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Single-Molecule Force Spectroscopy Measurements of Bond Elongation during a Bimolecular Reaction

Sri Rama Koti Ainavarapu,*,†,§ Arun P . Wiita,† Lorna Dougan,† Einar Uggerud,‡ and Julio M . Fernandez*,†

Department of Biological Sciences, Columbia University, New York, New York 10027, and Mass Spectrometry Laboratory and Centre for Theoretical and Computational Chemistry, Department of Chemistry, University of Oslo, P.O. Box 1033 Blindern, N-0315 Oslo, Norway

Received January 9, 2008; E-mail: koti@tifr.res.in; jfernandez@columbia.edu

Abstract: It is experimentally challenging to directly obtain structural information of the transition state (TS), the high-energy bottleneck *en route* from reactants to products, for solution-phase reactions. Here, we use single-molecule experiments as well as high-level quantum chemical calculations to probe the TS of disulfide bond reduction, a bimolecular nucleophilic substitution (S_N2) reaction. We use an atomic force microscope in force-clamp mode to apply mechanical forces to a protein disulfide bond and obtain force-dependent rate constants of the disulfide bond reduction initiated by a variety of nucleophiles. We measure distances to the TS or bond elongation (Δx), along a 1-D reaction coordinate imposed by mechanical force, of 0.31 $\pm$ 0.05 and 0.44 $\pm$ 0.03 Å for thiol-initiated and phosphine-initiated disulfide bond reductions, respectively. These results are in agreement with quantum chemical calculations, which show that the disulfide bond at the TS is longer in phosphine-initiated reduction than in thiol-initiated reduction. We also investigate the effect of solvent environment on the TS geometry by incorporating glycerol into the aqueous solution. In this case, the Δx value for the phosphine-initiated reduction is decreased to 0.28 $\pm$ 0.04 Å whereas it remains unchanged for thiol-initiated reduction, providing a direct test of theoretical calculations of the role of solvent molecules in the reduction TS of an S_N2 reaction. These results demonstrate that single-molecule force spectroscopy represents a novel experimental tool to study mechanochemistry and directly probe the sub-ångström changes in TS structure of solution-phase reactions. Furthermore, this single-molecule method opens new doors to gain molecular level understanding of chemical reactivity when combined with quantum chemical calculations.

Introduction

Knowledge of the transition state (TS), the family of configurations through which reactants evolve into products, is the key to molecular level understanding of chemical reactivity.[1] Chemical reactions that occur normally in the solution phase are more complex and involve many atoms, and there is growing evidence that solvent molecules also affect reaction kinetics by directly participating in TS structures.[2–6] This is particularly relevant for enzyme-catalyzed reactions, which are critical for biological function.[7] Although studies such as linear free energy relationships[8] or heavy atom kinetic isotope effects (KIE)[9] indirectly probe the potential TS structure of reactions in complex systems, atomic-level information about the TS has been until now only gained through quantum chemical calculations. Because there is no standard method available to directly determine the TS structure in solution,[1] there is a need to develop new experimental approaches that can directly probe TSs of complex chemical reactions in solution, and the results can then be compared with those of computational methods to further our understanding of chemical reactions.

Herein, we demonstrate that the reaction kinetics as well as structural details of the TS of a bimolecular reaction in solution can be obtained by using the recently developed single-molecule force-clamp atomic force microscope (AFM) technique.[10] The bimolecular reaction of interest in this study is the reduction of a disulfide bond. Disulfide bonds are critical to the structural integrity and the function of many proteins.[11,12] Mechanical properties of proteins containing disulfide bonds have previously

† Columbia University.
‡ University of Oslo.
§ Present address: Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai 400005, India.

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been investigated by using an AFM in constant-velocity mode (i.e., force-extension AFM).[13–17] In these studies, the reduction of disulfide bonds resulted in an increase in protein contour length, but single disulfide reduction events could not be identified.[13–17] Furthermore, these studies failed to directly probe the effect of stretching force on the disulfide bond reduction reaction. By using a variety of engineered polyproteins and force-extension AFM, Ainavarapu et al.[18] provided the first demonstration that disulfide bond reduction could indeed be observed at the single-molecule level. However, in these experiments where length and force varied simultaneously while extending the molecule, it was difficult to distinguish the dual effects of force on the kinetics of disulfide bond reduction: solvent exposure of the disulfide bond following mechanical unfolding and acceleration of the chemical reaction itself through the application of mechanical force. These issues were resolved by using the double-pulse protocol in force-clamp AFM introduced by Wiita et al.,[19] where the mechanical unfolding of the protein was kinetically separated from disulfide bond reduction by extending single molecules at a constant force. These experiments opened the door for other studies of the kinetics of force-dependent mechanochemical reactions.[20,21] Here, we use force-clamp AFM and quantum chemical calculations to further probe the detailed mechanochemistry of disulfide bond reduction. By incorporating a variety of small molecule chemical reducing agents as well as differing solvent environment conditions, we demonstrate that force-clamp AFM can reveal novel physicochemical properties of disulfide bond reduction and details of its TS geometry.

Materials and Methods

Materials. Disulfide bond reducing agents L-glutathione hydrochloride (GSH), β-mercaptoethanol (BME), 1,4-DL-dithiothreitol (DTT), 1,4-dithioerythritol (DTE), L-cysteine, tris(2-carboxyethyl) phosphine hydrochloride (TCEP), and tris(hydroxypropyl) phosphine (THP) are purchased from Sigma Chemicals and used without further purification. For aqueous solution experiments, the solutions of reducing agents were made in phosphate-buffered saline (PBS), pH 7.4. For glycerol incorporated solutions, the reducing agents were first dissolved in PBS solution, and then, glycerol was added to make the 30% v/v glycerol solution. The pH of the solution was then adjusted to 7.4. Design, construction, and purification of the $(I27_{G32C-A75C})_8$ polyprotein was described previously.[19,22]

AFM Experiments. The details of our custom-built AFM can be found elsewhere.[10,19] Silicon-nitride cantilevers are purchased from Veeco Corp., and their spring constants (15−30 pN/nm) were measured by using the equipartition theorem. All experiments were performed at room temperature (~25 °C). In our two-stage protocol, a force of 150 pN for 0.5 s (or 180 pN for 0.2 s) was applied to single molecules of polyprotein in the first stage, and in the second stage, the force was held constant in the range 100−400 pN for 5 s. The disulfide bond reduction is a slow process and is rarely observed in the first stage owing to the very low force applied over very short time.[19]

Data Analysis. Statistical analysis (average and standard error of the mean (SEM)) of the rate constants, $r(F)$, is performed by using the bootstrapping statistical method.[23] A randomly selected resampling set of extension profiles (extension versus time) from the original sample of single-molecule traces was averaged, and $r(F)$ was measured by fitting a single-exponential function. This procedure was repeated 200 times, and from the resulting distributions, $r(F)$ and SEM were estimated. The SEM of $r(F)$ was used as the weighting factor while fitting the $r(F)$ data to extract Δx and E_a in the Arrhenius model.

Computational Methods. Quantum chemical calculations were carried out by using the program system Gaussian 03.[24] All relevant critical points (reactants, TS structures, intermediates, and products) of the potential energy surface were characterized by complete optimization of the molecular geometries by using the hybrid density functional scheme B3LYP[25] with the 6-31G(d) basis set, which is abbreviated by B3LYP/6-31G(d). Relative energies were calculated by including unscaled zero-point vibrational energies. The fact that the reported TSs connect the indicated reactant and product structures was verified by calculating the intrinsic reaction coordinate paths.[26] Calculations including a polarizable continuum (water dielectricum) were conducted according to the self-consistent reaction field method reported by Tomasi et al.[27]

Results and Discussion

We have engineered two proximal cysteines in the 89-residue I27 protein, the 27th immunoglobulin-like domain of cardiac titin, by two point mutations (Gly32Cys and Ala75Cys) and constructed a polyprotein $(I27_{G32C-A75C})_8$ consisting of eight identical protein domains.[18,19,22] In this construct, each folded $I27_{G32C-A75C}$ domain contains a single disulfide bond (Cys32−Cys75), which is buried and inaccessible to the external solvent.[22] Wiita et al.[19] studied the kinetics of Cys32−Cys75 disulfide bond reduction in the presence of the reducing agent DTT with force-clamp AFM. Here, we study the kinetics of disulfide bond reduction by using a biologically relevant disulfide bond reducing agent GSH, a tripeptide with glutamic acid forming the amide linkage with the amino group of cysteine (see Table 1). The high concentration (0.1−10 mM) of GSH in cells is primarily responsible for the intracellular reducing environment, and most (>90%) of the nonprotein sulfur of the cell is in the form of GSH.[28] By using an AFM in force-clamp mode,[10] we extended single polyprotein molecules in 12.5 mM GSH dissolved in PBS by applying mechanical force in two stages (Figure 1). In the first stage, the polyprotein is stretched at a constant force (150 pN) to observe individual domains unfolding in a stepwise manner. In the second stage, the force on the protein molecule is held constant at a value in the range 100−400 pN. Each step in the second stage indicates the reduction of a single Cys32−Cys75 disulfide bond by the reducing agent GSH and immediate extension of the polypeptide

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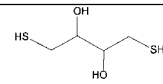
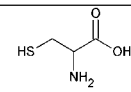
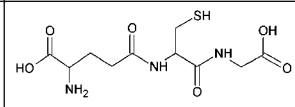
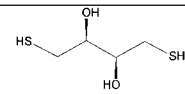
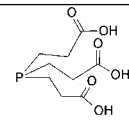
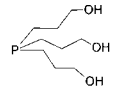
Table 1. Structures and Redox Properties of Disulfide Reducing Agents

disulfide reducing agent	structure	pK_a of RSH/PR_3H^+	redox potential, E (volt)*
DTT (1,4-DL-dithiothreitol)		9.2[55]	-0.33[55]
L-cysteine		8.3[56]	-0.22[57]
L-Glutathione (GSH) (γ-glutamyl-cysteinyl-glycine)		9.1[58]	-0.24[57]
DTE (1,4-dithioerythritol)		9.2[55]	-0.33[55]
BME (β-mercaptoethanol)		9.1[56]	-0.26[59]
TCEP (tris(2-carboxyethyl) phosphine)		7.6[32]	-
THP (tris(hydroxypropyl) phosphine)		7.22[32]	-

* Redox potentials are defined for reversible reactions only. Because phosphines reduce disulfide bonds via an irreversible pathway, redox potentials are not defined for them.

chain connecting Cys32 and Cys75. In such a two-stage protocol, the mechanical unfolding of the protein in the first stage is kinetically separated from disulfide bond reduction in the second stage, making it possible to directly study force-dependent disulfide bond reduction.[19] We can also rule out the mechanical rupture of the disulfide bond in our experiments as it is known to occur at very high forces (>1 nN).[29]

Figure 2A shows the force-dependent kinetic traces of disulfide bond reduction by GSH. We averaged many single-molecule recordings at each force and fit a single-exponential function to determine the force-dependent pseudofirst-order rate constant, $r(F)$, for disulfide bond reduction. The measured $r(F)$ demonstrates that the stretching force exponentially accelerates the reduction reaction (Figure 2B). This relationship is similar to the case of disulfide bond reduction by DTT under a stretching force, as studied by Wiita et al.[19] In a first approximation, these results can be described by a straightforward Arrhenius model,[30,31] where the force-dependent process is described by $r(F) = r_0 \exp(F\Delta x/k_BT)$. Here, r_0 is the rate constant at zero force ($F = 0$), T is absolute temperature, k_B is Boltzmann constant, F is the applied force, and Δx is the distance to the TS. Previous studies suggested that the experimentally measured Δx may directly correspond to the lengthening of the disulfide bond at the TS.[19] Fitting the Arrhenius model to the force-dependent rate constant data of GSH yields a distance to TS value of $\Delta x = 0.29 \pm 0.06$ Å (Figure 2B). This can be compared with $\Delta x = 0.34$ Å obtained by Wiita et al.[19] for the thiol-based reducing agent DTT. We further estimate the activation energy, E_a, by using the relationship $r_0 = [red]A \exp(-E_a/k_BT)$, where $[red]$ is the concentration of the reducing agent and A is the pre- or frequency factor (10^{12} $M^{-1}s^{-1}$). We estimate the activation barrier to reach the reduction TS to be 55.3 ± 1.0 kJ/mol for GSH. To compare the kinetic properties of GSH with those of other thiol-based disulfide bond reducing agents, we also performed experiments by using BME, DTE, and cysteine, and the force-dependent rate constants are obtained by using the Arrhenius model (Figure S1, Supporting Information). The results are presented in Table 2. Interestingly, these thiol-based reducing agents have a rather narrowly distributed set of values of Δx (0.29−0.35 Å) and E_a (53.5−55.3 kJ/mol), except cysteine, which has a smaller Δx (0.23 Å) and slightly larger E_a (56.3 kJ/mol). Such similarity in experimental values of Δx and E_a strongly suggests that the TS characteristics of disulfide bond reduction by these thiol-based reducing agents are structurally and energetically similar, irrespective of the structural and electrochemical differences in reducing agents (Table 1). These results led us to ask the following question: How does a reducing agent other than a thiol-based reagent affect the TS

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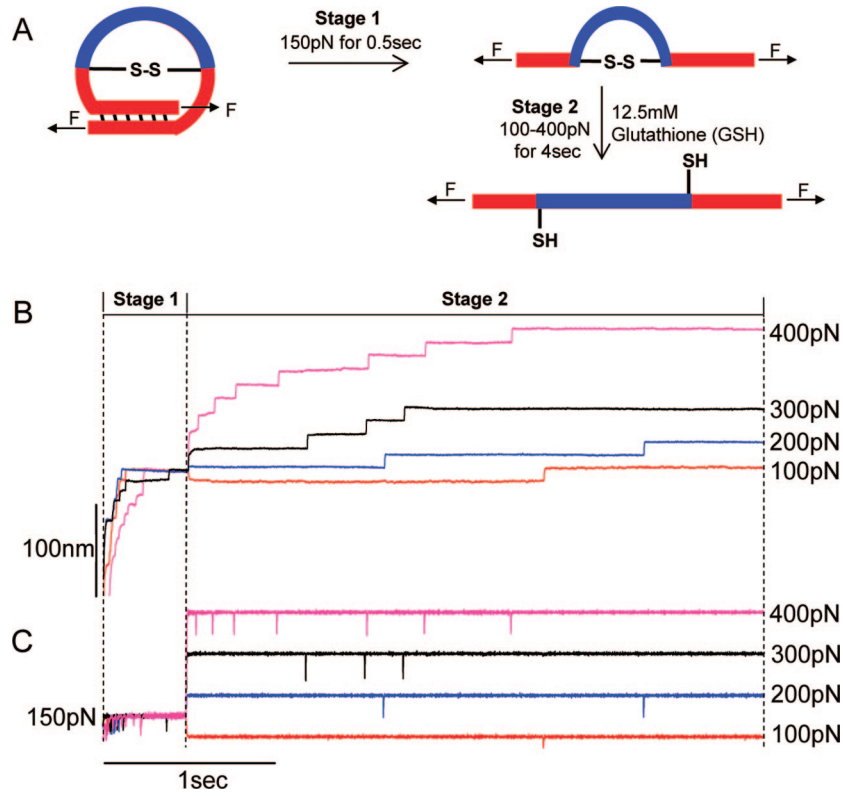

Figure 1. Reduction of protein disulfide bonds in the presence of a disulfide reducing agent observed by the single-molecule force-clamp technique. (A) Diagram showing the modified I27 protein, $I27_{G32C\text{-}A75C}$, with an engineered disulfide bond (Cys32−Cys75), being pulled by an AFM cantilever in two stages. Stage 1 includes the mechanical stretching of the protein and exposing the sequestered disulfide bond. This disulfide bond is reduced in the presence of a reducing agent in stage 2. (B) Temporal extension profile of the octameric protein, $(I27_{G32C\text{-}A75C})_8$, in 12.5 mM GSH (in PBS, pH 7.4) at various stretching forces. Unfolding steps (~10.5 nm) in stage 1 are due to the stretching of individual protein modules under force (150 pN) whereas the steps in stage 2 (13−15 nm at 100−400 pN) correspond to the reduction of individual disulfide bonds and stretching the remaining polypeptide between the cysteines. (C) Corresponding temporal profiles of stretching forces.

properties of disulfide bond reduction? To directly address this question, we studied the force-dependent kinetics of disulfide bond reduction with a different class of reducing agents, namely, phosphine-based reducing agents.

To investigate the effect of the attacking nucleophile on the TS properties, we conducted experiments with the phosphine-based reducing agents TCEP and THP (see Table 1 for structures). The force-dependent kinetics of disulfide bond reduction in the presence of TCEP is presented in Figures 2C,D and 3. In this experiment, the concentration of TCEP was chosen to be 2.5 mM (compared to 12.5 mM for thiol-based reducing agents) because the force-dependent (100−400 pN) rate constant $r(F)$ for the disulfide bond reduction would be in a range ($0.1-15\ s^{-1}$) that could be accurately measured with our experimental time window of 5 s. Furthermore, it has been shown that phosphines reduce disulfide bonds stoichiometrically.[33,34] Here, we have confirmed the first-order dependence of the disulfide bond reduction on [TCEP], demonstrating that the mechanochemical reduction of disulfide bonds is also a bimolecular reaction (Figure S2, Supporting Information), which is similar to the mechanochemical disulfide reduction by thiol-based reducing agents.[19] From Figures 2 and 3, it is apparent that the disulfide bond reduction by TCEP is also exponentially accelerated upon applying a mechanical force, similar to thiol-based reducing agents. When fitting the force-dependent rate constant with the Arrhenius model, we measure $\Delta x = 0.46 \pm 0.03$ Å, which is larger than that of thiol-based reducing agents (Table 2). Similarly, for the phosphine-based reducing agent THP, we measured a $\Delta x = 0.42 \pm 0.06$ Å larger than that measured for thiol-based reducing agents. Our experiments strongly suggest that the sub-ångström change in the measured value of Δx is directly related to the chemical reaction occurring in our system. Thus, the experimental results lend further support to the proposal that the measured value of Δx is related to disulfide bond elongation at the reaction TS, where phosphine- and thiol-initiated reduction reactions result in different TS geometries. On the basis of our measurements, we generated a simplified free energy landscape for the thiol- and phosphine-initiated disulfide bond reduction under a stretching force (Figure 4).

There is abundant evidence that the reduction of disulfide bonds proceeds via a bimolecular nucleophilic substitution (S_N2) mechanism.[32,34–38] According to this mechanism, the nucleophilic attack on the disulfide bond and the displacement of the leaving group occur simultaneously.[35,36] Previous experimental evidence suggested similarities between disulfide bond reduction

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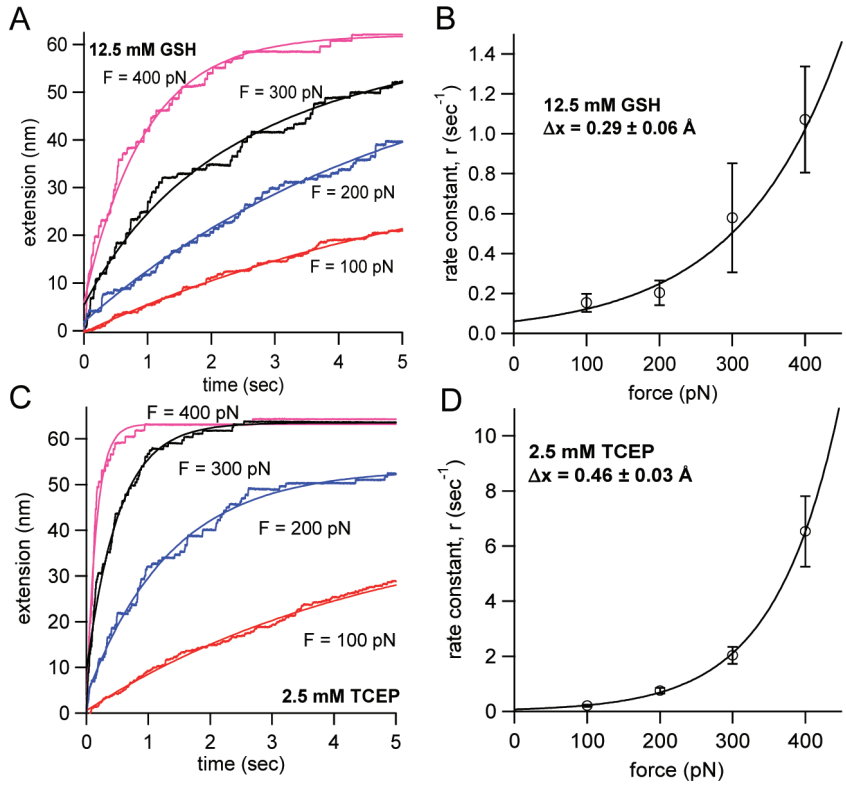

Figure 2. Force-dependent rate constants, $r(F)$, of the Cys32−Cys75 disulfide-bond reduction by GSH (A and B) and by TCEP (C and D). Panels A and C show stage 2, containing only reduction events averaged for many single-molecule recordings (n = 10−40) at each force. Rate constants, $r(F)$, are obtained by fitting the extension to a single-exponential function. It must be noted that the bootstrap error (SEM) in the rate constant is proportional to $r(F)$. The $r(F)$ increases exponentially with force (F), and so does the SEM. However, the increase in error may also be due to fewer data points (single-molecule traces) at high forces because the molecules detach more often at these forces. In panels B and D, the distance to the TS (Δx) is determined by using the Arrhenius model (fits are shown).

Table 2. TS Properties of the Disulfide Reduction with Different Chemical Reducing Agents

reducing agent	pK_a of RSH or PR_3H^+	conc. (mM)	[*red*][a] (mM)	Δx (Å)	E_a (kJ/mol)
GSH	9.1	12.5	0.2	0.29 ± 0.06	55.3 ± 1.0
BME	9.1	12.5	0.2	0.35 ± 0.05	54.5 ± 1.0
DTT	9.2	12.5	0.2	0.34 ± 0.05	54.3 ± 0.8
DTE	9.2	12.5	0.2	0.33 ± 0.05	53.5 ± 0.9
cysteine	8.3	12.5	1.4	0.23 ± 0.05	56.3 ± 0.8
TCEP	7.6	2.5	1.0	0.46 ± 0.03	58.3 ± 0.5
THP	7.22	2.5	1.5	0.42 ± 0.06	56.7 ± 1.4

[a] Calculated from pK_a and pH (= 7.4) by using pH = pK_a + log([*red*]/[red_{prot}]), where *red* and red_{prot} are deprotonated and protonated forms of the reducing agent, respectively. In the case of thiol-based reducing agents, no correlation between the redox potential (E) (see Table 1) and the distance to the TS (Δx) or activation energy (E_a) was found.

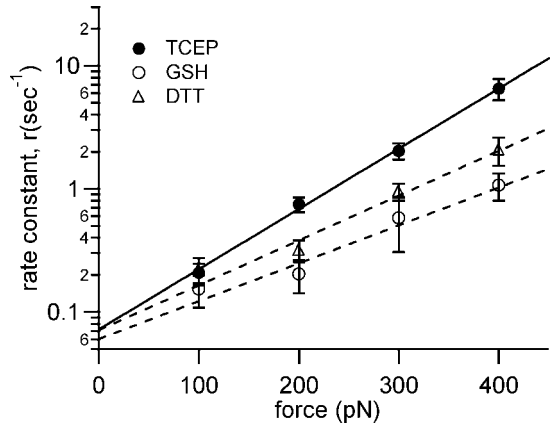

Figure 3. Arrhenius model fits to the force-dependent rate constants, $r(F)$, for different reducing agents. The steeper slope for phosphine indicates a higher force sensitivity and hence a larger Δx.

by thiol- and phosphine-based reducing agents.[32,34,38] Unlike thiol/disulfide exchange, which is a reversible reaction, the disulfide bond reduction by phosphines is irreversible and involves oxidation of the phosphorus atom. To investigate the mechanistic origin of disulfide bond reduction by phosphines and compare it with that of thiols, we performed quantum chemical calculations of the disulfide bond reduction TS. We studied the following prototype reaction in the presence of four extra water molecules, on the basis of the model of Fernandes and Ramos.[39]

$$CH_3SH + CH_3SSCH_3 \rightarrow CH_3SSCH_3 + CH_3SH \quad \text{(reaction1)}$$

The results are presented in Figure 5 (also see Scheme 1, Supporting Information). At the TS (Figure S3, Supporting Information), all hydrogen atoms linking the oxygens of water molecules and the arriving and departing sulfurs move in a clockwise fashion. This external hydrogen-transport wire is responsible for simultaneous deprotonation of the arriving sulfur and protonation of the departing sulfur. Coupled to this external proton transfer is the motion of the sulfur atom in the six o'clock position, representing the actual S_N2 type of displacement which

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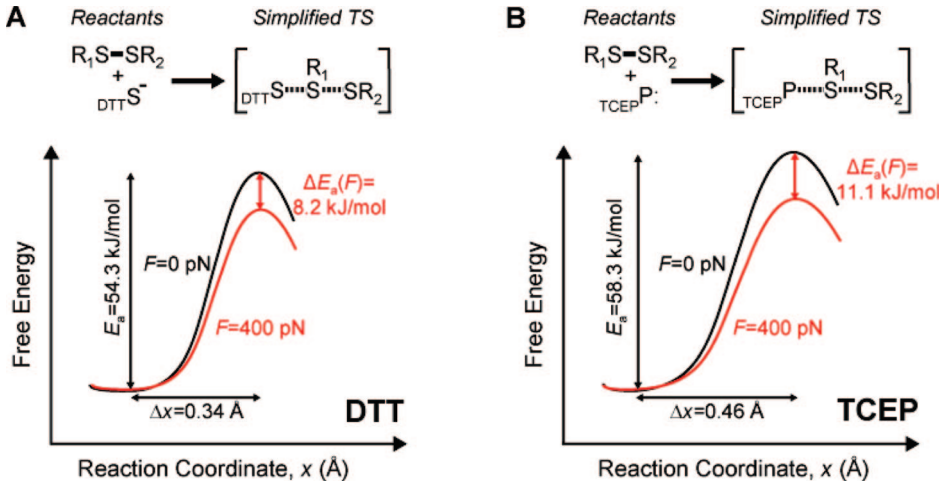

Figure 4. Simplified illustration of the free energy landscape of thiol- and phosphine-initiated disulfide-bond reduction. (A) In this model of DTT-catalyzed disulfide-bond reduction (top), one thiolate anion of DTT ($_{DTT}S^-$) initiates nucleophilic attack on the disulfide bond R_1S-SR_2. The estimated activation barrier (E_a) for the reaction at zero force is 54.3 kJ/mol, and the physical distance to the TS for the reaction along the force axis, Δx, is 0.34 Å. This is represented in the free energy landscape shown (bottom). An applied force of 400 pN lowers the activation energy barrier by $\Delta E_a(F) = 8.2$ kJ/mol, leading to an increase in the reduction rate under force. The factor Δx is interpreted as the length increase of the disulfide bond between R_1S and SR_2 at the TS of the reaction (top right) when compared to the equilibrium bond length (top left). (B) Same analysis for disulfide reduction as in (A) but here initiated by the lone pair of electrons on the phosphine-based reducing agent TCEP. For the phosphine-catalyzed reaction, the calculated E_a is higher but S−S bond lengthening at the TS is larger, leading to a more pronounced effect of force on the reaction kinetics. Note that the simplified TS only shows the important reactive atoms and does not include any solvent molecules.

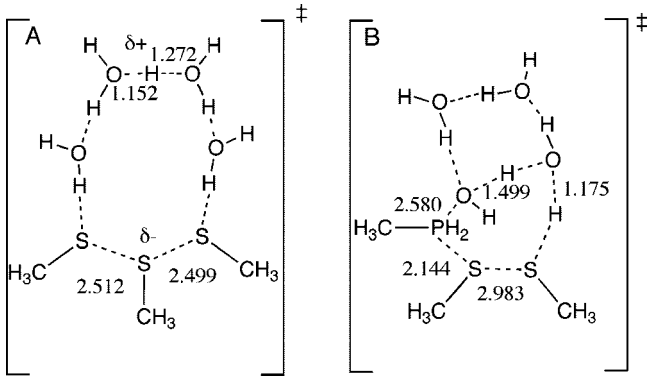

Figure 5. Geometries of the TSs found for the prototypical reduction by (A) a thiol and (B) a phosphine including the aqueous environment by using B3LYP/6-31G(d). Bond distances are in angström. For details, see the text and Supporting Information.

leads to reduction of the disulfide bond. The number of water molecules was carefully chosen to be sufficiently large to allow for proton transfer along a wire of unconstrained water molecules and small enough to permit calculation. Additional solvent effects were treated by imposing a self-consistent reaction field, which has a small influence on the calculated barrier height. See Supporting Information for more details. Because the mechanistic details of the reaction mechanism were mostly unknown, we decided to model the reaction quantum chemically for the following prototype reaction.

$$CH_3PH_2 + CH_3SSCH_3 + H_2O \rightarrow 2CH_3SH + (CH_3)H_2PO \quad \text{(reaction2)}$$

In order to make the model representing the phosphine reaction comparable to reaction 1, it was necessary to include the same number of explicit water molecules, namely, four. However, one water molecule is acting as a reactant (see reaction 2). and three additional water molecules are used to simulate the surrounding solvent. In the first phase of the reaction (see Scheme 1, Supporting Information), the P−S bond distance decreases whereas the S−S bond distance decreases; this is accompanied by a slight shortage of the P−O contact to one of the solvent waters. Before passage of the TS, this solvent water starts to transfer one of its hydrogens to a neighboring water molecule, which responds by transferring a hydrogen to the terminal sulfide which is to become expelled. At the TS, this atomic motion comprises the largest component of the reaction coordinate. At this stage, the S−S bond is effectively broken. Beyond the TS, the most noticeable change is the significant shortening of the P−O contact, completing the oxidation of the phosphorus atom. The key feature of the TS structure is that the disulfide bond to be broken for phosophine-initiated reduction is 2.983 Å, which is significantly longer than the corresponding bond in the TS for thiol-initiated reduction of 2.499 Å (Figure 5). The S−S bond distance in dimethyl disulfide is 2.090 Å prior to reaction. Thus, we find good qualitative agreement between experimental and quantum chemical calculations in terms of TS geometry, as the critical observation is that both the quantum chemical calculations of disulfide bond elongation and the experimental results of Δx confirm that the TS should be significantly longer for phosphine-initiated reduction when compared to thiol-initiated reduction. Our experimental results are also in agreement with previous studies of periodic trends in nucleophilicity, barrier heights, and bond-elongation factors, which all show that the TSs for phosphines have higher energy barriers and more elongated bonds compared to thiols.[40] Given these lines of evidence, our results here demonstrate that the experimental measure of Δx can serve as a direct probe of the TS geometry in the solution-phase reactions. In addition, our experimental methods serve as a new way to examine TS geometry and compare to quantum chemical calculations, which has not been investigated with other methods.

We note that the bond elongation values at the TS from quantum chemical calculations (0.41 Å for thiols and 0.89 Å for phosphines) are different from experimental values of Δx

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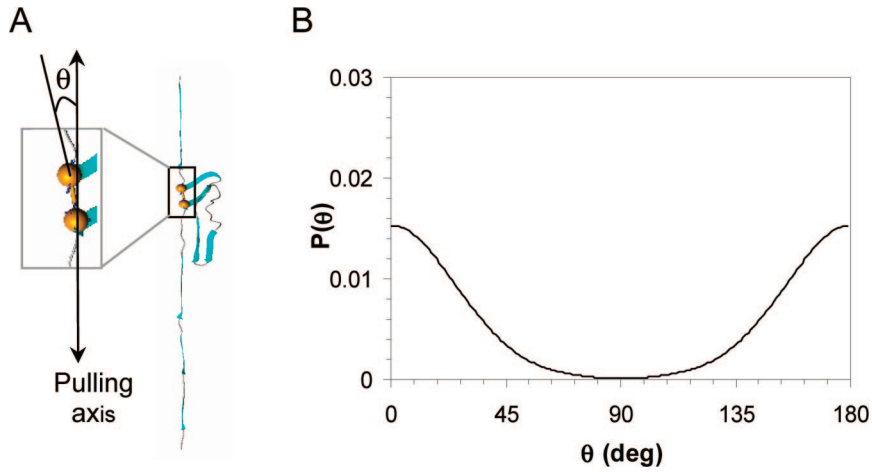

Figure 6. (A) Cartoon showing the unfolded protein with disulfide bond under mechanical force, in which the disulfide bond is oriented at an angle θ with respect to the pulling axis. (B) Probability density function, $P(\theta)$, of disulfide bond orientation, θ, calculated by using the freely jointed model.

(0.31 Å for thiols and 0.44 Å for phosphines). Therefore, although the qualitative trend in bond elongation is in agreement, we realize that a number of factors may need to be considered in order to gain full quantitative agreement between the experimental and computational results. For example, in the computationally intense quantum chemical calculations, we are forced to use a very simplified reaction to represent a highly complex system composed of a disulfide bond, extended polypeptide, and bulkier reducing agents. In addition, very little is known, from the theoretical standpoint, about how a mechanical force affects chemical processes in general. Thus, the effect of stretching force on the potential energy surface of the overall reduction reaction is not known, nor is the role of more sophisticated models[41] in describing the type of force-dependent chemical kinetics that we examine. Through the use of earlier molecular dynamics simulations,[21] we can infer that our experimental system consists of a disulfide bond in the stretched polypeptide that is not fully aligned with the pulling axis. In addition, this disulfide bond may further be subjected to the entropic elasticity of the protein and could dynamically sample many conformations during the reduction reaction.

We present a simple structural model which takes entropic elasticity of the polypeptide into account as a first step in incorporating some of these factors (Figure 6). Our experimental system consists of a disulfide bond that is formed between the side-chain thiols of cysteines and is exposed upon mechanical unfolding of protein. The polypeptide chains as well as disulfide bond are subjected to constant force (F) during the mechanochemical reduction of disulfide bond (Figure 6A). As a result of entropic elasticity, the polypeptide chains sample different conformations at any given force, also allowing the disulfide bond to sample different orientations.[21] We use a freely jointed chain (FJC) model[42] of polymer elasticity to estimate the distribution of disulfide bond orientations, θ, with respect to the pulling axis at a constant force of $F = 100$ pN. The probability density function, $P(\theta)$, defined by eq 1 gives the distribution of θ.

$$P(\theta) = \frac{e^{[Fb|\cos(\theta)|/k_BT]}}{\int_0^{180} e^{[Fb|\cos(\theta)|/k_BT]}\, d\theta} \qquad (1)$$

where $\theta = [0, 180]$, $b = 2.09$ Å is the quantum chemically calculated disulfide bond length, k_B is Boltzmann constant, and $T = 298$ K is the absolute temperature. We define $\theta = 90°$ as the angle perpendicular to the pulling direction (Figure 6A). The probability of disulfide bond orienting away from the pulling axis decreases from 0 to 90° as shown in Figure 6B.

We assume that the probability of nucleophile attack onto disulfide bond is equal in all orientations, θ, from the pulling axis (Figure 6A), and the disulfide bond lengthening at TS is independent of θ. However, the disulfide bond lengthening at TS projected onto the pulling axis, $\Delta x_{cal}(\theta)$, is dependent upon θ. In our experiments, we measure the average value of the distribution $\Delta x_{cal}(\theta)$ as the distance to TS (Δx). The theoretical disulfide bond lengthening at TS along the pulling axis, Δx_{cal}, can be calculated from the relationship given in eq 2.

$$\Delta x_{cal} = \langle \Delta x_{cal}(\theta) \rangle = \langle |\cos(\theta)| \rangle (b_{TS} - b) \qquad (2)$$

where b_{TS} is the quantum chemically calculated disulfide bond lengthening at TS (2.983 Å for phosphine and 2.499 Å for thiol, see Figure 5) and $\langle |\cos(\theta)| \rangle$ is given by eq 3.

$$\langle |\cos(\theta)| \rangle = \int_0^{180} |\cos(\theta)| P(\theta)\, d\theta \qquad (3)$$

From eq 2, we calculate Δx_{cal} to be 0.37 and 0.80 Å for thiol and phosphines, respectively. Upon using a simple polymer model to correct for the disulfide bond orientation, we find that the distance to TS values, Δx_{cal}, are smaller than the disulfide bond lengthening predicted by quantum chemical calculations (0.41 Å for thiol and 0.89 Å for phosphine). The Δx_{cal} for thiol is in agreement with the experimental value (0.31 ± 0.05 Å). However, for phosphines, the theoretical (0.80 Å) and experimental (0.44 ± 0.03 Å) values are closer to each other when compared to the uncorrected value from the quantum calculations (0.89 Å) but still far from being in good agreement. However, in this simple polymer model, we have ignored steric factors that will further restrict the geometry of the S_N2 reaction. This indicates that there might be additional corrections that need to be considered for the phosphine-initiated disulfide reduction to reach a quantitative agreement between calculations and the experiment. For example, geometrical/steric factors such as the bulky functional groups of the nucleophilic

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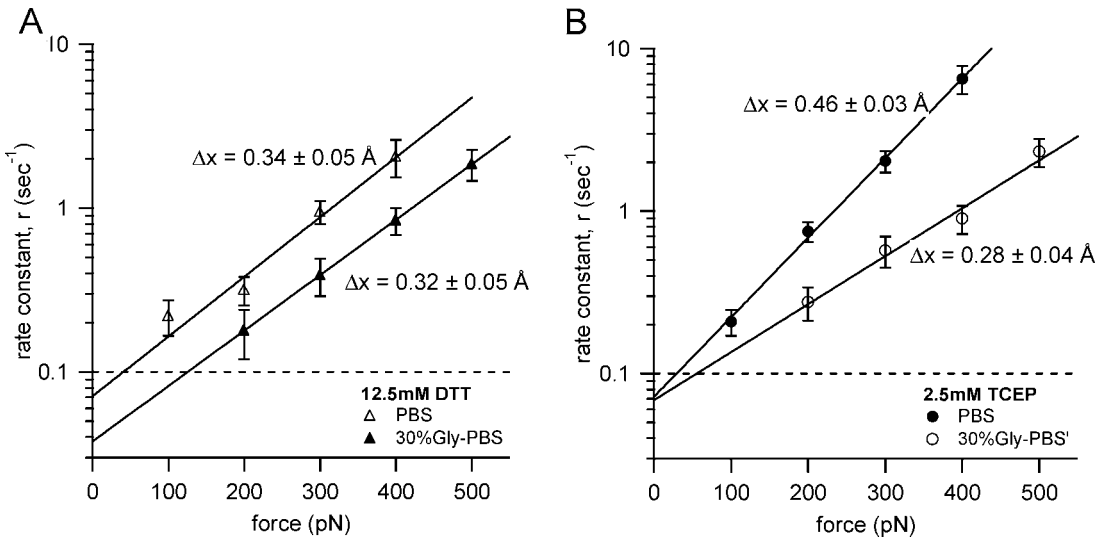

Figure 7. Comparison of force-dependent rate constants in aqueous and glycerol solutions. (A) The rate constant for the disulfide-bond reduction by DTT is approximately halved at each force when changing the solvent from aqueous to 30% v/v glycerol. Fitting with the Arrhenius model (thick line) gives the distance to the TS, Δx, which is largely unchanged, indicating that the TS structure remains unchanged. (B) The TS for the disulfide-bond reduction by TCEP is dependent on the solvent. When changing the solvent from aqueous to 30% v/v glycerol, the Δx for the disulfide reduction by TCEP decreased from 0.46 ± 0.03 to 0.28 ± 0.04 Å, indicating that the solvent induced changes in the TS structure. The dashed line in (A) and (B) marks the lower limit for the rate constant ($0.1\ s^{-1}$) that can be accurately measured in our force-clamp experiments.

center in phosphines (see Table 1 for structures) may limit the directions along which phosphine approaches onto the disulfide bond. Thus, phosphine may not access disulfide bonds oriented along the pulling axis (i.e., $\theta \approx 0$ and 180°). Therefore, when assuming that phosphine can access disulfide bonds oriented along $\theta = 15-165°$ instead of $\theta = 0-180°$, we calculate the theoretical Δx_{cal} for phosphine to be 0.42 Å, which is in agreement with the experimental value. Hence, considering all these factors, it is likely that the experimentally measured distance to the transition state, Δx, should always be less than or equal to that measured in quantum chemical calculations. Indeed, our experimental values of Δx are smaller than those obtained by quantum chemical calculations. Future studies, taking all the above factors into consideration, may lead to a more quantitative agreement between the experiment and calculations. Such advances will also provide new theoretical insight into how force affects chemical reactions in general. However, we note that our comparison here is an important first step in making the mechanochemical determination of reaction TS a general experimental tool.

Interestingly, our quantum chemical calculations on disulfide bond reduction by thiol and phosphine, which were done with explicit water molecules, indicate that solvent molecules are important in stabilizing the TS. Solvent molecules are organized in a network such that the concerted motion of atoms occurs during the reaction from initial nucleophilic attack to the final hydride transfer and leaving of sulfur from the disulfide bond (Figure 5). Previous theoretical studies also indicated the importance of solvent molecules in the identification of potential TS structures for disulfide bond reduction.[39,44] Fernandes and Ramos[39] studied the disulfide bond reduction of oxidized DTT by thiols and proposed that water molecules might be important in the disulfide bond reduction mechanism. In another study, Dmitrenko et al.[44] have also used quantum chemical calculations to investigate the phosphine-initiated reduction of a model disulfide bond, and it was mentioned that it was necessary to incorporate a network of water molecules in order to identify a potential TS structure. Taken together, these theoretical studies propose that solvent molecules are critical for the TS structure in disulfide bond reduction. Below, we demonstrate that the force-clamp technique can be used to experimentally investigate these theoretical predictions of the role of solvent molecules in the reaction TS.

We have performed disulfide bond reduction experiments by the thiol-based reducing agent DTT and phosphine-based reducing agent TCEP in a PBS solution containing 30% v/v glycerol. Force-dependent rate constants for disulfide bond reduction are obtained from averaged single-molecule recordings (Figure S5, Supporting Information), and the results are shown in Figure 7. For the disulfide bond reduction experiments in glycerol solution, the force-range is shifted to 200−500 pN because the reduction rate constants at 100 pN are too low (i.e., $<0.1\ s^{-1}$) to accurately measure in our experimental time window of 5 s. It is apparent that the disulfide bond reduction is sensitive to glycerol for both DTT and TCEP (Figure 7). However, the effect of glycerol on each reaction is markedly different. For DTT, the rate constant is approximately halved at each force (see at 200, 300, and 400 pN) in glycerol. On the other hand, for TCEP, addition of glycerol resulted in a decrease in the rate constant by 3-fold at 200 pN and by 8-fold at 400 pN. In both cases, though, the reduction is still accelerated upon application of a force, and the force-dependent rate constant data could be fitted with the Arrhenius model. We measure the distance to the TS for DTT, $\Delta x = 0.32 \pm 0.05$ Å, which is found to be similar to that in aqueous solution. However, in the case of TCEP, the Δx has decreased from 0.46 ± 0.03 Å in aqueous solution to 0.28 ± 0.04 Å in glycerol solution. These results indicate that the nature of the solvent significantly affects the TS structure for TCEP-initiated reduction, but its effect on DTT-initiated reduction is much less pronounced.

These experimental results raise an important question: How and why does a change in solvent, by incorporating glycerol into

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aqueous solution, affect the disulfide bond reduction by thiols and phosphines differently? In previous studies on the effect of solvent on S_N2 reactions, the changes in reaction rate were attributed to changes in the energetics of the reactants as well as the TS because of solvation.[5,6,45,46] The effect of changing the solvent from a PBS solution to a 30% v/v glycerol solution on the disulfide bond reduction by DTT and TCEP is very complex and markedly different. For example, the rate constant extrapolated by the Arrhenius model to zero force is halved for the DTT-initiated reaction whereas it is unchanged for TCEP-initiation reaction (Figure 7). This could be due to a change in the energetics of the reaction that might be solvent as well as nucleophile specific.[45,47,48] Further studies may explain the underlying mechanism. These overall changes in rate notwithstanding, in our measurements using force-dependent rate constants, we are most interested in measuring the distance to the TS, Δx, as a function of the solvent environment. Previous studies using a technique based on heavy atom KIE suggested a solvent rule to explain the effect of solvent medium on the S_N2 TS structure.[5,6]According to this solvent rule, there are two types of S_N2 TSs for which solvent effects are different. In the first type of TS, the charges on the two nucleophiles (i.e., attacking and leaving species) in the TS are the same. In this case, the solvent does not affect the S_N2 TS structure. In the second type of TS, oppositely charged nucleophiles are involved, and the solvent does affect the structure of the S_N2 TS. In the current S_N2 disulfide bond reduction by DTT, the charge on both the attacking nucleophile and leaving group is negative in the TS. In contrast, in TCEP-initiated disulfide bond reduction, the charge on the attacking nucleophile (phosphorus atom of the phosphine) is positive whereas the charge on the leaving group (sulfur atom of the disulfide bond) is negative in the TS. Therefore, according to the solvation rule for S_N2 reactions,[5,6] the TS for the disulfide bond reduction by DTT should be unaffected by a change in solvent whereas the TS for the disulfide bond reduction by TCEP should be affected by a change in solvent. Indeed, this is clearly the case in our experiments where we measure an unchanging Δx for DTT and a changing Δx for reduction by TCEP. Altogether, the experiments in glycerol solution strongly suggest that solvent molecules play an active role in the TS of disulfide bond reduction, demonstrating that force-clamp AFM can probe solvent effects on TS geometry. Although detailed structural properties of glycerol/water mixtures are not known, physical properties of these binary mixtures indicate the difference in their hydrogen-bonding properties different from those of water,[49] and this might be an important contributing factor in changing the TS geometry for the disulfide bond reduction. It is also possible that replacing water molecules with relatively large glycerol molecules might affect the arrangement and possibly the number of solvent molecules around the attacking and leaving groups in the TS (Figure 5). Future studies involving a systematic change in solvent composition of glycerol/water mixtures or binary mixtures of solvents with varying hydrogen-bonding properties will reveal further insight into the solvent effect on the TS.

Conclusions

The current study demonstrates that the experimentally measured distance to the TS, Δx, during mechanochemical disulfide bond reduction is related to bond elongation at the TS predicted by quantum chemical calculations. In force-clamp experiments, the mechanical force imposes a 1-D reaction coordinate (x) for the reaction, and the distance to the reduction TS measured by Δx directly reports on the progression along this reaction coordinate. Indeed, we are able to capture, with sub-ångström resolution, the changes in the TS geometry for the disulfide bond reduced by different chemical agents. The experimentally measured Δx values conclude that the disulfide bond at the TS lengthens significantly more in the presence of a phosphine-based reducing agent than in the presence of a thiol-based agent, which is in good agreement with quantum chemical calculations on the TS structure. Our quantum chemical calculations further support the proposal that by experimentally measuring Δx for a given chemical reaction, one can gain direct insight into the sub-ångström-scale changes in TS geometry. These insights will be further refined with new theoretical advances in understanding our experimental system. Also, direct participation of solvent molecules in the TS as proposed by theoretical calculations is experimentally determined. In the case of TCEP, the TS geometry is shown to be affected by changing the solvent with the incorporation of glycerol into the aqueous solution. Direct participation of solvent molecules in the TS has been reported before for chemical reactions[50,51] as well as more complex processes such as unfolding of proteins,[52] illustrating the importance of the solvent in reaction kinetics. In summary, single-molecule experiments with engineered polyproteins have made it possible to measure the TS properties of a bimolecular chemical reaction at the single-bond level. In the force-clamp experiment, a constant stretching force is applied to the chemical bond throughout the entire time evolution of the reaction, including the passage of the TS. We anticipate that mechanical activation of chemical reactions[53,54] by force-clamp AFM will become an important tool in the chemist's arsenal to probe structural information of short-lived TSs in solution-phase reaction.

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Supporting Information Available: Force-dependent kinetic data, [TCEP]-dependent rate constant data, details of TS structures from quantum chemical calculations, kinetic data of disulfide bond reduction in glycerol solution. This material is available free of charge via the Internet at http://pubs.acs.org.

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