Mechanical Properties

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Mechanical Activation Drastically Accelerates Amide Bond Hydrolysis, Matching Enzyme Activity

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Abstract: *Amide bonds, which include peptide bonds connecting amino acids in proteins and polypeptides, give proteins and synthetic polyamides their enormous strength. Although proteins and polyamides sustain mechanical force in nature and technology, how forces affect amide and peptide bond stability is still unknown. Using single-molecule force spectroscopy, we discover that forces of only a few hundred pN accelerate amide hydrolysis 10^9-fold, an acceleration hitherto only known from proteolytic enzymes. The drastic acceleration at low force precedes a moderate additional acceleration at nN forces. Quantum mechanochemical ab initio calculations explain these experimental results mechanistically and kinetically. Our findings reveal that, in contrast to previous belief, amide stability is strongly force dependent. These calculations provide a fundamental understanding of the role of mechanical activation in amide hydrolysis and point the way to potential applications from the recycling of macromolecular waste to the design of bioengineered proteolytic enzymes.*

The lifetime of amide and peptide bonds extends to several hundred years in aqueous solution.[1] This high stability is not only crucial for the structural integrity of proteins and biological functions like muscle contraction, it also gives synthetic polyamides, like nylon or Kevlar their fascinating endurance. For the efficient degradation of proteins, nature uses proteolytic enzymes, which accelerate peptide hydrolysis up to 10^{10}-fold.[1a] Without such enzymes, the hydrolysis of amide bonds and thus cleavage of peptides and proteins remains prohibitively slow, even at high temperatures and extreme pH.[2] Mechanical force provides an alternative route to accelerate chemical reactions.[3] It enhances the reactivity of selected reaction channels of a chemical reaction by deforming the potential energy surface (PES) in a controlled way,[3d] a process termed mechanical activation.[3,4] However, to date, the effect of mechanical force on amide and peptide bonds is virtually unknown. Among the vast number of studies on amide and peptide bond hydrolysis, only two address its mechanical activation.[4e,5] One study has even predicted that base-catalyzed peptide bond hydrolysis is insensitive to mechanical stress.[5b] In stark contrast, our single-molecule force spectroscopy (SMFS) experiments[3a,4g,6] combined with computational mechanochemistry[3d,e] and kinetic modeling reveal that astonishingly mild tensile forces dramatically decrease the lifetime of these bonds, akin to what is hitherto known only from enzymatic catalysis by proteases.

We formed individual amide bonds between an amine-terminated atomic force microscopy (AFM) tip and carboxymethylated amylose (CMA) grafted to an amine-terminated substrate, as depicted in Figure 1 A. We then picked up single CMA molecules and stretched them with a predefined force, until the connection between AFM tip and substrate broke. In this way, the survival time of the weakest bond connecting AFM tip and substrate was determined (see the Supporting Information (SI) for details). Figure 1 B shows a typical force vs. time trace at 0.6 nN clamp force which exhibits a survival time of 30.04 s. Upon a small increase in clamp force to 0.8 nN (Figure 1 C), the survival time drops to only 0.11 s. In Figure 1 D, the number of intact bonds in 58 force clamp experiments at 0.8 nN is plotted vs. time in a semi-logarithmic representation. By fitting an exponential function to this data, we obtain the mean bond lifetime $\tau_{0.8} = (0.171 \pm 0.025)$ s. This corresponds to a remarkable rate acceleration of 9 orders of magnitude, compared to thermal hydrolysis in the absence of external force.

To ensure that indeed the amide bond breaks in our experiments and not one of the bonds in the CMA linker, we conducted control experiments, where we replaced CMA with adipic acid or α,ω-biscarboxy-PEG, both of which can form amide bonds with the amine-terminated AFM tip. In both cases, the bond lifetime agreed within error limits with the value obtained with CMA (cf. SI, Figures S2 and S3). In

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Supporting information (including a detailed description of the materials used, the experimental and computational methods, the data analysis, as well as additional control experiments and experimental force- and temperature-dependent reaction rate constants and Arrhenius parameters) and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201902752.

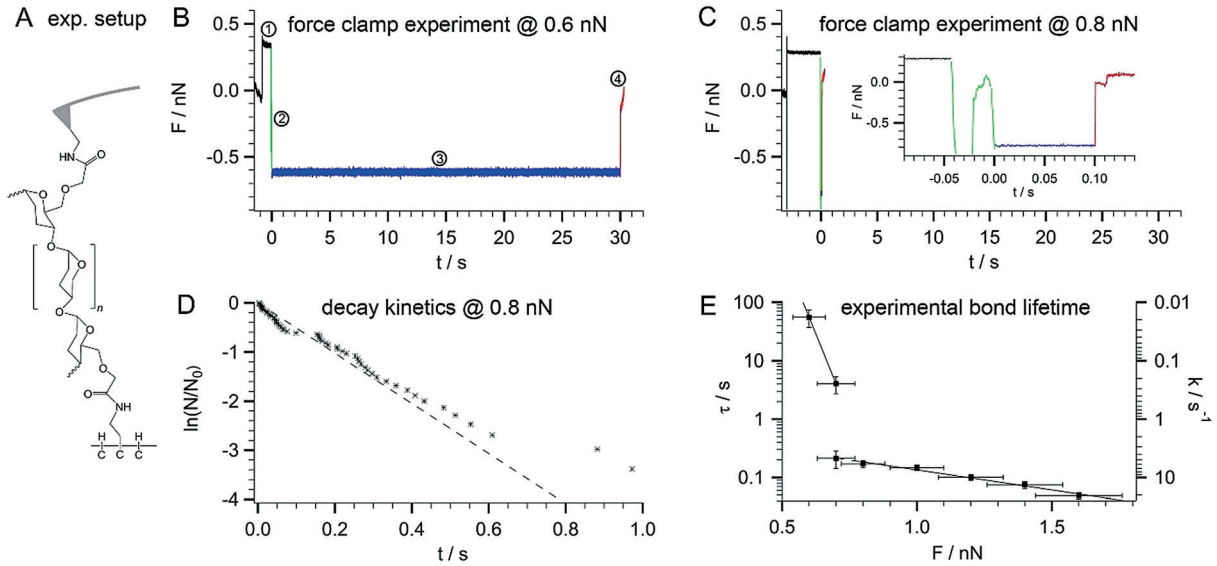


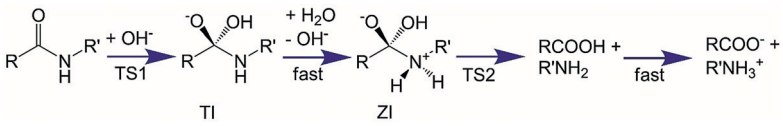
Figure 1. A) Covalent anchoring of CMA between AFM tip and substrate. B) Typical force clamp experiment at 0.6 nN: the tip approaches and touches the substrate to pick up CMA (black). The tip is withdrawn and the molecule stretched until the clamp force of 0.6 nN is reached (green). Force is kept constant (blue) until bond rupture occurs (red), yielding the bond survival time (here 30 s). C) Force clamp experiment at 0.8 nN with a survival time of 0.11 s. D) Exponential decay of the number of intact bonds at a clamp force of 0.8 nN vs. time. The fit (dashed line) yields $\tau = 0.17\ s^{-1}$. E) Mean bond lifetimes as a function of force. At 0.7 nN a biexponential decay is observed, rendering two values. Solid lines in (E) represent fits using the Bell model (cf. SI for details) from 0.6 to 0.7 nN and 0.8 to 1.6 N.

additional control experiments, we replaced the amine group on the tip and substrate by an alcohol, changing the amide to an ester bond upon reaction with CMA.[7] At a clamp force of 0.8 nN, the mean bond lifetime increased from (0.171 ± 0.025) s to (4.76 ± 0.45) s, again confirming that in the case of amine functionalization, the amide bond is in fact the weakest bond in the molecular chain connecting the AFM tip and substrate surface (cf. SI, Figure S4).

A second interesting discovery is that the force dependence changes abruptly and becomes much weaker above 0.8 nN, indicating a switch in the reaction mechanism. Figure 1E displays mean bond lifetimes from 0.6 to 1.6 nN. Between 0.6 and 0.7 nN, the lifetime decreases drastically. At a critical force of 0.7 nN, a biexponential decay, resulting in two lifetimes is observed, and above 0.8 nN, the bond lifetime continues to decrease, but with a much weaker force dependence. Such abrupt changes in the force dependence of mechanically activated covalent bond breaking reactions are rarely observed.[4e,h,8] In disulfide reduction, this has been attributed to force-induced steric hindrance.[9] Desolvation barriers or switching to different reaction channels beyond a critical force[4h,10] could be other explanations of the observed biphasic behavior. It is noteworthy that extrapolation of the bond lifetime from the low-force regime to zero force yields $\tau_0 \approx 3.5 \times 10^8$ s, roughly 11 years (cf. SI for details). This value compares favorably with literature values for thermal amide/peptide hydrolyses, which range from 10^6 to 10^{11} s.[1,11]

Aiming at a comprehensive molecular explanation of the extraordinary acceleration of amide hydrolysis and the discovered switching mechanism (Figure 1E), we first consider the thermal reaction mechanism of base-catalyzed amide hydrolysis, sketched in Scheme 1. It has two key steps: nucleophilic addition of OH^- (via transition state TS1) yielding an intermediate, and ensuing C–N bond dissociation via TS2.[12] Ab initio simulations[13] revealed that the intermediate consists of two rapidly interconverting states: an anionic tetrahedral (TI) and a zwitterionic (ZI) intermediate. The rates of the two key steps controlling the reaction kinetics depend on the height of the two activation barriers TS1 and TS2. At room temperature, it takes decades before thermal energy drives the reaction across both barriers. Force, however, distorts the PES and changes the activation energies in a distinct way. Upon mechanical activation, the key question is therefore the force sensitivity of TS1 versus TS2.

To address this question, we computed the mechanically activated base-catalyzed hydrolysis of a model amide bond using isotensional ab initio calculations.[4b] (see SI for computational methods and details). Figure 2A quantifies how the relevant energies along the thermal hydrolysis pathway ($F = 0$ nN) are affected by increasing the force to 1.8 nN. The first activation barrier (TS1) is essentially force insensitive, while TS2 exhibits a pronounced force depend-



Scheme 1. Base-catalyzed amide bond hydrolysis at moderate pH ($7 < pH < 10$) in the absence of mechanical activation: nucleophilic attack of the carbonyl carbon by OH^-(aq) via the first (lower-energy) transition state (TS1) generates the tetrahedral intermediate (TI), which is readily protonated by water to yield the zwitterionic intermediate (ZI). The C–N amide bond is cleaved in a subsequent step upon transcending the second (high-energy) transition state (TS2), followed by fast proton exchange to yield the charged cleavage products.

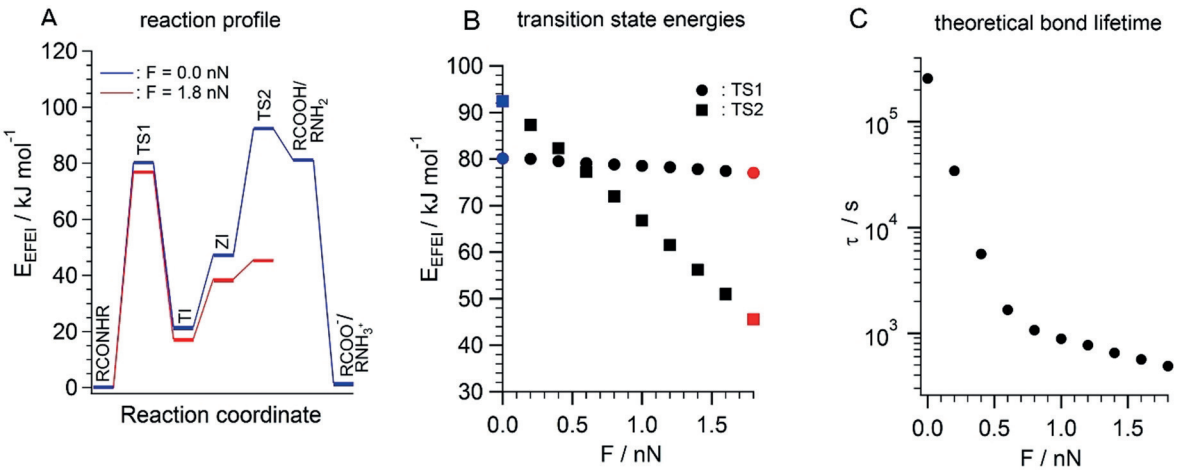


Figure 2. A) Reaction energy profiles of base-catalyzed amide bond hydrolysis in water (cf. Scheme 1) at $F=0$ and 1.8 nN, calculated from isotensional quantum mechanochemistry (cf. SI for details). B) Energies of the transition states TS1 and TS2 relative to reactants (cf. Scheme 1) provide the activation energies of the first and second reaction step, respectively, depending on the tensile force. C) Computed effective amide bond lifetimes τ as a function of force (cf. SI for details).

ence (cf. Figure 2B): the initial OH^- association (via TS1) proceeds nearly perpendicular to the pulling direction (cf. scheme in SI), while the final C–N bond dissociation (via TS2) occurs almost parallel to the applied force, providing for more effective mechanical coupling at this reaction step. Importantly, while TS2 is rate limiting at zero force, at approximately 0.6–0.8 nN it drops below the first barrier (TS1), making the forward reaction to C–N cleavage more probable than the backward reaction for forces above this critical value. Since the experimentally observed bond lifetime depends on the coupled kinetics of this two-step reaction, we considered the complete forward/backward kinetics involving all states depicted in Scheme 1 to compute the effective overall rate k_{hyd} and thus $\tau = 1/k_{hyd}$ as a function of force (cf. SI for details). In accord with experiment, between 0.6 and 0.8 nN, we observe a kink in the force dependence of τ (cf. Figure 2C). We can thus assign the reactivity switch to the drop of the second barrier (TS2) below the first one (TS1) beyond this critical force. Previously, only the force dependence of TS1 was considered, leading to the conclusion that base-catalyzed peptide/amide hydrolysis is insensitive to mechanical stress.[5b] However, it is the unexpected pronounced force sensitivity of the second transition state, TS2, which provides the mechanistic underpinnings of the outstanding acceleration of bond cleavage as discovered in our SMFS experiments.

For direct comparison of our experimental data to the theoretical activation energies, we carried out temperature-dependent measurements for five different forces between 0.8 and 1.6 nN and plotted the temperature-dependent reaction rate constants in an Arrhenius plot (Figure 3). The height of the first activation barrier (TS1) at these five forces can be derived from the slopes in the Arrhenius plot: The force-dependent activation energies are between (51 ± 13) kJ mol^{-1} at 1.6 nN and (61 ± 10) kJ mol^{-1} at 0.8 nN (cf. SI, Table S2 for the activation energies at the other forces). Extrapolation to zero force yields (69 ± 17) kJ mol^{-1} for TS1 in the thermal limit. Thus, within error limits, not only the small force sensitivity of the first barrier, but also its absolute value agree with our theoretical value of 80 kJ mol^{-1} at $F=0$ nN. Note that extrapolation to zero force from this high-force regime (0.8–1.6 nN) yields a rate of $k_{TS1} = 1/\tau_0 = (1.5 \pm 0.2)$ s^{-1} for crossing TS1 in the thermal limit. This would translate into a mean bond lifetime τ_0 as low as (0.65 ± 0.09) s if only the first barrier were present and thus emphasizes the crucial role of TS2 for the kinetic stability of amide and peptide bonds in proteins and polyamides.

Our single-molecule experiments disclose a hitherto unknown drastic, force-induced acceleration of amide/peptide bond cleavage under physiological conditions. This surprising discovery is in strong contrast to previous belief

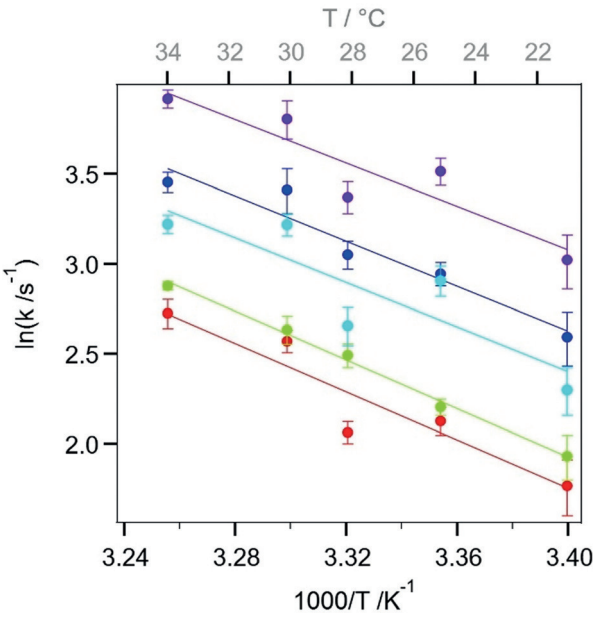


Figure 3. Arrhenius plots showing temperature-dependent reaction rate constants for clamp forces from 0.8 nN (red) to 1.6 nN (violet) in 0.2 nN steps. The slopes yield the force-dependent activation energies corresponding to the height of TS1 (cf. SI for details). Owing to thermal drift of the AFM cantilever, temperature dependent bond lifetimes could only be determined between 0.8 and 1.6 nN.

that base-catalyzed peptide hydrolysis is insensitive to mechanical stress. Moreover, a reactivity switch is detected at 0.7 nN, above which further force-induced acceleration becomes much weaker. Our force-dependent quantum chemical calculations convincingly explain these experimental observations, revealing that the initial acceleration and the switching behavior are caused by the distinct force dependences of the two prominent activation barriers TS1 and TS2. Taken together, these findings demonstrate the crucial role of mechanical forces in amide and peptide bond hydrolysis. They highlight the prominent role of the second activation barrier (TS2) for protein as well as polyamide stability. In future applications, our results may help to devise new energy-efficient strategies for example, to decompose and recycle macromolecular waste, by using mechanical instead of purely thermal activation. One might even envision harnessing mechanical energy in the design of bioengineered proteolytic enzymes.[14] Finally, there is increasing evidence that mechanical force indeed accelerates proteolysis in living systems, which has been attributed to the partial unfolding of proteins by force.[15] In view of our findings, it would be surprising if nature had not used mechanical activation to increase enzymatic efficiency also in a more direct way, that is, by lowering the activation energy.

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Conflict of interest

The authors declare no conflict of interest.

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