Among the challenges in this postgenomic era are reliable predictions of protein conformation and conformational changes. Because the crystallographic database is relatively small and intrinsically limited to statically equilibrated conformations of proteins, experimental data sets are clearly needed for key dynamic states and their energetics. To begin filling this knowledge gap, several new methods have emerged over the last decade, especially at the single-molecule level, where distinct dynamical states are not blurred by bulk averaging. In the present context, Nobel Laureate Steven Chu has pointed out the flaw of such blind averaging by saying that the average human is a mixed sex type, which obviously describes no one individual’s anatomical features.

With an atomic force microscope (AFM), one can grab and manipulate single proteins the way one might grip and distort almost any object to feel how it is made and to assess, with proteins at least, what conformations are allowed (1). It should be a given by now that force is central to the biological function of many key cytoskeletal, adhesion, motor, and matrix proteins. Even enzymes and transcriptional machines exert forces on their substrates (2, 3). Moreover, some of the biggest genes known translate to essential elastic proteins, such as dystrophin and titin, which highlight the genetic importance of such proteins. These proteins are incessantly exposed to physical forces in the body, whereas far less likely in vivo are the changes in temperature and solvent that are common in the many solution studies of protein folding. Various AFM methods of extending, compressing, and unwinding proteins are providing further opportunities to study biologically relevant, force-dependent conformational pathways together with other key aspects of the physical chemistry of proteins (4). In a recent issue of PNAS, Wiita et al. (5) exploit the exquisite distance and force sensitivity of constant force mode AFM to measure the force-dependent rate of reduction of a disulfide bond, a key motif in many proteins. The question that is addressed is rightly suggested to be more general.

In classic chemical kinetics, rates of reaction for covalent bonds are generally sensitive to pressure, and some "highly reactive" processes such as detonation are characterized by large "highly reactive" processes such as detonation are characterized by large

\[
\frac{\partial \ln k}{\partial P} = -\Delta V^*/k_B T + fcn(T),
\]

where \(\Delta V^*\) is the volume difference of the transition state or barrier species relative to the reactant(s). The reaction rate \(k\) increases with compression \((P > 0)\) only if the transition volume \(\Delta V^*\) decreases. In solution, \(\Delta V^*\) includes changes in solvent volume so that in water, as would be relevant to most studies of biomolecules, such transition state parameters lump together changes in hydration volumes. Viscosity might change with compression and also can artificially influence the reaction rate when diffusion dominates thermal activation. Expansion stresses \(\sigma\) can be applied in solution \((P = -\frac{1}{3} \sigma)\) and would tend to pull reactants apart faster if \(\Delta V^* > 0\). This inequality has been equated in the past to bond stretching by accounting for van der Walls volume, and typical experimental values suggest bond stretching of \(\sim 1\) Å. The pressure changes in pressure up to \(\sim 100,000\text{ atm (1 atm }=101.3\text{ kPa)}\) (6). In the gas phase, such a process follows standard Arrhenius kinetics with a rate constant \(k\) that decreases exponentially in the activation barrier’s free energy, \(\Delta G^*\), but the latter is a function of pressure \(P\). Differentiation with respect to \(P\) thus gives

\[
(\frac{\partial \ln k}{\partial P})_T = -\frac{\Delta V^*}{k_B T} + fcn(T),
\]

Conflict of interest statement: No conflicts declared.

See companion article on page 7222 in issue 19 of volume 103.

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or stress dependence of the reaction rate thus provides structural information on the transition state. These classical concepts of covalent kinetics now can be assessed one molecule at a time and in a more directed fashion by AFM. AFM allows one to focus forces on single molecules and to orient the stress \( \sigma \) along the pulling direction \( x \) (Fig. 1). Covalent bonds, noncovalent interactions, and thermal fluctuations of the hydrated protein all resist the pulling and collectively balance the thermally averaged force \( f \) imposed by deflection of the AFM cantilever tip. In other words, a \( y-z \) cross section through the molecule and the adjacent solvent shell will generally reveal a stress and even torsion distribution of varied origin. Such complexity is likely simplified in the presence of a reducing agent such as DTT under extension. The force \( f \) is the independent, controlled variable and needs to be such for the equivalent form of Eq. 1 to apply most directly. In either method, multidomain proteins with sequestered disulfides are extended to expose the S–S to a solution of reducing agent (Fig. 1). Past work on IgCAMs had confirmed with solution studies that the S–S was truly sequestered and would not reduce without unfolding. The forcibly distended protein rotates as it aligns its disulfide with \( f \) and exposes its hydrophobic core. Despite this complexity and the convoluting proximity of adjacent side chains, the disulfide bond seems likely to sustain much of the applied stress before it is broken by at least one of DTT’s two free thiol. This reduction by DTT under extension shows clear fingerprints in AFM, such as overextension of the reduced state. Moreover, the log-dependence on force also fits the one-dimensional theory of bond extension, giving \( \Delta x^* = 0.34 \) Å for the engineered titin domain (5). Although Wiita et al. (5) point out that it is not yet known how often any disulfide bond in vivo will be exposed to the force levels that they explored, it does seem likely that the sensitivity of any particular thiol/disulfide exchange reaction to a pulling force will depend very specifically on the environment surrounding the bond. Temperature already has been proven to strongly influence protein unfolding and refolding in AFM (11–13). This temperature effect is not because of the \( T \) dependence in Eq. 1 but largely because temperature influences the hydration environment around proteins.

Other proteins have been speculated to exhibit behavior in vivo similar to that documented in single-molecule AFM studies. Perhaps the most intriguing example is provided by the membrane protein CD4, which is an attachment factor in HIV-1 entry (14). Cysteines/disulfides in CD4’s Ig domains appear to be dynamic thiol structures, and thiol exchange could be enabled by thioredoxin, exposing free cysteines in a disulfide unfolded subpopulation. Thioreactive CD4 monomers on cells indeed have been captured by thiol-specific reagents. Previous AFM studies of Ig-CAMs have suggested that the highly conserved inter-\( \beta \)-sheet disulfides diminish the need for strong noncovalent interactions in folding, especially because lower forces are required to unfold Ig-CAMs versus titin Ig domains that lack the disulfide (1, 7, 8). Therefore, it has been predicted that a mechanical unraveling of CD4 domains could add extensional flexibility and provide a “shock absorber” structure to permit bonded CD4 ligation to the virus without membrane rupture. This unraveling might enable HIV’s gp120 to remain tethered to CD4 while permitting conformational rearrangements for coreceptor binding and membrane fusion of virus with the cell. Indeed, thiol exchange interactions involving both gp120 and CXCR4 as mediated by protein disulfide isomerase (PDI) have been implicated in prefusion entry of HIV. Pharmacological implications could be important in this case, and a broad understanding might translate to other adhesive interactions or even cytoskeletal dynamics under redox stress (6). Regardless, speculations such as these regarding HIV entry dynamics certainly motivate deeper studies of protein conformations and chemistry under force.

We thank H. Lu and S. Feng for simulation snapshots from ref. 9. Support from the National Science Foundation and the National Institutes of Health is gratefully acknowledged.