Real-Time Fluctuations in Single-Molecule Rotaxane Experiments Reveal an Intermediate Weak Binding State During Shuttling

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KEYWORDS: Single-Molecule Force Spectroscopy, Rotaxane, Molecular Motion, Hydrogen Bonds, Atomic Force Microscopy

ABSTRACT: We report on the use of AFM to identify and characterize an intermediate state in macrocycle shuttling in a hydrogen bonded amide-based molecular shuttle. The [2]rotaxane consists of a benzylic amide macrocycle mechanically locked onto a thread that bears both fumaramide and succinic amide-ester sites, each of which can bind to the macrocycle through up to four intercomponent hydrogen bonds. Using AFM-based single-molecule force spectroscopy we mechanically triggered the translocation of the ring between the two principal binding sites ('stations') on the axle. Equilibrium fluctuations reveal another interacting site involving the two oxygen atoms in the middle of the thread. We characterized the ring occupancy distribution over time, which confirms the intermediate in both shuttling directions. The study provides evidence of weak hydrogen bonds that are difficult to detect using other methods and shows how the composition of the thread can significantly influence the shuttling dynamics by slowing down the ring motion between the principal binding sites. More generally the study illustrates the utility that single-molecule experiments, such as force spectroscopy, can offer for elucidating the structure and dynamics of synthetic molecular machines.

INTRODUCTION

Rotaxanes are prototypical synthetic molecular machines consisting of a macrocycle threaded onto an axle.[1] They enable controlled large-amplitude movement and positioning of one mechanically interlocked component with respect to another.[2-7] Translocation of the ring can be triggered by a stimulus, resulting in its directional displacement along the thread. This movement is dependent on the relative interaction strengths between the ring and specific binding sites ('stations') in the thread. One well developed class of molecular shuttles features benzylic amide macrocycles threaded onto axles with two or more well-defined hydrogen bonding stations.[6,8] In these systems the translational co-conformers undergo exchange by the ring moving over relatively large distances between the two stations, often with excellent positional integrity.

Here, using single-molecule force spectroscopy (SMFS) by means of an atomic force microscope (AFM),[9,10] we investigate the shuttling motion of the ring in such a hydrogen bonded [2]rotaxane (Figure 1A). Several SMFS experiments have been recently reported[11-15] that illustrate the efficacy of this technique in deciphering precise molecular behaviors and dynamic processes in rotaxanes and catenanes. In this particular [2]rotaxane (Figure 1B), the benzylic amide macrocycle is mechanically locked onto the thread by bulky diphenylethyl groups situated at either end of the axle.[8] The thread bears both fumaramide and succinic amide-ester sites, each of which can bind to the macrocycle through up to four intercomponent hydrogen bonds (Figure 1B). The benzylic amide macrocycle has a significantly higher affinity for the fumaramide (*fum*) station than the succinic amide-ester (*succ*) one.[8d] The occupancy of the ring on each station (*fum*:*succ* = 95:5 in 1,1,2,2-tetrachloroethane) corresponds to the $\Delta\Delta G$ values between the co-conformers.[8d] SMFS has previously been used to pull the ring along the axle, away from the thermodynamically favored binding site, through which it was demonstrated that the ring was able to travel back to the original site against an external mechanical load.[11] Here we investigate in greater detail the mechanically-induced shuttling motion of the ring. We performed pulling and relaxing cycles to monitor the ring motion and observed the existence of an intermediate step that we attribute to the formation of weak hydrogen bonds with the axle along the shuttling path.

RESULTS AND DISCUSSION

The rotaxane was chemically modified for its interfacing between a surface and an AFM tip following the previously described strategy.[11] A poly(ethylene oxide) (PEO) chain attached to the molecular ring serves as a tether for the tip attachment during force experiments. The tip was brought into contact with the substrate in 1,1,2,2-tetrachloroethane and then withdrawn from the surface in a controlled manner so that the molecular ring is mechanically pulled away from its preferred *fum* station to reach the *succ* one. The mechanically-induced shuttling motion is identified by a characteristic force peak (Figure 1C) presenting a revealed length of ~ 4 nm, easily distinguishable from the usual stretching and desorption of the tether. The intensity of the peak, about 40 pN (Figure 1D), reflects the force required to break the H-bonds between the ring and the *fum* station, matching the force values already observed for these interactions under similar conditions.[11,16]

The revealed length—obtained from the contour length variation ΔL_c of the worm-like chain (WLC) model[17]— corresponds well to the distance between the *fum* and the *succ* stations (details in SI). The relaxing part of the experiment is performed by releasing the force applied on the ring by moving the tip towards the surface. We observed a similar deviation in the relaxing curve evidencing the shuttling of the ring back to its preferred *fum* station (Figure S2). During both pulling and relaxing curves, *i.e.* respectively withdrawal and approach of the tip, we observed many fluctuations between the two states (Figure 2B and S2). This behavior, also known as a *hopping* phenomenon,[13,14,18,19] is indicative of a rapid movement of the ring between the two stations.

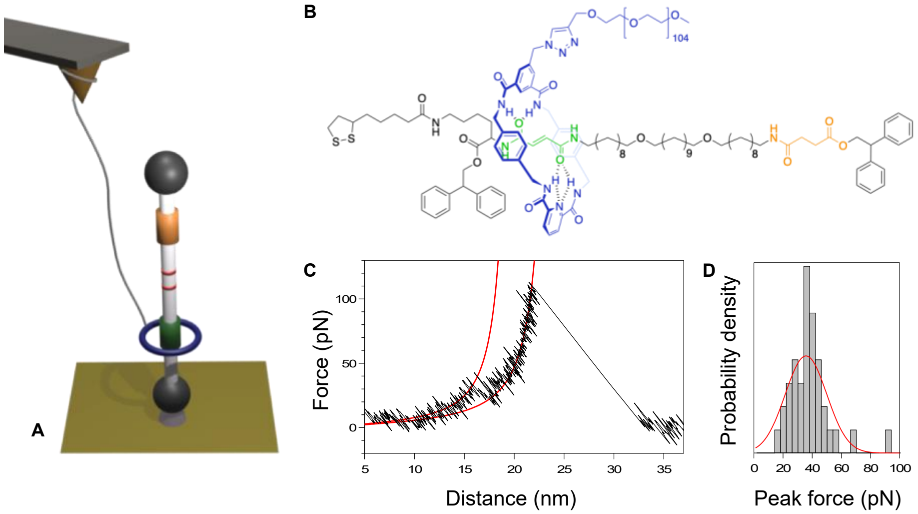

Figure 1. Single-molecule force spectroscopy experiment on a hydrogen bonded [2]rotaxane. (A) Scheme of the experimental setup representing the rotaxane chemically attached to a gold-coated silicon surface and the AFM mechanical pulling using a tether attached to the ring. (B) Chemical structure of the [2]rotaxane. The benzylic amide macrocycle (in blue) can shuttle from the fumaramide station (in green) to the succinic amide-ester station (in orange). (C) Typical force-distance curve showing a rupture peak with $\Delta L_c = 4$ nm, followed by the final PEO detachment peak. Worm-like chain (WLC) fits are added in red. (D) Distribution of the peak force, *i.e.* rupture of intramolecular hydrogen bonds between the ring and the fumaramide station. Gaussian analysis returns $F_{peak} = 36 \pm 5$ pN.

In addition to the macrocycle binding to the *fum* and *succ* stations, we were able to identify an intermediate state during the shuttling of the ring along the molecular thread. The distribution of revealed lengths during pulling-relaxing experiments shows a further population at 1.8 ± 0.2 nm (Figure 2A). This corresponds to the ring being trapped in the middle of the thread instead of shuttling to the second station. It is worth noting that, unlike force-clamp experiment performed previously on a related rotaxane[13], here the relative proportion of the populations in Figure 2A does not reflect the ring occupancy on the different stations, given that the pulling-relaxing experiments were performed under a force ramp with a variable threshold force.

The intermediate state is also apparent from the occupancy analysis shown in Figure 2B. During the pulling (Figure 2B, top) the ring shuttles from the *fum* station (in green) to the *succ* station (in orange). The transition from one station to the other is not straightforward, but rather is interrupted by another state right in the middle of the thread, represented by the red region. This intermediate state manifests itself as a third population in the ΔF distribution (Figure 2B, right). This ΔF distribution is obtained from the deviation from the experimental force ramp (see details in SI). The ΔF value does not represent the binding force of the ring with the thread but simply reflects the presence of interacting states during the pulling and relaxing movements. Similar extension variation (Δd) graphs are routinely constructed for force-clamp experiments on folding proteins as evidence for intermediate conformations.[18-21] Identical behavior is observed during the relaxing part (Figure 2B, bottom), highlighting the presence of this intermediate step during the shuttling of the ring in both directions.

We inspected the evolution of the ring occupancy with time under increasing and decreasing force regimes. Figure 3 shows that the ring has a high probability of occupying a central position on the thread during its shuttling movements, *i.e.* again providing evidence for the intermediate step.

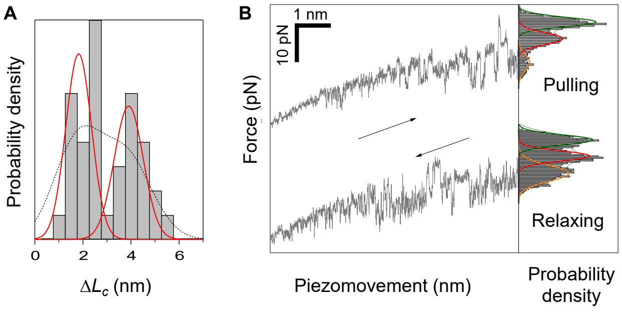

Figure 2. Evidence of an intermediate state during the ring shuttling. (A) Distribution of ΔL_c values measured on pulling and relaxing curves. The first population centered at 1.8 ± 0.2 nm corresponds to the intermediate state, *i.e.* the interaction between the ring and the oxygens in the middle of the thread. The second population centered at 3.9 ± 0.3 nm corresponds to the complete shuttling from *fum* station to *succ* station (or reversibly on relaxing curves). (B) Two force-displacement curves corresponding to subsequent pulling and relaxing movements of the tip, both showing the presence of an intermediate state. The ring shuttles from the *fum* station (green), through the intermediate state (red),

to the *succc* station (orange). The distributions based on the force deviation from the force ramp, ΔF, are shown on the right with three populations reflecting the existence of three different occupied states.

As the occupancy over time was measured under a force ramp, the movement kinetics are not constant under the experimental conditions. The implementation of force-clamp experiments by AFM on such small molecules (< 5 nm) is not straightforward and requires technical developments that are currently in progress in our lab. Nevertheless, both occupancy graphs over time show the appearance of the intermediate state and are almost symmetrical, assuring again the reversibility of the shuttling process.

The chemical structure of the thread between the two main stations comprises three short alkyl regions joined by two ether oxygen atoms (Figure 1B and S1). Given the central position of the ring in this intermediate state, as evidenced by the revealed length population at 1.8 nm (Figure 2A), we suggest that the benzylic amide ring forms hydrogen bonds between the amide N–H groups of the ring and the two oxygens of the thread. Amides are excellent H-bond donors,[22] and although ethers are modest H-bond acceptors,[22] they should be able to form weak noncovalent interactions that stabilize the ring in this central region rather than desolvate the amide groups completely in the 1,1,2,2-tetrachloroethane solvent.

The squared average end-to-end distance of a linear molecular chain in solution (corresponding here to the distance between the two oxygens) can be approximated by the formula:

$$\langle r^2 \rangle = 2nl^2$$

where n is the number of C-C bonds and l the length of the bond. The result gives an end-to-end distance of 7.5 Å, consistent with the distance between the opposite amide groups in the macrocycle. As a comparison, the distance between the hydrogen atoms of opposite amide groups in the macrocycle bound to a fumaramide station is about 6.6 Å.[23] We can exclude the formation of two degenerate H-bonds with only one oxygen atom. Indeed, the existence of two intermediate steps would appear as two additional (red) populations and would show rapid shuttling fluctuations. The intermediate step takes place at the central position of the thread where H-bonds on both sides can be created, the formation of only one H-bond being presumably too weak to temporarily capture the ring.

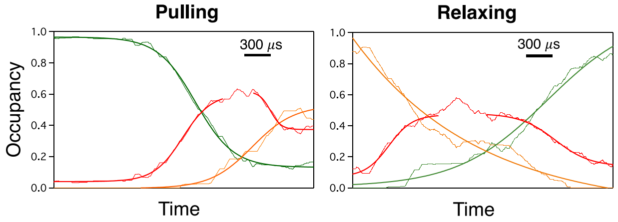

Figure 3. Relative occupancy of the molecular ring on the fumaramide (green), succinic amide-ester (orange) and intermediate (red) stations over time during a pulling-relaxing cycle. The intermediate step is clearly evidenced in the pulling (left) with the increase of the red curve once the green occupancy drops. An inverse behavior is observed in the relaxing part (right), evidence for the intermediate step in each direction. Exponential fits are added as a guide for the eyes.

Ibarra *et al.*[13] previously studied the dynamics of a related rotaxane modified to contain a very long thread so that the molecule could be investigated using optical tweezers. Force-clamp experiments under low external forces were made possible due to the high stability of the trapping and allowed them to measure the dynamics of the rotaxane in aqueous medium. They observed a much lower rupture force of 8.5 pN for the breaking of H-bonds between the macrocycle and the fumaramide station, in agreement with the relative strengths of hydrogen bonding in water and organic solvents,[11] intercomponent hydrogen bonds being stronger in less polar solvents.[22] *Hopping* events were also evidenced between the fumaramide and succinic amide-ester stations, but no intermediate states were observed. Since these experiments were performed in aqueous conditions, we suggest that the formation of weak H-bonds between the macrocycle and the polyether thread would be much less favorable, and so under those experimental conditions the intermediate states may not be significant. Furthermore, the detection of such close intermediate states is difficult given the spatial resolution of optical tweezers.

Finally, we performed an occupancy analysis on successive pulling-relaxing cycles, *i.e.* when one molecule is trapped and the ring is successively moved from one station to the other. Figure 4 reveals the presence of the intermediate state during almost all of the shuttling motions. Each population of the ΔF distributions was attributed to the corresponding state given its ΔL_c value. We note that each occupancy graph is different, reflecting the stochastic behavior of individual molecules during the shuttling cycles.

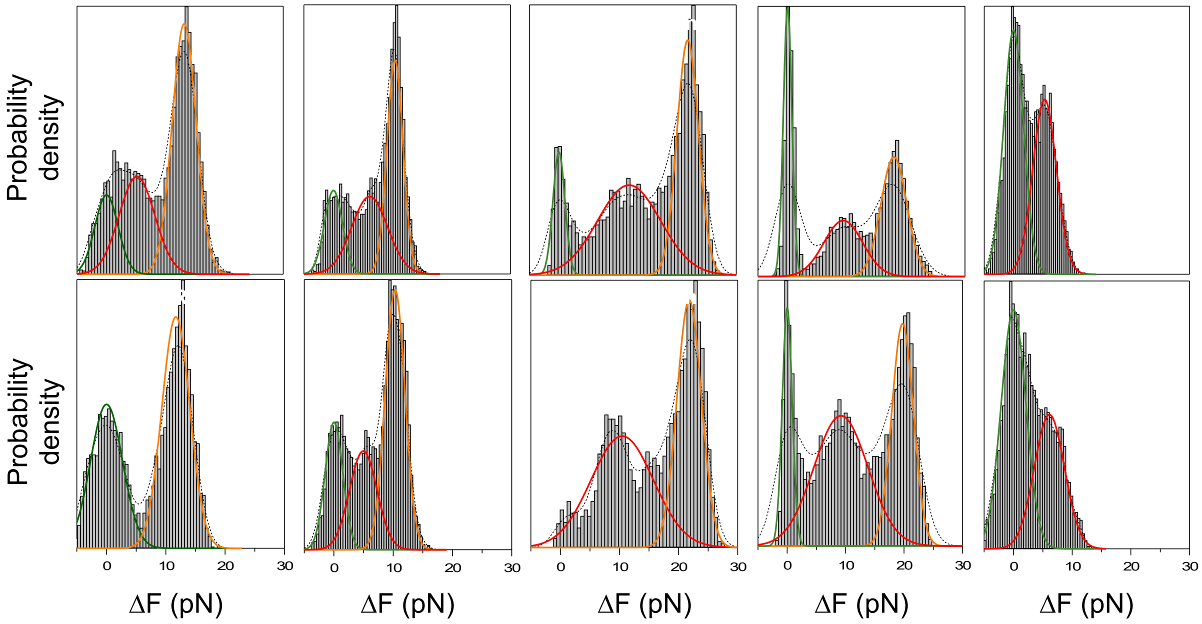

Figure 4. Deviation of the measured force from the force ramp (ΔF) obtained from successive pulling-relaxing curves, reflecting the existence of three different occupied states. The data are fitted by Gaussian distributions. ΔF is determined and normalized from the *fum* state at the beginning of the pulling. The intermediate state is always evidenced, excepted during relaxing 1. The *succ* station was not attained during the last cycle. The ΔF value associated with the intermediate and the *succ* states are different from one cycle to another, reflecting the stochastic behavior of a single molecule trapped.

CONCLUSION

The detailed SMFS investigation of the mechanically-triggered motion of a macrocycle between two stations on a molecular thread reveals an intermediate state during the shuttling in 1,1,2,2-tetrachloroethane. The intermediate probably arises from the formation of weak hydrogen bonds between the ring and two oxygen atoms in the central region of the thread. The revealing of the intermediate state was made possible by the spatial and force resolutions of the AFM along with experimental conditions specifically chosen to allow the detection of fluctuations during pulling-relaxing experiments. The experiments demonstrate that the two oxygen atoms in the thread impacts the shuttling kinetics, slowing down the ring motion between the two main stations. The results illustrate the efficacy of single-molecule force spectroscopy in identifying and characterizing individual molecular motions within complex nanoscale architectures.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

AFM-based experiment details and data analysis (PDF)

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENT

This research was funded by the FRS-FNRS (PDR T.0244.16 to A.-S.D. and C.-A.F.), the Engineering and Physical Sciences Research Council (EPSRC; EP/P027067/1) and the European Research Council (ERC Advanced Grant 786630). D.S. is a Postdoctoral Researcher of the F.R.S.-FNRS (Chargé de recherches).

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