nature portfolio

Peer Review File

AFM observation of protein translocation mediated by one unit of SecYEG-SecA complex

Corresponding Author: Professor Tomoya Tsukazaki

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The paper by Kanaoka et al. reports the use of high-speed atomic force microscopy (HS-AFM) to study protein translocation by the SecA ATPase and the SecYEG complex. Previous studies have reported crystal and cryo-EM structures of the complex as well as a wealth of biochemical data. However, the mechanism by which SecA moves polypeptides into the SecY channel remains controversial. Specifically, it has been shown before that the PPXD of SecA undergoes a conformational change to generate open and closed conformations of SecA, but whether these changes reflect binding of SecA to SecYEG and substrate, or ATPase-dependent changes of SecA that push the polypeptide into the channel is unclear.

In this paper, the authors claim that they have visualized by HS-AFM the movement of a polypeptide through the channel. Unfortunately, the videos that supposedly show this movement are not convincing to this reviewer. The resolution of AFM does not seem to be sufficient to demonstrate substrate translocation. The authors go on to analyze different nucleotide-dependent stages, but again, this reviewer is not convinced that definitive conclusions can be drawn from the noisy images. There is no question that the authors have analyzed the data by state-of-the art methods, but the AFM technique does not seem to be suitable to distinguish between the different models proposed in the literature. I therefore regret that I cannot recommend this paper for publication.

Reviewer #2

(Remarks to the Author)

Kanaoka et al. use a combination of high-speed atomic force microscopy and AFM image simulations to directly visualize motor protein SecA-mediated (post-translational) protein translocation through the bacterial SecYEG complex embedded in lipid nano-discs (ND), in real-time and on the single molecule level. Substrate translocation through the ND embedded protein conducting channel and distinct domain motions on SecA were observed in an ATP-dependent manner. Interpretation of the latter in terms of conformational changes between known high-resolution SecA structures was facilitated by comparison with simulated AFM images generated thereof. The (likely) corresponding nucleotide-specific states of the PPXD domain and estimated ATP hydrolysis rates were found to be in agreement with FRET experiments conducted earlier by another group (Ref 38 of the manuscript).
The study provides the first real-time HS-AFM images of substrate translocation through a membrane protein by employing upright (with respect to the support) oriented lipid nano discs to enable a side-view on the complex, which is certainly outstanding from a methodological point of view.
However, apart from the methodological advance, it is not clear to the reviewer how the study, “enhances our understanding of protein transport mechanisms across membranes” or “shed new light on the dynamic conformational changes in SecA during ATP hydrolysis” as stated in the conclusion section. This and the suggestions listed below should be addressed in a possible revision.
Specific comments:
1) Line 159: The authors found that the height of the SecYAEG-NDs is approx. 4 nm lower than the theoretical value which they attributed to deformation caused by the adsorption to the substrate, or the mechanical deformation introduced by the AFM tip. Would an analysis of the cross-sectional area and/or the volume of the particles in experiment vs. simulation give a

better accordance than the height?

2) Line 165: The authors determined the orientation of PPXD mutant by correlating distinct orientations of simulated AFM images of a SecYA(PPXD)EG-ND structural model and HS-AFM images of this mutant and found a maximum correlation for a rotational angle of ~ 80°. Why should the NDs carrying the full-length SecA have the same orientation, couldn't it be that the presence of the PPXD in the full-length SecA modifies the interaction with the surface and thus the orientation of the SecYAEG-ND?

3) The end-to-end distance of the substrate is determined to be 22 +/- 0.4 nm. How does this length correspond to the theoretical value of the substrate construct? The histograms Fig. 2A and B suggest a rather broad distance distribution ranging from 10 to 50 nm which is not compatible with a standard deviation of only 0.4 nm?

4) Discrepancy between text and figure, line 185: "Approximately 16% of 262 observed molecules were accompanied by…" this equals approx. n=42, however, the respective histograms (Fig. 2A and B) each contain n=85 events.

5) Larger overview images (as supplementary Figures) or movies would help to demonstrate the percentage of SecYAEG-ND having a substrate bound or does who have not (e.g. when using the 3Q mutation of proOmpA)

6) Does the probability of observing a substrate bound to SecYAEG-ND depend on the particular substrate (proOmpA-sfGFP, preMglB.sfGFP, prePhoA-sfGFP) used?

7) For the other two substrates: Does a length difference exist between SecYAEG-ND -bound / free substrate as observed for proOmpA. Might the initial insertion depth (under nucleotide free conditions) depend on the substrate sequence?

8) The translocated substrate can hardly be seen in the last image of Fig. 2C but is much better visible in Movie 4. I would suggest adding a corresponding panel of images from Movie 4 as additional panel to Fig. 2.

9) Line 205: How did the authors quantify the time-dependent elongation of the substrate polypeptide? How does the elongation depend on time?

10) The authors found a translocation rate of 2.2 aa per second which was much slower than what was reported for E. coli (40 aa/s). A possible explanation could be that the rate would be increased at elevated temperatures, however, there might be an alternative explanation: SecAs N terminus was suggested to function as a membrane-tether (Bauer et al. doi: 10.1016/j.cell.2014.03.063) that keeps SecA in place during the short nucleotide-free time span in every ATP-hydrolysis cycle (Winkler et al. doi: 10.1039/D0NA00427H). While the linker between SecY and SecA used in the SecYAEG construct might play a similar role, the rotational alignment and thus the interaction of SecA sitting on SecY might be altered as compared to the wild type SecA in which the amphipathic N-terminus can more freely align and bind to lipids in the vicinity of SecY. A potential alteration of the SecA-SecYEG interaction could very well be responsible for the observation of a different translocation rate.

11) Line 251: Does that mean that the wide-open state is induced/stabilized by the mica surface?

12) Line 275: possible typo "fold the substrate tightly…"

13) Line 284: Please include histograms of the transition times for the Apo and 5 mM ATP as supplementary Figures.

14) General question: Would it be possible to orient the ND so that SecA can be watched from top?

Reviewer #3

(Remarks to the Author)
In the manuscript from Kanaoka et al., the authors use high-speed AFM to visualize translocation of a substrate protein by the SecA-SecYEG complex. They use MD to produce simulated AFM images to match to the experimental images. They observe different conformational states of SecA in a nucleotide-dependent matter.

This is a very interesting study. I appreciate the effort the authors went through to maximize their ability to interpret the very-low-resolution data, especially the creative use of simulated structures to create simulated AFM images. With that in mind, I have a number of questions and suggestions.

1) Maybe I missed it, but what is the precision of the AFM? I assume it's sub-nm, but it would be good to know exactly to know how to interpret differences in the height profiles. For instance, Figure S2 indicates the difference between open and closed is only 2 Å (1.9 vs 1.7 nm). How reliable is this?

2) In Figure 2A, two regions are interpreted as the GFP and signal sequence, respectively. However, they appear to be basically the same size in the image. How can this be?

3) In Figure 2C, a small region is interpreted as the translocated substrate. But how many residues is this supposed to represent? When watching the movie, I sometimes see other similarly sized and shaded regions that are presumably noise (or at least irrelevant material). Therefore, how can the authors be sure what they are seeing at 118 s is substrate?

4) On Page 10, line 214, the inability to observe substrate for 20/43 particles is interpreted as half of them not exhibiting translocase activity. But isn't it likely that the substrate just isn't observed in all cases? If the authors want to substantiate a quantitative claim like this, they need to somehow demonstrate they should be able to always observe substrate.

5) On Page 8, line 159, the authors note that the experimental AFM images are ~4 nm lower than the simulated images. This is a big difference! It would be nice if the authors could use the simulations to explain this. For example, one hypothesis is that the AFM tip squeezes the nanodisc. Can't the authors try to simulate an analogous process to see what a squished nanodisc looks like?

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have addressed all my earlier concerns/suggestions and have significantly improved the manuscript. I only have two minor comments left:

1) Please add some details about how the model of a deformed ND was created.

2) Regarding the ‘time from ATP addition to confirmation of membrane protein translocation’ used to calculate the translocation rate: Does this also include the time required for ATP to diffuse to the SecYEG-ND site? This would certainly reduce the rate calculated using the given formula. Estimating and subtracting this time would improve the rate estimate.

Reviewer #3

(Remarks to the Author)
I am satisfied with the authors' response to my concerns.

Reviewer #1

> *Comments:*
> *The paper by Kanaoka et al. reports the use of high-speed atomic force microscopy (HS-AFM) to study protein translocation by the SecA ATPase and the SecYEG complex. Previous studies have reported crystal and cryo-EM structures of the complex as well as a wealth of biochemical data. However, the mechanism by which SecA moves polypeptides into the SecY channel remains controversial. Specifically, it has been shown before that the PPXD of SecA undergoes a conformational change to generate open and closed conformations of SecA, but whether these changes reflect binding of SecA to SecYEG and substrate, or ATPase-dependent changes of SecA that push the polypeptide into the channel is unclear.*
> *In this paper, the authors claim that they have visualized by HS-AFM the movement of a polypeptide through the channel. Unfortunately, the videos that supposedly show this movement are not convincing to this reviewer. The resolution of AFM does not seem to be sufficient to demonstrate substrate translocation. The authors go on to analyze different nucleotide-dependent stages, but again, this reviewer is not convinced that definitive conclusions can be drawn from the noisy images. There is no question that the authors have analyzed the data by state-of-the art methods, but the AFM technique does not seem to be suitable to distinguish between the different models proposed in the literature. I therefore regret that I cannot recommend this paper for publication.*

We sincerely appreciate your thoughtful review of our manuscript. All three reviewers raised concerns regarding the quality of the HS-AFM images. Based on your and their comments, we conducted additional experiments to improve the quality of HS-AFM videos demonstrating how the substrate protein translocates across the SecYAEG-ND complex. We have replaced the original images and videos with new data that provide a clearer view of the intermediate stages of translocation through the SecYAEG-ND complex (Fig. 2 and Movie S3 and S4). We also updated the image and movies of the complex of SecYAEG-ND with the substrate after membrane translocation (Fig. S7). Even with the new movie, you may still have concerns that the term ‘visualization’ might be too strong. Therefore, we have revised the title to "AFM observation of protein translocation mediated by one unit of SecYEG-SecA complex" to better reflect our findings.

Regarding the suitability of HS-AFM for distinguishing between the various models reported in other papers, we understand your concerns. However, we believe that our findings offer significant insights into the dynamics of protein translocation. While HS-AFM has limitations, it uniquely allows real-time observation of conformational changes, complementing static structural data from techniques such as X-ray crystallography and cryo-EM. We are confident that this study represents a significant example of extracting meaningful data from low-resolution images. The ability to capture the dynamic behavior of proteins as they function is crucial for understanding the Sec pathway. We believe that these updated materials more effectively illustrate the dynamics of the translocation process.

Reviewer #2

> *Comments:*
> *Kanaoka et al. use a combination of high-speed atomic force microscopy and AFM image simulations to directly visualize motor protein SecA-mediated (post-translational) protein translocation through the bacterial SecYEG complex embedded in lipid nano-discs (ND), in real-time and on the single molecule level. Substrate translocation through the ND embedded protein conducting channel and distinct domain motions on SecA were observed in an ATP-dependent manner. Interpretation of the latter in terms of conformational changes between known high-resolution SecA structures was facilitated by comparison with simulated AFM images generated thereof. The (likely) corresponding nucleotide-specific states of the PPXD domain and estimated ATP hydrolysis rates were found to be in agreement with FRET experiments conducted earlier by another group (Ref 38 of the manuscript).*
> *The study provides the first real-time HS-AFM images of substrate translocation through a membrane protein by employing upright (with respect to the support) oriented lipid nano discs to enable a side-view on the complex, which is certainly outstanding from a methodological point of view.*
> *However, apart from the methodological advance, it is not clear to the reviewer how the study, "enhances our understanding of protein transport mechanisms across membranes" or "shed new light on the dynamic conformational changes in SecA during ATP hydrolysis" as stated in the conclusion section. This and the suggestions listed below should be addressed in a possible revision.*

We sincerely appreciate your positive comments and suggestions for improving our manuscript. The feedback has greatly guided our revisions, helping to enhance the clarity and impact of our work. We have carefully addressed each of the reviewers' comments and made substantial revisions to the manuscript. The revised manuscript provides a clearer and more detailed explanation of how our real-time HS-AFM observations capture the intricate dynamics of substrate translocation and SecA's conformational changes during ATP hydrolysis. In particular, we have replaced the original images and videos with new data—a groundbreaking achievement—that provide a clearer view of the intermediate stages of translocation through the SecYAEG-ND complex (Fig. 2 and Movie S3 and S4). Our findings provide direct evidence of ATP-dependent movements of SecA's PPXD domain, which are crucial for driving polypeptide translocation through the SecYEG channel. Additionally, our HS-AFM observations not only support previous FRET studies but also offer a more nuanced view of the mechanistic steps involved in protein transport. These achievements mark a significant step forward in understanding SecA-mediated translocation and pave the way for future research in this field.

> *1) Line 159: The authors found that the height of the SecYAEG-NDs is approx. 4 nm lower than the theoretical value which they attributed to deformation caused by the adsorption to the substrate, or the mechanical deformation introduced by the AFM tip. Would an analysis of the cross-sectional area and/or the volume of the particles in experiment vs. simulation give a better accordance than the height?*

We sincerely appreciate your suggestion to clarify the height difference between the HS-AFM and simulated images. In the manuscript, we noted that the ~4-nm lower height, compared to theoretical estimation, could be attributed to deformation caused by adsorption to the mica substrate or mechanical deformation

introduced by the AFM tip. To address this, we created a model of a scaled and elliptical-deformed nanodisc structure and generated a corresponding simulated AFM image. We then compared the height of the simulated image with that from the HS-AFM data (Fig. S3), which showed that the two were similar. Additionally, analysis of the actual HS-AFM image alongside the simulated deformed nanodisc showed a strong match in nanodisc size and volume, further supporting our explanation for the observed lower height. We have revised the main text in lines 167-172.

> *2) Line 165: The authors determined the orientation of ΔPPXD mutant by correlating distinct orientations of simulated AFM images of a SecYA(ΔPPXD)EG-ND structural model and HS-AFM images of this mutant and found a maximum correlation for a rotational angle of ~ 80°. Why should the NDs carrying the full-length SecA have the same orientation, couldn't it be that the presence of the PPXD in the full-length SecA modifies the interaction with the surface and thus the orientation of the SecYAEG-ND?*

We greatly appreciate your valuable feedback. The electrostatic potential calculation of SecYAEG-ND demonstrated that the region in contact with the mica substrate at the 80-degree angle is highly positively charged (Fig. S2B). This positively charged region remains present even in the ΔPPXD mutant, as the deletion does not affect its distribution (Fig. S2A). Since the mica substrate is negatively charged, electrostatic interactions predominantly govern the orientation of the observed complex, ensuring consistent alignment. Additionally, the structural changes in the flexible region (PPXD) of SecA consistently occur at the same location on each particle (as shown in Fig. 3), further supporting this conclusion. We believe these phenomena provide a likely explanation for the observed orientation. We have revised the main text in lines 155-160.

> *3) The end-to-end distance of the substrate is determined to be 22 ± 0.4 nm. How does this length correspond to the theoretical value of the substrate construct? The histograms Fig. 2A and B suggest a rather broad distance distribution ranging from 10 to 50 nm which is not compatible with a standard deviation of only 0.4 nm?*

We sincerely appreciate the valuable feedback. Since proOmpA is an unstructured peptide, determining the precise length is challenging, making it difficult to compare the actual peptide length from HS-AFM measurements with the theoretical length. Although we manually measured the end-to-end distances in this study, the lengths (Xo) of the preproteins in Figs. 2A and 2B are distinguishable.

Regarding the histogram, we acknowledge an error in our initial description: the value of 0.4 nm referred to the fitting error rather than the standard deviation of the length distribution. We have corrected this in the revised manuscript (lines 191-192).

> *4) Discrepancy between text and figure, line 185: "Approximately 16% of 262 observed molecules were accompanied by…" this equals approx. n=42, however, the respective histograms (Fig. 2A and B) each contain n=85 events.*

The statement 'Approximately 16% of 262...' in the main text simply estimated the ratio of the complex particles. Actually, we observed a far greater number of particles—so many that they were difficult to count

accurately. The counts in Fig. 2A and 2B are not related to the 262 particles, as they are from a separate experiment. The distances were extracted from snapshots of the HS-AFM movies, and we have added this information in the figure legend for 2A and 2B.

> *5) Larger overview images (as supplementary Figures) or movies would help to demonstrate the percentage of SecYAEG-ND having a substrate bound or does who have not (e.g. when using the 3Q mutation of proOmpA)*

We appreciate the suggestion. In response, we have obtained images of substrate complexes over a wider area. In Fig. S5, out of the nine molecules observed, two formed substrate complexes (indicated by red arrows). It is evident that the molecules not forming a complex do not have the substrate bound to SecA. We included the data as supplemental data and revised the main text (Fig. S5 and lines 194-197).

> *6) Does the probability of observing a substrate bound to SecYAEG-ND depend on the particular substrate (proOmpA-sfGFP, preMgIB.sfGFP, prePhoA-sfGFP) used?*

The answer is yes. As mentioned in the main text, we observed similar substrate complex formation with the different substrates. However, the rate of complex formation for preMgIB-sfGFP and prePhoA-sfGFP is significantly lower, at around 2%. To conduct similar analyses using these substrates to those using proOmpA-sfGFP, further optimization of conditions may be necessary, as these substrates are less familiar to our group.

> *7) For the other two substrates: Does a length difference exist between SecYAEG-ND -bound / free substrate as observed for proOmpA. Might the initial insertion depth (under nucleotide free conditions) depend on the substrate sequence?*

We greatly appreciate your question. First, we measured the end-to-end distance of the two substrate proteins in their free state, not bound to SecYAEG-ND. The Xo values of preMglB and prePhoA are 20 ± 6 nm and 20 ± 9 nm, respectively. These results indicated that the distance was approximately 2 nm shorter than proOmpA but not consistent with the length of precursor proteins: prePhoA>proOmpA~preMglB. Due to the differing properties of the proteins, comparing the lengths of different proteins on HS-AFM images is considered problematic. As mentioned in our above response (to comment 6), the number of substrate complexes bound to SecYAEG-ND was quite limited. Therefore, creating a meaningful histogram from the data for these substrates is challenging.

> *8) The translocated substrate can hardly be seen in the last image of Fig. 2C but is much better visible in Movie 4. I would suggest adding a corresponding panel of images from Movie 4 as additional panel to Fig. 2.*

We are grateful for your suggestion. To provide a clearer view of the intermediate stages of substrate translocation, we have conducted additional experiments and obtained new images (Fig. 2C). We also captured images of the substrate after translocation (Fig. S7). We believe these revisions enhance the clarity of our manuscript. We have revised the main text (lines 212-216) and replaced Figure 2C, Movie S3 and S4 with the

new results.

> *9) Line 205: How did the authors quantify the time-dependent elongation of the substrate polypeptide? How does the elongation depend on time?*

We appreciate your comment regarding the analysis of time-dependent elongation of the substrate polypeptide. We defined the time dependency of substrate elongation using the following equation:

Substrate translocation rate = {(Length of the substrate extending from the SecYEG-ND side) + (Total distance of SecYAEG structure: 10 nm)} / (Time from ATP addition to membrane translocation confirmation)

The equation has been added to the legend of Fig. S7.

> *10) The authors found a translocation rate of 2.2 aa per second which was much slower than what was reported for E. coli (40 aa/s). A possible explanation could be that the rate would be increased at elevated temperatures, however, there might be an alternative explanation: SecAs N terminus was suggested to function as a membrane-tether (Bauer et al. doi: 10.1016/j.cell.2014.03.063) that keeps SecA in place during the short nucleotide-free time span in every ATP-hydrolysis cycle (Winkler et al. doi: 10.1039/D0NA00427H). While the linker between SecY and SecA used in the SecYAEG construct might play a similar role, the rotational alignment and thus the interaction of SecA sitting on SecY might be altered as compared to the wild type SecA in which the amphipathic N-terminus can more freely align and bind to lipids in the vicinity of SecY. A potential alteration of the SecA-SecYEG interaction could very well be responsible for the observation of a different translocation rate.*

We are grateful for your insightful suggestion. While it is possible that the Sec machinery functions normally and SecA changes conformation without restrictions, we agree that there is also the possibility of constraints. The studies by Bauer et al. and Winkler et al. provide compelling evidence that SecA's N-terminus acts as a membrane tether, potentially limiting its movement during the nucleotide-free phase of the ATP-hydrolysis cycle. The SecYAEG construct, where the linker between SecA and SecY may affect the rotational alignment of the SecA N-terminus compared to the wild-type, could alter their interactions. We have updated the citation and revised the main text to reflect this consideration (lines 223-227).

> *11) Line 251: Does that mean that the wide-open state is induced/stabilized by the mica surface?*

To eliminate any potential effects from the mica substrate, we utilized end-up HS-AFM images of SecYAEG-ND, where SecA was observed from the top side using the immobilization method described by Haruyama et al. (DOI: 10.1016/j.str.2018.09.005). The top-view images are shown in Fig. 1G of that paper. Using similar end-up images, one of the authors (T. Mori) is currently developing a computational quantitative method to automatically discern whether the images show a wide-open or closed state. This approach allowed us to estimate whether the observed images corresponded to the wide-open or closed structures. As a preliminary result, most images were classified as wide-open structures. While he is still refining this new method, including the development of various algorithms, and plans to publish the complete approach elsewhere, we are unable to

release the program at this time. However, even in its preliminary version, the program indicates that most end-up images correspond to the wide-open structure. Thus, we concluded that the mica substrate does not significantly impact the SecA states.

> *12) Line 275: possible typo "fold the substrate tightly…"*

The typo has been corrected (line 294).

> *13) Line 284: Please include histograms of the transition times for the Apo and 5 mM ATP as supplementary Figures.*

We sincerely appreciate your suggestion to include the transition event histogram as supplemental information. Unfortunately, the images' resolution approaches the limits of high-speed AFM, and there is not enough accumulated data to create a histogram. Instead, we have included a typical time-course graph of the Low-High state transitions (Fig. S10). The transitions of structural changes between low and high states are faster with ATP than without ATP.

> *14) General question: Would it be possible to orient the ND so that SecA can be watched from top?*

Yes, it is. As mentioned above, we have successfully established the end-up observation method in Haruyama et al. Briefly, by labeling the two periplasmic loop regions (i.e., the bottom side) of SecYAEG with biotin and allowing it to interact with streptavidin in a two-dimensional crystal formed on a lipid bilayer containing biotinylated lipids, we were able to observe the end-up view of the SecYAEG-ND complex and MgtE (a Mg ion transporter)-ND.

Reviewer #3

> *Comments:*
> *In the manuscript from Kanaoka et al., the authors use high-speed AFM to visualize translocation of a substrate protein by the SecA-SecYEG complex. They use MD to produce simulated AFM images to match to the experimental images. They observe different conformational states of SecA in a nucleotide-dependent matter.*
>
> *This is a very interesting study. I appreciate the effort the authors went through to maximize their ability to interpret the very-low-resolution data, especially the creative use of simulated structures to create simulated AFM images. With that in mind, I have a number of questions and suggestions.*

Thank you for your encouraging feedback and positive comments. We are delighted that you found our study interesting and truly appreciate your recognition of our efforts, especially in using simulated AFM images to interpret low-resolution data. We have carefully addressed your questions and suggestions in the revised manuscript.

> *1) Maybe I missed it, but what is the precision of the AFM? I assume it's sub-nm, but it would be good to know exactly to know how to interpret differences in the height profiles. For instance, Figure S2 indicates the difference between open and closed is only 2 Å (1.9 vs 1.7 nm). How reliable is this?*

Typically, the vertical resolution of AFM is around 0.1 nm, and, as you point out, a height change of 0.2 nm is quite subtle. For instance, when comparing two different molecules with a height difference of about 0.2 nm, it may be difficult to draw a clear conclusion without statistical analysis. However, when observing height changes within the same molecule over time, or when comparing different structural regions of the same molecule, a 0.2-nm change can be distinctly identified as a clear contrast in the images and recognized in the height profiles.

In this study, based on previous X-ray structural analyses, we interpret the high state as the wide-open state and the low state as the closed state, and discuss the conformational transitions of SecA. This concept is described in detail starting from line 250.

> *2) In Figure 2A, two regions are interpreted as the GFP and signal sequence, respectively. However, they appear to be basically the same size in the image. How can this be?*

We appreciate your question regarding the size of GFP and the signal sequence. The previous figure captured the signal sequence in a compact state; however, during observation, the signal sequence was observed to move flexibly, alternately entangling and unfolding. To clarify this, we have replaced Fig. 2A and revised the accompanying text (lines 188-189).

> *3) In Figure 2C, a small region is interpreted as the translocated substrate. But how many residues is this supposed to represent? When watching the movie, I sometimes see other similarly sized and shaded regions that are presumably noise (or at least irrelevant material). Therefore, how can the authors be sure what*

> *they are seeing at 118 s is substrate?*

We sincerely appreciate your question regarding the reliability of identifying the translocated substrate in the HS-AFM image. In response, we have conducted additional experiments and obtained new data, which more clearly capture the substrate post-translocation (Fig. 2C, S7; Movie S3, S4). We believe these images illustrate protein translocation in real time. The manuscript has been revised (lines 212-216), and Fig. 2C and Movies S3 and S4 have been updated to reflect these new results.

> *4) On Page 10, line 214, the inability to observe substrate for 20/43 particles is interpreted as half of them not exhibiting translocase activity. But isn't it likely that the substrate just isn't observed in all cases? If the authors want to substantiate a quantitative claim like this, they need to somehow demonstrate they should be able to always observe substrate.*

We sincerely appreciate your comments and understand your concern. We have removed and revised the phrase 'exhibited translocation activity' to a more cautious expression. Now, we simply report the number of particles that likely achieved protein translocation (lines 230-231).

> *5) On Page 8, line 159, the authors note that the experimental AFM images are ~4 nm lower than the simulated images. This is a big difference! It would be nice if the authors could use the simulations to explain this. For example, one hypothesis is that the AFM tip squeezes the nanodisc. Can't the authors try to simulate an analogous process to see what a squished nanodisc looks like?*

We sincerely appreciate your suggestion to clarify the height difference between the HS-AFM and simulated images. In the original manuscript, we noted that the approximately 4-nm lower height, compared to theoretical estimation, could be attributed to deformation caused by adsorption to the mica substrate or mechanical deformation introduced by the AFM tip. To address this, we created a model of a scaled and elliptical-deformed nanodisc structure and generated a corresponding simulated AFM image. We then compared the height of the simulated image with that from the HS-AFM data (Fig. S3), which showed similar heights. Additionally, analysis of the actual HS-AFM image alongside the simulated deformed nanodisc showed a strong match in nanodisc size and volume, further supporting our explanation for the observed lower height. We have revised the main text in lines 167-172.

Reviewer #2

> *Comments:*
> *The authors have addressed all my earlier concerns/suggestions and have significantly improved the manuscript. I only have two minor comments left:*

We sincerely appreciate your thoughtful review of our manuscript. We answered the minor comments as shown below.

> *1) Please add some details about how the model of a deformed ND was created.*

Thank you for pointing this out. The process of making the deformed ND model was added in the legend of Supplementary Fig. 4.

> *2) Regarding the 'time from ATP addition to confirmation of membrane protein translocation' used to calculate the translocation rate: Does this also include the time required for ATP to diffuse to the SecYEG-ND site? This would certainly reduce the rate calculated using the given formula. Estimating and subtracting this time would improve the rate estimate.*

After adding ATP, we immediately performed pipetting. The diffusion time of ATP is estimated to be at most a few seconds. In contrast, protein translocation across the membrane occurs on the order of minutes. Therefore, the error caused by diffusion can be considered negligible. This explanation has been added in Methods of HS-AFM observation to avoid misunderstanding.

Reviewer #3

> *Comments:*
> *I am satisfied with the authors' response to my concerns.*

Thank you very much for taking the time to review our work.