a discrete protein conformation. In summary, therefore, we expect that the basic results of this study of the Pf3 binding reversibly to a single site of YidC with a K_D of 1 μM will also be relevant for the physiological process. However, the role of the membrane surface, which is the subject of ongoing studies, in this process has yet to be ascertained.

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