

Force-dependent chemical kinetics of disulfide bond reduction observed with single-molecule techniques

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The mechanism by which mechanical force regulates the kinetics of a chemical reaction is unknown. Here, we use single-molecule force-clamp spectroscopy and protein engineering to study the effect of force on the kinetics of thiol/disulfide exchange. Reduction of disulfide bonds through the thiol/disulfide exchange chemical reaction is crucial in regulating protein function and is known to occur in mechanically stressed proteins. We apply a constant stretching force to single engineered disulfide bonds and measure their rate of reduction by DTT. Although the reduction rate is linearly dependent on the concentration of DTT, it is exponentially dependent on the applied force, increasing 10-fold over a 300-pN range. This result predicts that the disulfide bond lengthens by 0.34 Å at the transition state of the thiol/disulfide exchange reaction. Our work at the single bond level directly demonstrates that thiol/disulfide exchange in proteins is a force-dependent chemical reaction. Our findings suggest that mechanical force plays a role in disulfide reduction *in vivo*, a property that has never been explored by traditional biochemistry. Furthermore, our work also indicates that the kinetics of any chemical reaction that results in bond lengthening will be force-dependent.

atomic force microscopy | mechanochemistry

The intersection of force and chemistry has been studied for over a century, yet not much is known about this phenomenon compared with more common methods of chemical catalysis (1). There are a number of reasons for this discrepancy, but one of the most important factors remains that it is quite difficult to directly measure the effect of force on a bulk reaction. This difficulty arises because an applied force is not a scalar property of a system; it is associated with a vector. As a result, it is often not possible to directly probe the effect of force on a particular reaction because of heterogeneous application of force and a distribution of reaction orientations (1). To fully quantify the effect of an applied force on a chemical reaction, it is necessary to generate an experimental system where the reaction of interest is consistently oriented with respect to the applied force. Thus, recent advances in single-molecule techniques are particularly well suited to address this problem. The direct manipulation of single molecules allows for the application of force in a vector aligned with the reaction coordinate (2), avoiding the heterogeneity of bulk studies. Earlier works using single-molecule techniques have described the rupture forces necessary to cleave single covalent bonds (reviewed in ref. 1), including Si–C bonds in polysaccharide attachment (3), Au–Au bonds in nanowires (4, 5), and Ni²⁺–NTA attachments (6). However, these studies have not been able to describe the effect of force on the kinetics of these reactions, nor have they examined more complex chemical reactions beyond simple bond rupture.

To test the hypothesis that mechanical force can directly influence the kinetics of a chemical reaction, we studied thiol/disulfide exchange, the reduction of disulfide bonds in a protein (7). The disulfide bond itself is a covalent bond formed between the thiol groups of two vicinal cysteine residues. In the first step of thiol/disulfide exchange, a new disulfide bond is formed between a thiolate anion of the reducing molecule (in this case DTT) and one cysteine on a protein, whereas the sulfur of the

other cysteine reverts to the free thiolate state. This reaction has been extensively studied and is known to be important in the function and folding processes of proteins (8–10). This reaction is also of particular interest because it is known that many proteins that are exposed to mechanical stress *in vivo* contain disulfide bonds (11–13). Thus, the effect of force on this reaction could be of significance in biological systems.

Disulfide bonds have been studied in previous atomic force microscopy (AFM) experiments where a protein molecule is stretched at a constant velocity whereas the applied force varies (force-extension AFM). Most of these experiments could identify the presence or absence of a disulfide bond (14–17) but could not determine when the disulfide reduction reaction occurred. In recent work from our laboratory, we used engineered disulfide bonds to precisely correlate disulfide reduction events with increases in protein contour length, developing a molecular fingerprint for identifying individual chemical reactions (S.R.K.A. and J.M.F., unpublished data). In the present work, we use this fingerprint to investigate the kinetics of thiol/disulfide exchange as a function of pulling force using force-clamp AFM (18, 19). This method provides the only direct means by which to observe the exponential chemical kinetics of thiol/disulfide exchange under a calibrated pulling force. This technique has been used to study the unfolding kinetics (19) as well as refolding (20) of single protein molecules as a function of force, offering insight into the link between protein dynamics and force. By using force-clamp AFM, we directly demonstrate that thiol/disulfide exchange, a bimolecular chemical reaction (21), is catalyzed by mechanical force. The force-dependency of the reaction rate is determined by the structure of the transition state, a result that may be generalized to other chemical reactions. These findings demonstrate that force-clamp AFM is a powerful tool with which to study chemistry at the single-molecule level.

Results and Discussion

In our studies, we use the 27th immunoglobulin-like domain of cardiac titin (I27), an 89-residue, β -sandwich protein with well characterized mechanical properties (22, 23). Through cysteine mutagenesis, we engineered a disulfide bond in the I27 domain between the 32nd and 75th residues, which are closely positioned in space as determined by the NMR structure of wild-type I27 (Protein Data Bank ID code 1TIT). We constructed and expressed an eight-repeat polyprotein (22) of this modified domain, (I27_{G32C–A75C})₈, and used single-molecule force-clamp spectroscopy (19) to manipulate and stretch single polyproteins. Under force-clamp conditions, stretching a polyprotein results in a well defined series of step increases in length, marking the

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Abbreviations: AFM, atomic force microscopy; I27, 27th immunoglobulin-like domain of cardiac titin.

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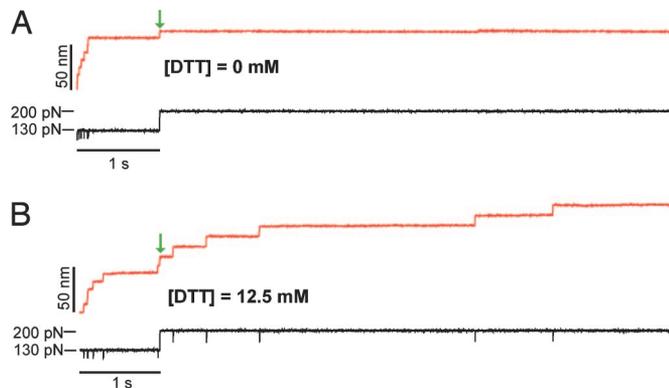


Fig. 2. A double-pulse protocol separates the unsequestered unfolding from disulfide reduction events. (A) Typical double-pulse force-clamp experiment pulling the $(I27_{G32C-A75C})_8$ protein first at 130 pN for 1 s and then stepping to a force of 200 pN for 7 s (black trace). In this experiment, the first pulse to 130 pN causes a series of seven unsequestered unfolding events (10.6-nm steps). Upon increasing the force to 200 pN (green arrow), we observed an elastic step elongation of the protein. In the absence of DTT, no further steps are observed. (B) Repeating the same experiment in the presence of 12.5 mM DTT, we again observed six unsequestered unfolding events at 130 pN. Upon stepping to 200 pN and after the elastic elongation of the protein (green arrow), we then observed a series of five steps of ≈ 14.2 nm corresponding to the disulfide reduction events. Notice the rapid exponential time course followed by the unsequestered unfolding events at 130 pN and the much slower reduction events observed during the second pulse at 200 pN.

four recordings. The green trace in Fig. 3B was obtained following similar procedures with the second force pulse to 300 pN. By comparison, the blue trace in Fig. 3B corresponds to the averaging of four traces obtained with the second force pulse set to 200 pN and in the absence of DTT. It is apparent from Fig. 3B that protein unfolding during the first pulse to 130 pN is

independent of DTT, following a similar exponential time-course in all cases at this force. However, thiol/disulfide exchange during the second pulse appears both DTT- and force-dependent. The double-pulse protocol as shown here effectively separates protein unfolding from the disulfide-bond reduction events. Hence, in the subsequent analysis we ignore the unsequestered unfolding observed during the first pulse and only analyze thiol/disulfide exchange events in the second pulse.

We conducted multiple double-pulse experiments, all with an identical first force pulse to 130 pN lasting 1 s. Fig. 4A shows multiple (>25) trace averages of only the second pulse at four different forces (100, 200, 300, and 400 pN) and at a constant DTT concentration of 12.5 mM. Only traces that included a clear unsequestered unfolding fingerprint in the first pulse and that contained only disulfide reduction events in the second pulse were included in this analysis. By fitting a single exponential to the traces shown in Fig. 4A, we define the observed rate of thiol/disulfide exchange as $r = 1/\tau_r$, where τ_r is the time constant measured from the exponential fits. In Fig. 4B, we show that r is exponentially dependent on the applied force (12.5 mM DTT) ranging from 0.211 s^{-1} (100 pN) up to 2.20 s^{-1} (400 pN). Fig. 4C shows the second-pulse averages (>20) of experiments conducted at 200 pN, at different concentrations of DTT (0, 1.25, 12.5, 31, 50, 83, and 125 mM). In Fig. 4D, we show that r has a first-order dependence on the concentration of DTT, demonstrating that the thiol/disulfide exchange reaction in our system is bimolecular.

Given these observations, we derive an empirical relationship $r = k(F)[\text{DTT}]$, where $k(F)$ depends exponentially on the applied force and is given by a Bell-like (25) relationship: $k(F) = A \exp((F\Delta x_r - E_a)/k_B T)$. In this equation, A is a constant with units of $\text{M}^{-1}\text{s}^{-1}$, Δx_r is the distance to the transition state for the reaction, and E_a is the activation energy barrier for the thiol/disulfide exchange at zero force. Fitting this equation to the data presented in Fig. 4B, we obtain a value of $\Delta x_r = 0.34 \text{ \AA}$ and a

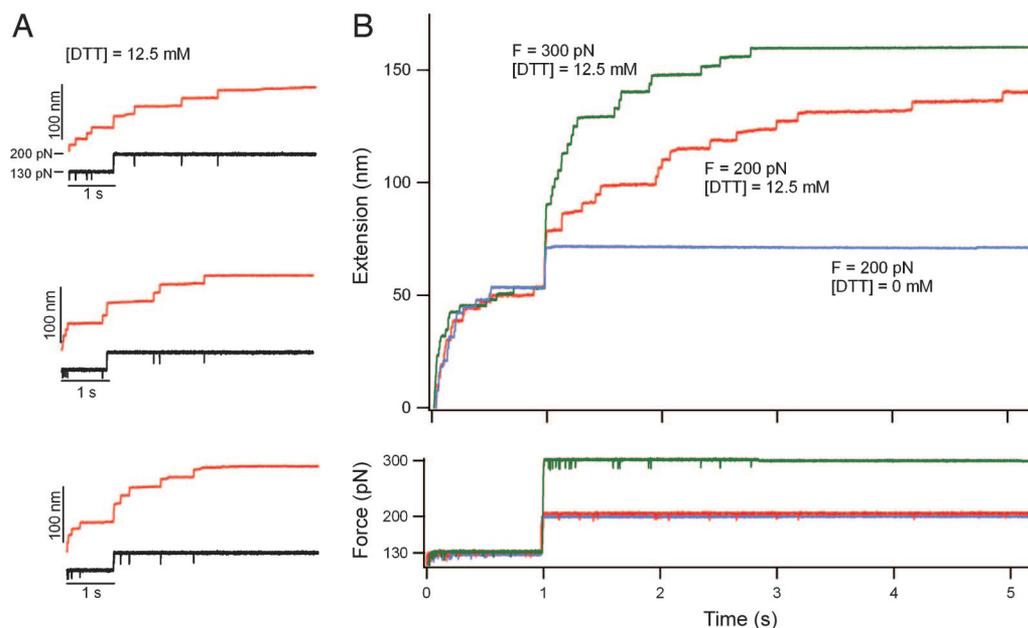


Fig. 3. Ensemble measurements of the kinetics of thiol/disulfide exchange. (A) Three recordings are shown of single $(I27_{G32C-A75C})_8$ polyproteins that were extended with the same double-pulse protocol shown in Fig. 2B: 12.5 mM DTT, $F = 130$ pN for 1 s, and then $F = 200$ pN. The stochastic nature of both the unsequestered unfolding events as well as of the thiol/disulfide exchange events becomes apparent when comparing these recordings. (B Upper) A four-trace average (red trace) of the double-pulse experiments shown in A and Fig. 2B demonstrates the methods used to build up an ensemble of recordings under a set of conditions. Similar four-trace averages are shown for data obtained under two other conditions: 12.5 mM DTT, $F = 130$ pN for 1 s then $F = 300$ pN (green trace); and 0 mM DTT, $F = 130$ pN for 1 s then $F = 200$ pN (blue trace). Notice that the time course of unsequestered unfolding during the first pulse is similar under all conditions. (B Lower) The averaged force traces are shown.

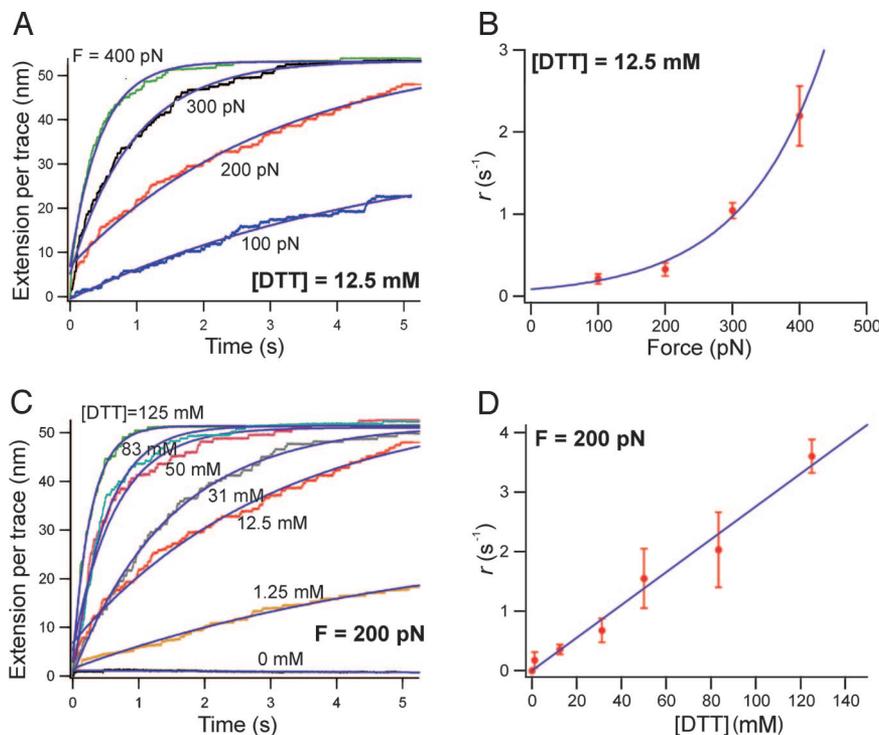


Fig. 4. The thiol/disulfide exchange chemical reaction is force and [DTT] dependent. Multiple-trace averages ($n > 20$ in each trace) of thiol/disulfide exchange events measured by using the double-pulse protocol as a function of force and DTT concentration are shown. Only the second pulse averages are shown; time = 0 s denotes the start of the second pulse. (A) A set of trace averages measured at a constant concentration of DTT (12.5 mM), while varying the force of the second pulse between 100 and 400 pN. Single-exponential fits (continuous lines) measure the time constant, τ_r , of thiol/disulfide exchange. (B) Plot of the rate of thiol/disulfide exchange, $r = 1/\tau_r$, as a function of the pulling force at [DTT] = 12.5 mM. The solid line is an exponential fit to the data. (C) Trace averages measured at a constant pulling force ($F = 200$ pN) and at various DTT concentrations. (D) Plot of r as a function of [DTT]. The solid line is a linear fit to the data. The linear (first-order) dependence on [DTT] demonstrates that the thiol/disulfide exchange reaction in our system is bimolecular. This reaction can be described empirically from B and D by the simple rate equation $r = k(F)[\text{DTT}]$, where $k(F)$ is exponentially dependent on the pulling force and $k(200 \text{ pN}) = 27.6 \text{ M}^{-1}\text{s}^{-1}$ from the linear fit in D.

value of $k(0) = 6.54 \text{ M}^{-1}\text{s}^{-1}$, which is similar to the second-order rate constant for DTT reduction of disulfide bonds in insulin at neutral pH [$k = 5 \text{ M}^{-1}\text{s}^{-1}$ (26)]. Other studies have found that under certain solution conditions, thiol/disulfide exchange can occur with a second-order rate constant as high as $132,000 \text{ M}^{-1}\text{s}^{-1}$ (27) but also can be up to 6 orders of magnitude slower, in accord with our observations. From the linear fit in Fig. 4D, we see that an applied force alters the second-order rate constant in our system; $k(200 \text{ pN}) = 27.6 \text{ M}^{-1}\text{s}^{-1}$, a 4-fold increase from zero force. Assuming that A ranges from 10^5 to $10^{12} \text{ M}^{-1}\text{s}^{-1}$, we estimate E_a to be in the range of 30–65 kJ/mol. The upper values in this range overlap with the calculated energy barriers for a number of thiol/disulfide exchange reactions in solution [60–66 kJ/mol (28)], and each 100 pN of force lowers the energy barrier by ≈ 2 kJ/mol.

Although our empirical Bell-like model (2, 25) is a useful first approximation to examine our data, it may not hold over all combinations of DTT and force. It also cannot completely describe the effect of a force on the thiol/disulfide exchange reaction. The force constant for an S–S bond, found by vibrational spectrum in the gas phase, is 4.96 N/cm (29). As a result, an applied force of 400 pN will stretch this bond by only 0.008 Å, which is a negligible effect on the geometry of the S–S bond. However, as pointed out by Beyer (30), the reactivity of a stretched molecule is likely to depend on the pulling force despite only minor changes in bond geometry. Furthermore, a reorganization of the energy landscape of the bond is likely to occur during bond lengthening (31). These effects are not accounted for by our model. Hence, further theoretical developments on the effect of a mechanical force on the thiol/

disulfide exchange reaction will be required to fully understand our experiments. These limitations notwithstanding, we can still extract useful parameters from our analysis. For example, the sensitivity of the rate of reduction to a pulling force is well represented by the measured value of Δx_r , which can be contrasted to that of unfolding the unsequestered region of the protein. By fitting a single exponential to an average of traces containing solely unsequestered unfolding events of the type shown in Fig. 1B (no DTT), we measured the rate of unfolding, $\alpha_u = 1/\tau_{u_i}$, at different pulling forces (see Fig. 7, which is published as supporting information on the PNAS web site). Fig. 5A shows a semilogarithmic plot of both α_u and r as a function of the pulling force. The dashed line corresponds to a fit of $\alpha_u(F) = \alpha_u(0) \exp(F\Delta x_u/k_B T)$ (19), obtaining $\Delta x_u = 1.75 \text{ Å}$ for the unsequestered unfolding. The solid line is a fit of the Bell-like model described earlier, where $\Delta x_r = 0.34 \text{ Å}$ for the thiol/disulfide exchange reaction. Fig. 5A confirms the difference in force sensitivity between the unsequestered unfolding and the thiol/disulfide exchange reaction, which are two distinct processes occurring within the same protein.

From our measurements above we obtained a preliminary description of the energy landscape for the thiol/disulfide exchange chemical reaction under a stretching force (Fig. 5B). Recent theoretical calculations have proposed that the length of an S–S bond at the transition state of a simple S_N2 thiol/disulfide exchange reaction in solution increases by 0.36 Å (32) or 0.37 Å (28). These values suggest that the value of Δx_r that we have measured experimentally corresponds to the lengthening of the S–S bond during an S_N2 reaction with a DTT molecule (Fig. 5C). Furthermore, in these theoretical studies, varying the reaction

pulse times are to reduce thiol/disulfide events during the first pulse), then the force increased to 200 pN for 5–7 s. The interaction between protein and cantilever/coverslide is non-specific. Thus, in most cases fewer than eight domains were unfolded when a molecule was stretched.

Data Analysis. The fingerprint of a single $(I27_{G32C-A75C})_8$ was considered to be two well resolved steps of ≈ 10.5 nm during the first pulse. No traces that included unsequestered unfolding events during the second pulse were included in the analysis. Such mixed spectra were very rarely observed ($<1\%$) at forces of 200 pN or greater because of the very rapid kinetics of unsequestered unfolding at these forces. At a second pulse force of 100 pN, such mixed spectra were observed $\approx 15\%$ of the time; such traces were not included in the averaging analysis because the unsequestered unfolding steps would corrupt the time course of disulfide reduction. We assume that disulfide reduction in our protein is Markovian (i.e., each reduction event is independent of all others); thus, averaging

traces with different numbers of reduction steps will result in invariant exponential kinetics. Error bars in Figs. 4 B and D and 5A were obtained by partitioning the entire set of traces into random subsets (for example, if 20 total single-molecule traces were used, then two subsets of 10 traces each were constructed). These traces in these subsets then were averaged and fit with a single exponential. The rate of thiol/disulfide exchange (Fig. 4 A and B) or rate of unsequestered unfolding (Fig. 5A) was determined as described from the exponential fit. The standard deviation of the rate was then calculated with $n =$ the number of subsets (in the case of the example, $n = 2$). This value then was used as the magnitude of the error bar shown in the figures.

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1. Beyer, M. K. & Clausen-Schaumann, H. (2005) *Chem. Rev.* **105**, 2921–2948.
2. Evans, E. & Ritchie, K. (1997) *Biophys. J.* **72**, 1541–1555.
3. Grandbois, M., Beyer, M., Rief, M., Clausen-Schaumann, H. & Gaub, H. E. (1999) *Science* **283**, 1727–1730.
4. Marszalek, P. E., Greenleaf, W. J., Li, H., Oberhauser, A. F. & Fernandez, J. M. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 6282–6286.
5. Rubio-Bollinger, G., Bahn, S. R., Agrait, N., Jacobsen, K. W. & Vieira, S. (2001) *Phys. Rev. Lett.* **87**, 026101.
6. Conti, M., Falini, G. & Samori, B. (2000) *Angew. Chem. Int. Ed.* **39**, 215–218.
7. Sevier, C. S. & Kaiser, C. A. (2002) *Nat. Rev. Mol. Cell. Biol.* **3**, 836–847.
8. Kadokura, H., Katzen, F. & Beckwith, J. (2003) *Annu. Rev. Biochem.* **72**, 111–135.
9. Hogg, P. J. (2003) *Trends Biochem. Sci.* **28**, 210–214.
10. Barford, D. (2004) *Curr. Opin. Struct. Biol.* **14**, 679–686.
11. Yan, B. & Smith, J. W. (2001) *Biochemistry* **40**, 8861–8867.
12. Chen, S. & Springer, T. A. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 950–955.
13. Mayans, O., Wuerges, J., Canela, S., Gautel, M. & Wilmanns, M. (2001) *Structure (London)* **9**, 331–340.
14. Bustanji, Y. & Samori, B. (2002) *Angew. Chem. Int. Ed.* **41**, 1546–1548.
15. Carl, P., Kwok, C. H., Manderson, G., Speicher, D. W. & Discher, D. E. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 1565–1570.
16. Bhasin, N., Carl, P., Harper, S., Feng, G., Lu, H., Speicher, D. W. & Discher, D. E. (2004) *J. Biol. Chem.* **279**, 45865–45874.
17. Li, H. & Fernandez, J. M. (2003) *J. Mol. Biol.* **334**, 75–86.
18. Oberhauser, A. F., Hansma, P. K., Carrion-Vazquez, M. & Fernandez, J. M. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 468–472.
19. Schlierf, M., Li, H. & Fernandez, J. M. (2004) *Proc. Natl. Acad. Sci. USA* **101**, 7299–7304.
20. Fernandez, J. M. & Li, H. (2004) *Science* **303**, 1674–1678.
21. Kuwajima, K., Ikeguchi, M., Sugawara, T., Hiraoka, Y. & Sugai, S. (1990) *Biochemistry* **29**, 8240–8249.
22. Carrion-Vazquez, M., Oberhauser, A. F., Fowler, S. B., Marszalek, P. E., Broedel, S. E., Clarke, J. & Fernandez, J. M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 3694–3699.
23. Marszalek, P. E., Lu, H., Li, H., Carrion-Vazquez, M., Oberhauser, A. F., Schulten, K. & Fernandez, J. M. (1999) *Nature* **402**, 100–103.
24. Beyer, M. K. (2000) *J. Chem. Phys.* **112**, 7307–7312.
25. Bell, G. I. (1978) *Science* **200**, 618–627.
26. Holmgren, A. (1979) *J. Biol. Chem.* **254**, 9627–9632.
27. Snyder, G. H., Cennerazzo, M. J., Karalis, A. J. & Field, D. (1981) *Biochemistry* **20**, 6509–6519.
28. Fernandes, P. A. & Ramos, M. J. (2004) *Chem. Eur. J.* **10**, 257–266.
29. Lide, D. R., ed. (1995) *CRC Handbook of Chemistry and Physics* (CRC, Cleveland).
30. Beyer, M. K. (2003) *Angew. Chem. Int. Ed.* **42**, 4913–4915.
31. Marcus, R. A. & Sutin, N. (1985) *Biochim. Biophys. Acta* **811**, 265–322.
32. Csaszar, P., Csizmadia, I. G., Viviani, W., Loos, M., Rivail, J. L. & Perczel, A. (1998) *J. Mol. Struct. Theochem.* **455**, 107–122.
33. Boggon, T. J., Murray, J., Chappuis-Flament, S., Wong, E., Gumbiner, B. M. & Shapiro, L. (2002) *Science* **296**, 1308–1313.
34. Graves, B. J., Crowther, R. L., Chandran, C., Rumberger, J. M., Li, S., Huang, K. S., Presky, D. H., Familletti, P. C., Wolitzky, B. A. & Burns, D. K. (1994) *Nature* **367**, 532–538.
35. Wierzbicka-Patynowski, I. & Schwarzbauer, J. E. (2003) *J. Cell Sci.* **116**, 3269–3276.
36. Schrijver, I., Liu, W., Brenn, T., Furthmayr, H. & Francke, U. (1999) *Am. J. Hum. Genet.* **65**, 1007–1020.
37. Xie, L., Chesterman, C. N. & Hogg, P. J. (2001) *J. Exp. Med.* **193**, 1341–1349.
38. Ryser, H. J. & Fluckiger, R. (2005) *Drug Discov. Today* **10**, 1085–1094.
39. Matthias, L. J., Yam, P. T., Jiang, X. M., Vandegraaff, N., Li, P., Pombourios, P., Donoghue, N. & Hogg, P. J. (2002) *Nat. Immunol.* **3**, 727–732.
40. Maier, B., Koomey, M. & Sheetz, M. P. (2004) *Proc. Natl. Acad. Sci. USA* **101**, 10961–10966.
41. Aktah, D. & Frank, I. (2002) *J. Am. Chem. Soc.* **124**, 3402–3406.
42. Houk, J., Singh, R. & Whitesides, G. M. (1987) *Methods Enzymol.* **143**, 129–140.
43. Hansen, R. E., Ostergaard, H. & Winther, J. R. (2005) *Biochemistry* **44**, 5899–5906.
44. Wouters, M. A., Lau, K. K. & Hogg, P. J. (2004) *BioEssays* **26**, 73–79.
45. Singh, R. & Whitesides, G. M. (1990) *J. Am. Chem. Soc.* **112**, 6304–6309.