Optical Switches

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Light-Induced Control of Protein Translocation by the SecYEG Complex**

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The convergence of molecular biology and synthetic chemistry has opened new avenues that enable, beyond the understanding of biological phenomena, the reproduction, control, and engineering of functions of naturally occurring systems.[1,2] This approach has been extended to the exploration of biological motors and the incorporation of molecular switches in proteins. Illustrative are the use of biomolecular motors interfaced with synthetic systems,[3] the allosteric control of a glutamate-sensitive protein by photochemical switching,[4] and the design of a light-actuated nanovalve derived from the protein MscL, which controls ion flow through a lipid bilayer.[5]

In nature many proteins synthesized in the cell need to cross or be incorporated into lipid bilayers. In bacteria, a membrane protein channel, SecYEG, together with a motor protein, SecA, is responsible for these processes. Once a hydrophobic signal-sequence-containing protein (preprotein) has been synthesized,[6] its conformation is modified by the molecular chaperone SecB to facilitate its recognition by SecA. SecA then initiates cycles of adenosine-5′-triphosphate (ATP) hydrolysis to translocate the preprotein across the SecYEG channel.[7] The main subunit of this complex, SecY, comprises two sets of five transmembrane segments (TMs), which are arranged as a clamshell-like structure encompassing a central hourglass-shaped pore[8–10] (Figure 1 a). The pore harbors a lateral gate or hydrophobic crevice between TM2 and TM7 (Figure 1 a). The lateral gate provides an opening of the central pore to the interior of the lipid membrane. It is believed to widen upon the binding of the motor protein SecA and the ATP-dependent insertion of the signal sequence and unfolded preprotein substrate into the translocation pore. Recently, we showed that when the lateral gate is constrained by the specific introduction of a disulfide bridge or a chemical cross-link spanning 5 Å or less, the translocation activity of the SecYEG complex is blocked. However, when cross-linkers are introduced with a span of 10 Å or larger, the pore is fully active.[11] Thus, it seems that the lateral gate does need to open during preprotein translocation. Indeed, analysis of the SecY structure revealed that the distance between the sulfur atoms of the introduced cysteine residues in the lateral gate in the closed state is about 5 Å, whereas in the preopen state it is about 13 Å (Figure 1 a).[8–10] The disadvantage of such a chemical cross-linking approach is that the channel is irreversibly immobilized in a single and specific conformation. However, the proposed conformational-switching behavior of the lateral gate of SecY makes it a good candidate for modification with an optical switch to control its activity. It should thus be possible to determine the overall channel flexibility in a reversible and noninvasive manner. Herein we report the introduction of an optical switch into the lateral gate of the SecYEG protein-translocating channel.

Among the organic molecules known to undergo a large geometrical change triggered by the application of an external stimulus, azobenzenes have proven useful not only in materials science,[1] but also for the induction of changes in protein conformation in biological studies.[12] We therefore synthesized an azobenzene derivative (4,4′-bis(bromomethyl)azobenzene, DBAB; see Figure S1 in the Supporting Information)[13] that can switch reversibly between the *trans* and *cis* configuration upon irradiation with UV and visible light (Figure 1 b). In the *trans* isomer, the two aromatic rings are planar, whereas in the *cis* isomer, they are closer together and tilted. In this way, the distance between the substituents in the *para* positions of the aryl groups shifts from approximately 13 Å in the *trans* isomer to 5–9 Å in the *cis* isomer. The azobenzene was functionalized with two bromine atoms to enable the introduction of the optical switch between two specific cysteine positions engineered in TM2 and TM7 of SecY; these cysteine residues are part of the lateral gate.

Escherichia coli inner-membrane vesicles (IMVs) containing overexpressed levels of the SecY(S87C/F286C)EG

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Supporting information for this article, including experimental details of the synthesis and characterization of the optical switches, the modification of the protein-conducting channel, and biochemical assays, is available on the WWW under http://dx.doi.org/10.1002/anie.201002243.

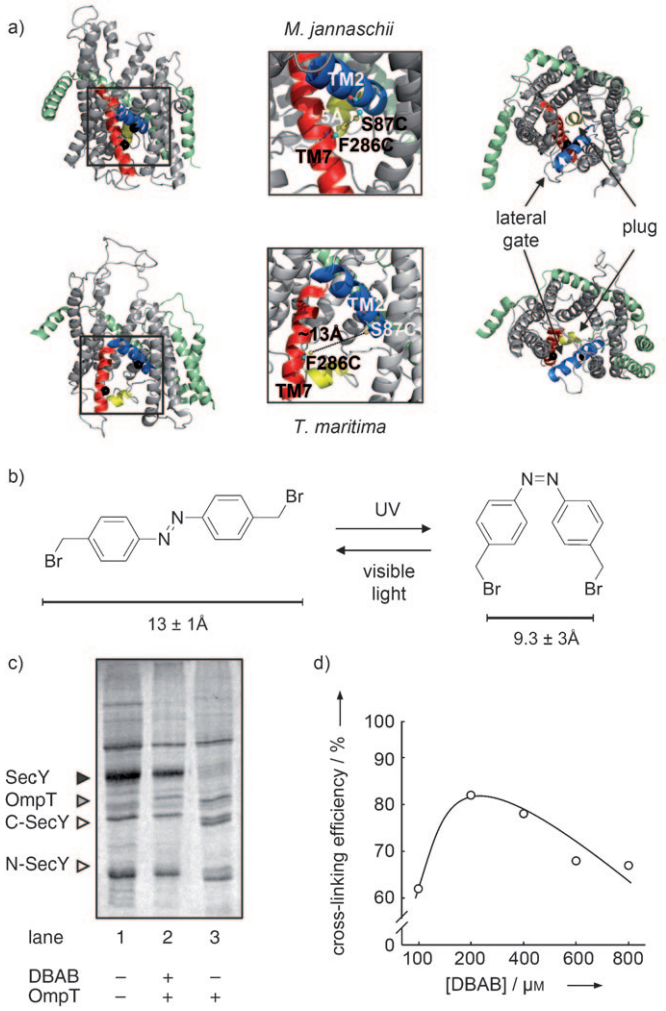

Figure 1. a) Comparison of the structures the SecYEG complex from *Methanococcus jannaschii* (1RHZ.pdb) and *Thermotoga maritima* (3DIN.pdb) as viewed from the side (left) and from the cytosolic face of the membrane (right). The central panels highlight structural details of the side view of the lateral gate of SecY. TM2 and TM7 of SecY are indicated in blue and red, respectively, and the plug domain is indicated in yellow. Black spheres indicate the positions corresponding to cysteine mutations S87C and F286C of *E. coli*. SecE and SecG subunits are indicated in pale green. The lateral gate is closed in the *M. jannaschii* structure[8] and in a preopen state in the *T. maritima* SecYEG structure, which is in a complex with the SecA protein (not displayed).[9] The figures were generated with PyMOL (www.pymol.org). b) Scheme showing the *cis*/*trans* isomerization of DBAB. c) OmpT assay of IMVs containing the SecY(F286C/S87C)EG complex incubated with DBAB. OmpT-treated SecY migrates as the uncleaved protein (lane 2). In the presence of tris(2-carboxyethyl)phosphane, SecY is cleaved (lane 3). N-SecY and C-SecY indicate the N-terminal and C-terminal fragment, respectively. d) The cross-linking efficiency was optimized by the incubation of IMVs containing the SecY(F286C/S87C)EG complex with increasing amounts of DBAB at 37 °C for 2 h, followed by OmpT cleavage to determine the extent of the cross-linking.

mutant were incubated with increasing amounts of DBAB. After this treatment, the efficiency of bifunctional incorporation into SecY was assessed by the use of a specific protease, OmpT.[11] This protease cleaves SecY at a double-arginine motif in cytoplasmic loop 4. Successful cleavage of SecY results in the formation of N- and C-terminal degradation products that can be identified by SDS-PAGE and staining with Coomassie brilliant blue (Figure 1c, lane 3). By contrast, when the two cysteine residues in TM2 and TM7 were linked covalently by DBAB, the OmpT-treated SecY protein migrated as a full-length protein on an SDS-PAGE gel (Figure 1c, lane 2). Under the same conditions, the SecYEG complex without cysteine residues is cleaved by OmpT both in the absence and in the presence of DBAB (see Figure S2 in the Supporting Information); thus, the OmpT activity is not blocked by DBAB. The maximal modification efficiency was approximately 80% at a DBAB concentration of 200 μM (Figure 1d). With this proteolytic assay, the efficiency of incorporation of the switch into SecYEG could be determined, and the use of mass spectrometry to assess the incorporation of the switch in a very hydrophobic part of the protein could be avoided.

To determine whether *cis*/*trans* isomerization took place after incorporation of the azobenzene into SecY, we purified the DBAB-derivatized SecYEG complex by Ni–nitrilotriacetic acid affinity chromatography and analyzed the protein by UV/Vis spectroscopy. Free DBAB in the *trans* configuration in dimethyl sulfoxide (DMSO) has a characteristic absorption at 340 nm that decreases in intensity upon UV irradiation ($\lambda_{max} = 365$ nm; Figure 2a). The absorption spectrum of the SecY–DBAB complex showed the characteristic absorption at 340 nm, which decreased in intensity upon UV irradiation and reappeared upon irradiation with visible light[15] (Figure 2b,c), as observed for the *trans* form of the free azobenzene in DMSO (Figure 2a); this absorption behavior is similar to that of previously reported azobenzene-containing peptides.[15,16] These results indicate that the light-induced *trans*/*cis* and *cis*/*trans* isomerization of DBAB is retained when the optical switch is conjugated to SecY.

To analyze the effect of the isomerization of DBAB on the SecYEG translocation activity, we exposed IMVs containing the SecY–DBAB conjugate to UV or visible light and used them subsequently in an in vitro translocation reaction with the preprotein proOmpA as the substrate (for an explanation of the translocation assay, see Figure S3 in the Supporting Information). IMVs containing the *trans*-DBAB-conjugated SecY subunit translocated proOmpA with similar efficiency to that of IMVs containing nonconjugated SecY (Figure 3a, lanes 2 and 4). By contrast, UV irradiation of the IMVs containing *trans*-DBAB-conjugated SecY resulted in a three-to-fivefold decrease in the translocation of proOmpA as a result of the formation of excess[14] *cis*-DBAB, which causes contraction of the channel (Figure 3a, lane 3). *cis*-DBAB was found to be thermally stable under our measurement conditions.[14] UV irradiation of IMVs containing nonconjugated SecY did not significantly affect translocation (Figure 3a, lane 5). These IMVs also contained endogenous levels of the wild-type (wt) SecYEG complex. The translocation activity of wild-type IMVs (Figure 3a, lane 6) was similar to the activity of UV-irradiated IMVs containing the overexpressed DBAB-conjugated SecY

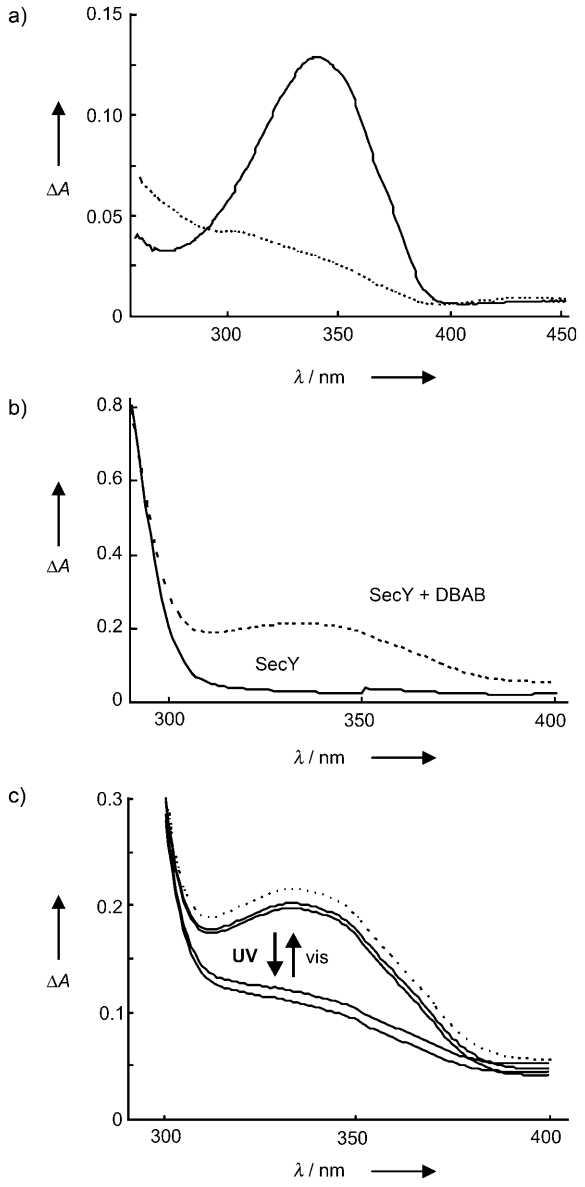

Figure 2. Spectral analysis of the optical switching of free DBAB in solution and DBAB conjugated to SecY. a) UV/Vis spectra of the molecular switch DBAB dissolved in DMSO after irradiation with white light (solid line) and 365 nm UV light (dashed line). b) UV/Vis spectra of the purified SecYEG complex without (solid line) and with cross-linking by DBAB (dashed line). c) UV/Vis spectra of the SecY–DBAB complex during cycles of irradiation with UV and visible light.

(compare lanes 6 and 3). This result suggests that the low residual activity is largely due to the presence of endogenous wild-type SecY, which does not react with DBAB. However, the incomplete *trans*/*cis* photostationary state may also contribute to the residual activity, and the possibility of some photodegradation of the switch during the irradiation cycles can not be entirely excluded.[14]

To confirm that the decrease in translocation efficiency was indeed due to the *trans*/*cis* isomerization of DBAB in the bifunctionally modified SecY subunit upon irradiation, we analyzed proOmpA translocation in IMVs in which only one of the two cysteine positions in the lateral gate of SecY was labeled with the optical switch. To this end, we synthesized a monobromoazobenzene derivative[14] (4-bromomethyl-4′-methylazobenzene, BAB) and incorporated it into SecY mutants containing a single cysteine residue either in TM2 (S87C) or in TM7 (F286C). The labeling efficiency of the two SecY mutants by BAB indicated different accessibilities of the cysteine residues in TM2 and TM7. At a concentration of 200 μM, the incorporation of BAB appeared to be approximately 70% for C87 in TM2, whereas C286 in TM7 showed negligible labeling (see Figure S2 in the Supporting Information). This result suggests that the accessibility of the C286 residue in TM7 is hindered, possibly because it is part of an α helix that is oriented towards the interior of the pore. Since DBAB is able to react with both cysteine residues in TM2 and TM7 in a bimodal fashion, it appears that DBAB initially reacts with the more accessible cysteine residue in TM2 and only then gains access to the cysteine residue in TM7 to form a covalent cross-link between these two α helices. Importantly, the incorporation of BAB (Figure 3b) and DBAB (data not shown) into the SecY(S82C)EG complex had no effect on the translocation of proOmpA upon irradiation with visible or UV light. However, as noted previously,[11] the introduction of the S87C mutation led to a slight decrease in the activity of the SecYEG complex relative to that of the native complex.

We analyzed the reversibility of the UV-induced inactivation of the protein-translocation activity of DBAB-conjugated SecY. To this end, IMVs containing the SecY–DBAB hybrid were irradiated sequentially with visible and UV light and tested for translocation activity directly after exposure to each light source. After illumination with visible light (Figure 3c, lane 1), subsequent irradiation of SecY–DBAB IMVs with UV light inhibited proOmpA translocation (lane 2). Next, illumination with visible light reactivated these IMVs for proOmpA translocation nearly to the original level (Figure 3c, lane 3). A second irradiation with UV light again inhibited proOmpA translocation (Figure 3c, lane 4). These results show that the light-induced opening and closing of the lateral gate is reversible.[14]

Our observations demonstrate the importance of an opened lateral gate between TM2 and TM7 for translocation. When these two transmembrane segments are constrained relative to each other at a distance of 5 Å, as induced by the *cis* geometry of the optical switch, the translocation channel cannot open, and translocation is therefore blocked. The SecA motor utilizes ATP to drive preprotein translocation, an activity termed translocation ATPase. When SecA was tested for the translocation of proOmpA, the translocation ATPase activity decreased when the pore was in the UV-induced closed conformation in comparison to the translocation ATPase activity when the pore was in the visible-light-induced open conformation or with non-cross-linked SecY (Figure 3d). This decrease in ATPase activity is not as large as that observed for the oxidized SecY(S87C/F286C)EG complex, in which the lateral gate is maximally constrained by a disulfide bridge, but compares well with that observed for a dibromobimane-cross-linked lateral gate.[11] Thus, the presence of the bridging azobenzene switch in its *cis* configuration hinders the movement of the lateral gate to a sufficient extent

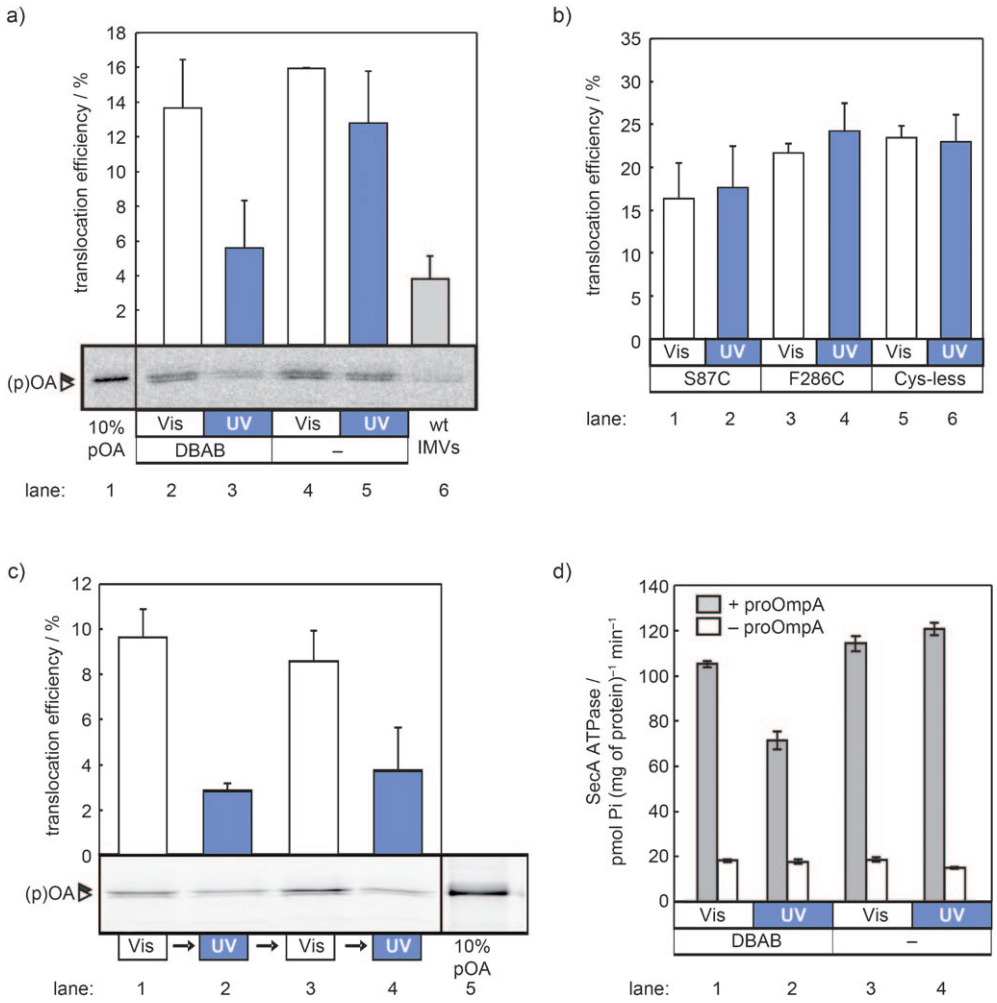

Figure 3. a) Light-induced control of the SecY lateral gate. The translocation of fluorescein-labeled proOmpA (pOA) was assayed with IMVs containing DBAB-cross-linked SecY after irradiation with white light (lane 2) and UV light (lane 3); with IMVs containing untreated SecY after irradiation with white light (lane 4) and UV light (lane 5); and with IMVs containing wild-type levels of SecY (lane 6). The translocation efficiency was calculated on the basis of the intensity of the in-gel fluorescence of the fluorescein-labeled proOmpA that remained after proteinase treatment. The intensity of proOmpA fluorescence in each lane was compared to the fluorescence intensity of 10% of the fluorescein-labeled proOmpA used per assay (lane 1; without treatment with proteinase K). Error bars refer to the standard deviation of three independent experiments. b) Control mutants SecY(S87C)EG and SecY(F286C)EG, and SecY without cysteine residues (Cys-less) were labeled with monobromoazobenzene (BAB) and tested for translocation after irradiation with UV and visible light. c) Multiple rounds of light-induced opening and closing of the SecY lateral gate. The translocation of fluorescein-labeled proOmpA was assayed with IMVs containing overexpressed levels of the SecY(S87C/F286C)EG complex cross-linked with DBAB. The cross-linked complex was exposed to visible light (lanes 1 and 3) and 365 nm UV light (lanes 2 and 4) in consecutive cycles. The efficiency of translocation was quantified relative to the 10% standard of fluorescein-labeled proOmpA (lane 5). d) SecA ATPase assay of SecY(S87C/F286C)EG IMVs labeled with DBAB (lanes 1 and 2) and untreated SecY(S87C/F286C)EG IMVs (lanes 3 and 4) after irradiation with visible (lanes 1 and 3) and UV light (lanes 2 and 4). White bars indicate the basal SecA ATPase activity in the absence of proOmpA. Pi = inorganic phosphate.

to interfere with the activation of the SecA translocation ATPase. With the optical switch, some flexibility seems to be retained to enable decreased ATPase activity, but this activity is insufficient to support translocation.

This study shows that the incorporation of a photoswitchable compound into the lateral gate of the protein-conducting channel SecYEG enables reversible optical control of the opening and closing of the SecY pore, and thus a controlled translocation event. These results provide strong support for the enlargement of the distance between TM2 and TM7 as an essential step during protein translocation. Furthermore, this first example of the direct control of the activity of a membrane protein translocation channel represents a step toward a biological system with added functionality and potential applications in structural-biology research, which benefits from the external control of conformational changes without perturbation of the system.

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