

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

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19 evidence of weak hydrogen bonds that are difficult to detect using other methods and shows how the composition of the thread can 20 significantly influence the shuttling dynamics by slowing down the ring motion between the principal binding sites. More generally, 21 the study illustrates the utility that single-molecule experiments, such as force spectroscopy, can offer for elucidating the structure 22 and dynamics of synthetic molecular machines.

23 INTRODUCTION

²⁴ Rotaxanes are prototypical synthetic molecular machines ²⁵ consisting of a macrocycle threaded onto an axle.¹ They ²⁶ enable controlled large-amplitude movement and positioning ²⁷ of one mechanically interlocked component with respect to ²⁸ another.^{2–7} Translocation of the ring can be triggered by a ²⁹ stimulus, resulting in its directional displacement along the ³⁰ thread. This movement is dependent on the relative interaction ³¹ strengths between the ring and specific binding sites ³² ("stations") in the thread. One well-developed class of ³³ molecular shuttles features benzylic amide macrocycles ³⁴ threaded onto axles with two or more well-defined hydrogen ³⁵ bonding stations.^{6,8} In these systems, the translational ³⁶ coconformers undergo exchange by the ring moving over ³⁷ relatively large distances between the two stations, often with ³⁸ excellent positional integrity.

³⁹ Here, using single-molecule force spectroscopy (SMFS) by ⁴⁰ means of atomic force microscopy (AFM),^{9,10} we investigate ⁴¹ the shuttling motion of the ring in such a hydrogen bonded ⁴² [2]rotaxane (Figure 1A). Several SMFS experiments have been ⁴³ recently reported¹¹⁻¹⁵ that illustrate the efficacy of this ⁴⁴ technique in deciphering precise molecular behaviors and ⁴⁵ dynamic processes in rotaxanes and catenanes. In this ⁴⁶ particular [2]rotaxane (Figure 1B), the benzylic amide macrocycle is mechanically locked onto the thread by bulky 47 diphenylethyl groups situated at either end of the axle.⁸ The 48 thread bears both fumaramide and succinic amide-ester sites, 49 each of which can bind to the macrocycle through up to four 50 intercomponent hydrogen bonds (Figure 1B). The benzylic 51 amide macrocycle has a significantly higher affinity for the 52 fumaramide (fum) station than the succinic amide-ester (succ) 53 station.^{8d} The occupancy of the ring on each station (fum/succ 54 = 95:5 in 1,1,2,2-tetrachloroethane) corresponds to the $\Delta\Delta G$ 55 values between the coconformers.^{8d} SMFS has previously been 56 used to pull the ring along the axle, away from the 57 thermodynamically favored binding site, through which it 58 was demonstrated that the ring was able to travel back to the 59 original site against an external mechanical load.¹¹ Here, we 60 investigate in greater detail the mechanically induced shuttling 61 motion of the ring. We performed pulling and relaxing cycles 62 to monitor the ring motion and observed the existence of an 63

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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 $F_{\text{peak}} = 36 \pm 5$ pN.

64 intermediate step that we attribute to the formation of weak 65 hydrogen bonds with the axle along the shuttling path.

66 RESULTS AND DISCUSSION

67 The rotaxane was chemically modified for its interfacing 68 between a surface and an AFM tip following the previously 69 described strategy.¹¹ A poly(ethylene oxide) (PEO) chain 70 attached to the molecular ring serves as a tether for the tip 71 attachment during force experiments. The tip was brought into 72 contact with the substrate in 1,1,2,2-tetrachloroethane and 73 then withdrawn from the surface in a controlled manner so 74 that the molecular ring is mechanically pulled away from its 75 preferred fum station to reach the succ station. The 76 mechanically induced shuttling motion is identified by a 77 characteristic force peak (Figure 1C) presenting a revealed 78 length of ~4 nm, easily distinguishable from the usual 79 stretching and desorption of the tether. The intensity of the 80 peak, about 40 pN (Figure 1D), reflects the force required to 81 break the H-bonds between the ring and the fum station, 82 matching the force values already observed for these 83 interactions under similar conditions.^{11,16} The revealed ⁸⁴ length—obtained from the contour length variation ΔL_c of 85 the worm-like chain (WLC) model¹⁷—corresponds well to the 86 distance between the fum and the succ stations (details in the 87 SI). The relaxing part of the experiment is performed by 88 releasing the force applied on the ring by moving the tip 89 toward the surface. We observed a similar deviation in the 90 relaxing curve evidencing the shuttling of the ring back to its 91 preferred fum station (Figure S2). During both pulling and 92 relaxing curves, i.e., respectively withdrawal and approach of 93 the tip, we observed many fluctuations between the two states 94 (Figures 2B and S2). This behavior, also known as a *hopping* 95 phenomenon,^{13,14,18,19} is indicative of a rapid movement of the 96 ring between the two stations.

97 In addition to the macrocycle binding to the *fum* and *succ* 98 stations, we were able to identify an intermediate state during 99 the shuttling of the ring along the molecular thread. The 100 distribution of revealed lengths during pulling-relaxing

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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 the *fum* station to the *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 *succ* 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.

experiments shows a further population at 1.8 ± 0.2 nm 101 (Figure 2A). This corresponds to the ring being trapped in the 102 middle of the thread instead of shuttling to the second station. 103 It is worth noting that, unlike the force-clamp experiment 104 performed previously on a related rotaxane,¹³ here the relative 105 proportion of the populations in Figure 2A does not reflect the 106 ring occupancy on the different stations, given that the 107 pulling—relaxing experiments were performed under a force 108 ramp with a variable threshold force.

The intermediate state is also apparent from the occupancy 110 analysis shown in Figure 2B. During the pulling (Figure 2B, 111 top) the ring shuttles from the *fum* station (in green) to the 112 *succ* station (in orange). The transition from one station to the 113

114 other is not straightforward but rather is interrupted by $\langle r^2 \rangle = 2nl^2$ 115 another state right in the middle of the thread, represented by 116 the red region. This intermediate state manifests itself as a problem of the red region.

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115 another state right in the middle of the thread, represented by 116 the red region. This intermediate state manifests itself as a 117 third population in the ΔF distribution (Figure 2B, right). This 118 ΔF distribution is obtained from the deviation from the 119 experimental force ramp (see details in the SI). The ΔF value 120 does not represent the binding force of the ring with the thread 121 but simply reflects the presence of interacting states during the 122 pulling and relaxing movements. Similar extension variation 123 (Δd) graphs are routinely constructed for force-clamp 124 experiments on folding proteins as evidence for intermediate 125 conformations.^{18–21} Identical behavior is observed during the 126 relaxing part (Figure 2B, bottom), highlighting the presence of 127 this intermediate step during the shuttling of the ring in both 128 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 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.

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 requires technical developments that are use currently in progress in our lab. Nevertheless, both occupancy use 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 the stations comprises three short alkyl regions joined by two ether two ether stations (Figures 1B and S1). Given the central position the ring in this intermediate state, as evidenced by the two ether two ether two ether 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 two ethers are modest H-bond acceptors,²² and although the thers are modest H-bond acceptors,²² they should be able to the form weak noncovalent interactions that stabilize the ring in this central region rather than desolvate the amide groups the completely in the 1,1,2,2-tetrachloroethane solvent.

The squared average end-to-end distance of a linear ns6 molecular chain in solution (corresponding here to the ns7 distance between the two oxygens) can be approximated by ns8 the formula where *n* is the number of C–C bonds and *l* is the length of the 159 bond. The result gives an end-to-end distance of 7.5 Å, 160 consistent with the distance between the opposite amide 161 groups in the macrocycle. As a comparison, the distance 162 between the hydrogen atoms of opposite amide groups in the 163 macrocycle bound to a fumaramide station is about 6.6 Å.²³ 164 We can exclude the formation of two degenerate H-bonds with 165 only one oxygen atom. Indeed, the existence of two 166 intermediate steps would appear as two additional (red) 167 populations and would show rapid shuttling fluctuations. The 168 intermediate step takes place at the central position of the 169 thread where H-bonds on both sides can be created, the 170 formation of only one H-bond being presumably too weak to 171 temporarily capture the ring. 172

Ibarra et al.¹³ previously studied the dynamics of a related 173 rotaxane modified to contain a very long thread so that the 174 molecule could be investigated using optical tweezers. Force- 175 clamp experiments under low external forces were made 176 possible due to the high stability of the trapping and allowed 177 them to measure the dynamics of the rotaxane in aqueous 178 medium. They observed a much lower rupture force of 8.5 pN 179 for the breaking of H-bonds between the macrocycle and the 180 fumaramide station, in agreement with the relative strengths of 181 hydrogen bonding in water and organic solvents,¹¹ inter- 182 component hydrogen bonds being stronger in less polar 183 solvents.²² Hopping events were also evidenced between the 184 fumaramide and succinic amide-ester stations, but no 185 intermediate states were observed. Since these experiments 186 were performed in aqueous conditions, we suggest that the 187 formation of weak H-bonds between the macrocycle and the 188 polyether thread would be much less favorable, and so under 189 those experimental conditions, the intermediate states may not 190 be significant. Furthermore, the detection of such close 191 intermediate states is difficult given the spatial resolution of 192 optical tweezers. 193

Finally, we performed an occupancy analysis on successive 194 pulling—relaxing cycles, *i.e.*, when one molecule is trapped and 195 the ring is successively moved from one station to the other. 196 Figure 4 reveals the presence of the intermediate state during 197 f4 almost all of the shuttling motions. Each population of the ΔF 198 distributions was attributed to the corresponding state given its 199 ΔL_c value. We note that each occupancy graph is different, 200 reflecting the stochastic behavior of individual molecules 201 during the shuttling cycles. 202

The detailed SMFS investigation of the mechanically triggered 204 motion of a macrocycle between two stations on a molecular 205 thread reveals an intermediate state during the shuttling in 206 1,1,2,2-tetrachloroethane. The intermediate probably arises 207 from the formation of weak hydrogen bonds between the ring 208 and two oxygen atoms in the central region of the thread. The 209 revealing of the intermediate state was made possible by the 210 spatial and force resolutions of the AFM along with 211 experimental conditions specifically chosen to allow the 212 detection of fluctuations during pulling—relaxing experiments. 213 The experiments demonstrate that the two oxygen atoms in 214 the thread impact the shuttling kinetics, slowing down the ring 215 motion between the two main stations. The results illustrate 216 the efficacy of single-molecule force spectroscopy in identifying 217

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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 values associated with the intermediate and the *succ* states are different from one cycle to another, reflecting the stochastic behavior of a single molecule trapped.

218 and characterizing individual molecular motions within 219 complex nanoscale architectures.

220 ASSOCIATED CONTENT

321 Supporting Information

222 The Supporting Information is available free of charge at 223 https://pubs.acs.org/doi/10.1021/jacs.0c12161.

AFM-based experiment details and data analysis (PDF)

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Notes

The authors declare no competing financial interest. 249

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