

# Probing protein dynamics using temperature jump relaxation spectroscopy

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There have been recent advances in initiating and perturbing chemical reactions on very fast timescales, as short as picoseconds, thus making it feasible to study a vast range of chemical kinetics problems that heretofore could not be studied. One such approach is the rapid heating of water solutions using laser excitation. Laser-induced temperature jump relaxation spectroscopy can be used to determine the dynamics of protein motion, an area largely unstudied for want of suitable experimental and theoretical probes, despite the obvious importance of dynamics to protein function. Coupled with suitable spectroscopic probes of structure, relaxation spectroscopy can follow the motion of protein atoms over an enormous time range, from picoseconds to minutes (or longer), and with substantial structural specificity.

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## Abbreviations

<b>G3P</b>	DL-glycerol 3-phosphate
<b>LDH</b>	lactate dehydrogenase
<b>NAD<sup>+</sup></b>	oxidized β-nicotinamide adenine dinucleotide
<b>NADH</b>	reduced β-nicotinamide adenine dinucleotide
<b>TIM</b>	triose-phosphate isomerase
<b>T-jump</b>	temperature jump

## Introduction

A useful method of determining atomic and molecular motion on all relevant timescales is relaxation spectroscopy. In this approach, an equilibrium of interconverting chemical species is suddenly perturbed, forcing the system to establish a new equilibrium point. As atomic motion relevant to function can take place on timescales from subpicoseconds to minutes, approaches that initiate chemistry on very fast timescales are essential. Some recent work has focused on creating laser-induced temperature jumps (T-jumps) in water, as fast T-jumps are possible using pulsed laser excitation of the near-IR solvent absorbancies. Vibrational relaxation takes place on the picosecond timescale both in water and in proteins; therefore complete thermalization of solvent and solute occurs in 10–20 ps [1,2]. The motion of the system as it evolves in response to the T-jump can be monitored provided suitable probes of structure are available that can follow the kinetic changes. This approach and its application to proteins are the subject of this review.

In a sense, this review is both late and early. The use of fast T-jump methods to displace chemical equilibrium and measure relaxation rates was pioneered some time ago by Eigen and De Maeyer [3], using rapid capacitance discharge to produce Joule heating in conducting solutions. Some studies on the dynamics of ligand binding to proteins and other biomolecules have been performed, but with limited time domains and structural specificity (c.f. [4,5]). However, technology has improved substantially over the past four decades in several directions, making it feasible to perform kinetic studies on proteins on timescales from 10 ps to minutes (some 15 decades in time) and now with substantial structural specificity. Although T-jump relaxation spectroscopy has been used with great success in studying the fast dynamics of protein folding [6,7], there has been scant use concerning the dynamics of folded proteins. It is clear, however, that the approach is feasible and has sufficient sensitivity so that the time course of single molecular groups within large proteins can be monitored.

## Temperature jump relaxation spectroscopy

T-jump relaxation spectroscopy relies on the existence of an enthalpic difference between the old and new equilibrium points of a chemical equilibrium, so that the change in temperature can induce kinetics. The approach ultimately also requires a kinetic model to interpret the results. Let us assume the simplest model, a two-state equilibrium:  $[A] \leftrightarrow [B]$ . It can be shown [8] that the change in equilibrium between the two interconverting species for a given jump in temperature is given by:

$$\frac{\Delta K}{K} = 5.67 \cdot 10^{-3} \Delta H \cdot \Delta T \quad (1)$$

where  $K$  is the equilibrium constant,  $\Delta H$  is the enthalpy difference between the two species (in kcal/mol) and  $\Delta T$  is the T-jump (in degrees Kelvin). This equation shows explicitly that an enthalpy difference is required for there to be any measurable change in the chemical composition of the equilibrium. It also suggests that sensitivity depends on entropy as well, as shown in Equation 1 via the equilibrium constant. Trace amounts of a minor species are difficult to observe. Assuming  $K \approx 1$ , an average T-jump of 15°C (typical) and a sensitivity to changes in  $K$  of approximately 4% (quite possible), then any two states separated by as little as 0.5 kcal/mol in enthalpy can be observed (note that thermal energy at room temperature is  $kT = 0.6$  kcal/mol).

Analysis of the kinetic results also requires a kinetic model to relate the observed relaxation times to the rate constants of the model reaction. Again assuming the simplest case of two interconverting species, one relaxation time is

observed, which is the sum of the forward and reverse rate constants. Bimolecular reactions can be discerned from unimolecular reactions using measurements as a function of concentration. In general, a minimal kinetic model involves one more chemical species than the number of observed relaxation times. Complicated reaction schemes can be solved numerically. In the past, the equations have been linearized, which reduces the problem to an eigenvalue/eigenvector problem that can be solved analytically (the eigenvalues being the relaxation times as functions of the rate constants of the kinetic model). This has been worked out in substantial detail [4,5,8]. T-jumps of 10–20°C are too large to assume that the equations can be linearized. However, simulations comparing solutions from a linearized approach with numerical solutions from the nonlinear equations have shown that often the error is small, even for T-jumps of this magnitude.

The dynamics of any reversible system can be characterized by T-jump relaxation spectroscopy provided that the general concepts discussed above are met and also that there is a suitable probe of structure. Many different kinetic systems have been developed (e.g. [8]). One example is  $[ES] \leftrightarrow [EP]$ , where ES and EP represent the Michaelis complexes of an enzyme, E, from the substrate and product sides, respectively. Typically, there will be an enthalpic difference between the substrate and product sides because the chemical nature of the two species differs. If the chemical equilibrium can be set up in solution under saturating substrate conditions, it is more often the case than not that the equilibrium constant of  $[ES] \leftrightarrow [EP]$  conversion is close to one. A second example is the binding of ligand to protein, that is,  $P + L \leftrightarrow P-L$ . Generally, this is a too simple model of the kinetics; the binding pathway almost certainly must include, at minimum, the formation of an encounter complex(es):  $P + L \leftrightarrow (P-L)_{\text{encounter}} \leftrightarrow P-L$ . Although such encounter complexes may be present only in trace concentrations at equilibrium, their presence can be observed; as the T-jump triggers (typically) the dissociation of the ligand from the protein, the system must pass through the encounter complex at a concentration equal to the amount of produced free ligand.

### Spectrometers

For technical reasons, three different T-jump relaxation spectrometers are now needed to span the time range from 10 ps to minutes (or longer). Two spectrometer systems, covering 10 ps to 10 ns and 10 ns to 10 ms, are based on a laser-induced T-jump. The time required for thermalization of absorbed light fixes the ultimate ‘dead time’ of the laser-induced T-jump approach at about 5–10 ps. Typically, given the specific types of cells and their dimensions and configurations, the heat generated by the pump laser diffuses out of the laser-irradiated volume with a time constant of 5–50 ms. Hence, there is a ‘longest’ time, varying from setup to setup, over which the laser-induced temperature change lasts. The method of regulating the time between the ‘pump’ laser pulse and the ‘probe’ light

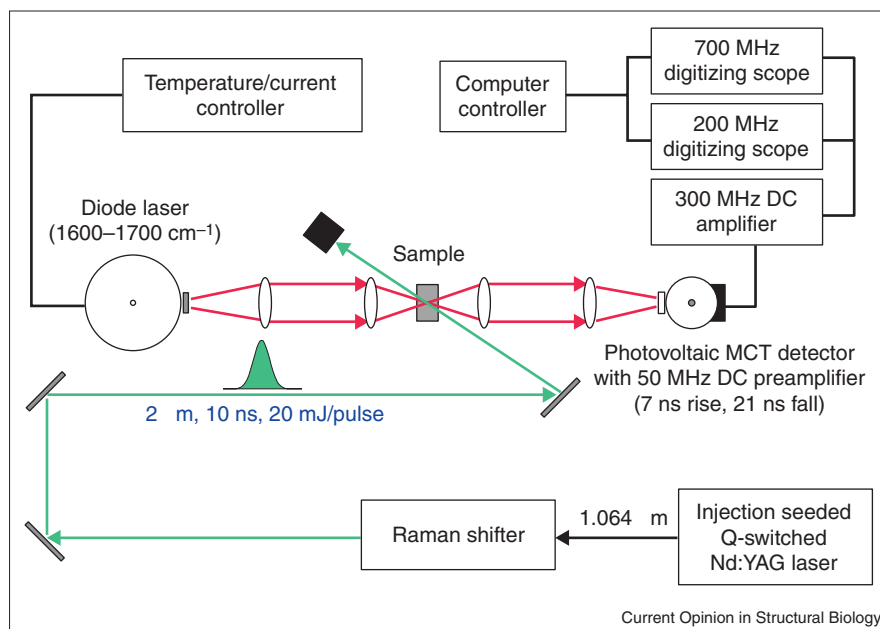
used in the various spectroscopy methods (optical absorption and fluorescence, CD and IR absorption) differs for the 10 ps to 10 ns spectrometer compared to the 10 ns to 10 ms spectrometer. In the former, optical delay lines are used; in the latter, the timing is controlled by electronics. The third apparatus, covering the 1 ms to minutes (or longer) range, is required to probe ‘slow’ dynamics. Several methods may be used to study T-jump-induced kinetics during these times. One is simply based on conventional stopped-flow mixing technology. In this case, two solutions held at two separate temperatures,  $T_1$  and  $T_2$ , are mixed with a dead time of  $\sim 1$  ms to produce a solution at  $T_3$ .

Figure 1 shows a schematic of a 10 ns to 10 ms instrument using IR absorption as the structural probe [6,9]. A tunable continuous wave IR laser beam irradiates the sample. The change in transmission induced by the T-jump is detected in real time. In this way, a very accurate baseline is automatically provided and quite small (one part in  $10^4$ ) transmission changes can be determined. The pump laser energy is absorbed by water and the temperature of the volume of water reaches its maximum value within  $\sim 10$ – $20$  ns (twice the fwhm [full width at half-maximum] of the pump pulse), as thermalization of the absorbed laser light is much faster, as mentioned above. Several instruments, conceptually similar to the spectrometer in Figure 1, have been reported that employ UV/visible fluorescence and/or absorption as probes of structure [7,10,11]. By limiting the pulse width of the laser used to bring about the T-jump to the picosecond timescale, a picosecond T-jump spectrometer is built; the use of such a system has been reported [6,12].

Relaxation spectroscopy offers exceptional time resolution ( $\sim 10$  ps) and dynamical range — 10 ps to minutes can be equally probed. The major drawback of the approach is structural specificity. It is relatively easier to characterize the chemical kinetics of a system as opposed to measuring its structural evolution. Chromophores in the UV/visible region (such as tryptophan emission, absorbing prosthetic groups and so forth) yield useful but limited information. In this regard, IR absorption, coupled with isotope editing approaches, has the potential to characterize the structural evolution of a system down to a range of specific bonds.

Isotope editing schemes in Raman and IR vibrational studies of proteins [13] involve tagging a specific bond with a stable isotope (typically  $^{13}\text{C}$ ,  $^{18}\text{O}$  or  $^{15}\text{N}$ ). The isotope shifts the vibrational frequency of this bond and the difference spectrum derived from the unlabeled protein complex minus that of the labeled complex consists of only vibrational modes that are affected by the label. All the unaffected modes subtract out. This approach has led to the structure-specific assignment of the vibrational spectra of proteins and their bound ligands for single specific bonds within an  $\sim 100$  kDa protein. This analogous approach to structure-specific measurements in kinetic studies has recently been demonstrated in a series

Figure 1



Schematic showing a nanosecond laser T-jump time-resolved apparatus employing IR absorption as a probe of structure.

of studies [14<sup>\*</sup>] of the NAD–pyruvate adduct complexed with lactate dehydrogenase (LDH). Although the isotope editing approach has been limited to the study of protein-bound ligands because small molecules are easily labeled, segmental labeling of proteins changes this dramatically. Segmental isotopic labeling refers to the site-specific introduction of stable atomic isotopes into the primary sequence of a protein. This has been developed for solution NMR studies of large proteins [15]. Segmental isotopic labeling, in principle, allows isotope edited IR and Raman spectroscopy to be applied to protein groups as well. It should be possible to selectively observe defined regions of a protein using T-jump time-resolved techniques coupled with IR absorption spectroscopy.

## Examples

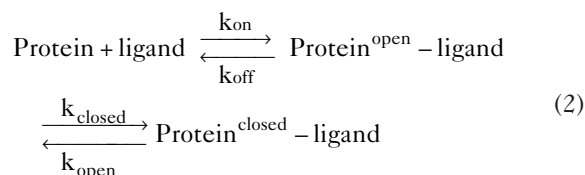
### Ligand binding

Several studies in the late 1960s and early 1970s were performed to determine the fast steps of the binding of ligands to proteins. A substantial amount of our present knowledge of these kinetics comes from these early studies. Much of this literature is reviewed in [4]. A more recent study investigated the binding of NADH to LDH with substantially better time resolution compared to these earlier studies [16<sup>\*</sup>]. The bimolecular rate process, at about 290 ns, was easily observed, as were multiple faster events (with relaxation times of 200 ns, 3.5 ns and 24 ns), revealing the rich dynamical nature of the binding step. The results show that there are multiple structures of bound enzyme–ligand complexes, some of which are likely to be far from the catalytically productive structure. The results have important implications for interpreting the thermodynamics of the binding of ligands to LDH, suggesting, for example,

that the binding constant of LDH with NADH has a rather complicated interpretation.

### Loop motion in proteins

In LDH, binding of substrate must be accompanied by the closure of a mobile loop of the protein (residues 98–110) over the protein–substrate complex before efficient catalysis can occur. This motion desolvates the binding site and brings key residues into proper contact with the substrate. Holbrook and co-workers [17] employed T-jump relaxation spectroscopy to determine the loop motion kinetics. The data, including the dependence of the observed relaxation rates on the concentration of free protein and ligand, could be well modeled by the following kinetic model:

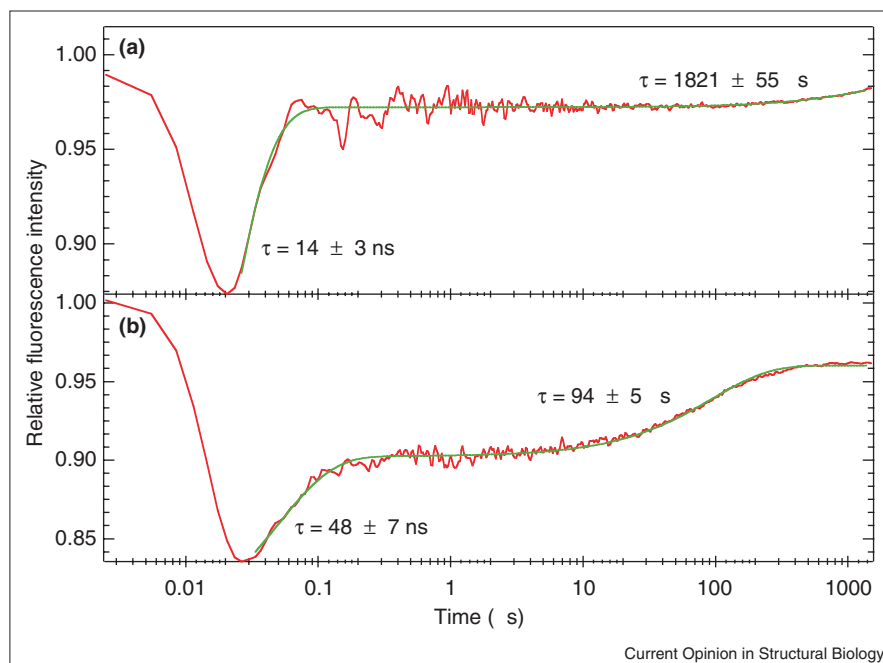


where the formation of the encounter complex with the loop open was assumed to be fast compared to the loop closure motion. By varying the amount of free oxamate and of the binary complex LDH–NADH, the following rates are derived:  $K = k_{\text{off}}/k_{\text{on}} = 0.156 \text{ mM}$ ,  $k_{\text{closed}} = 580 \text{ s}^{-1}$ ,  $k_{\text{open}} = 3020 \text{ s}^{-1}$ , at 23°C. More recent measurements showed that at least two faster steps on the microsecond timescale, in addition to loop closure, also accompany the binding of substrate to LDH [14<sup>\*</sup>].

In unpublished work (R Desamero, S Rozovsky, A McDermott, R Callender, unpublished data), we

**Figure 2**

The emission at 340 nm in response to a 20°C (5 to 25) T-jump of a solution of 20  $\mu$ M TIM: (a) apo-TIM; (b) TIM plus 5 mM G3P (see text for further details). Red line, data; green line, fit to data. The observed 14–50 ns transient response is not well understood.

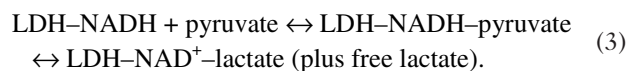


have characterized the kinetics of loop movement in triose-phosphate isomerase (TIM). To study the loop dynamics, a double mutant of *Saccharomyces cerevisiae* TIM (Trp90Tyr and Trp157Phe) that only has one tryptophan, Trp168, was used. Trp168 is located at the edge of the mobile loop in TIM (residues 166–176) that closes over the bound substrate, desolvating the active site and bringing substrate into contact with key protein residues. Tryptophan residues are notably valuable fluorescence probes as the indole ring is sensitive to its environment. We expected Trp168 to be sensitive to loop closure as the indole ring solvation is significantly modulated during this motion. Figure 2 shows the kinetic response to a 20°C T-jump of the emission of the indole ring as stimulated at 275 nm and measured at 340 nm for the apo-protein (Figure 2a) and for TIM in the presence of G3P, which is a nonreactive substrate analog (Figure 2b). Common to both plots is the abrupt decay of fluorescence as the system is heated, followed by an immediate rise in intensity. The decay may be attributed to temperature-induced quenching of fluorescence. The life-time of this decay is at the response time of the electronics and the pulse width of the heating beam, about 15 ns. The immediate rise in intensity that follows the decay is not so well understood. Another common feature is the rise in intensity at around 10 ms, which is attributed to cooling of the irradiated volume by thermal diffusion. The only part of the relaxation kinetic profile influenced by the presence of the ligand is the region from about 1  $\mu$ s to about 1 ms. In all measurements, the concentration of TIM was set at  $250 \pm 20 \mu$ M. For unligated TIM (Figure 2a), this region is flat, a period of constant intensity, much like the kinetic profile for tryptophan in solution by itself (data not

shown). The relaxation kinetics at 20°C for TIM ligated with 5 mM G3P (Figure 2b) could be fitted to a single exponential equation and had a life-time of  $94 \pm 5 \mu$ s. A detailed study of the TIM–G3P system revealed that the observed relaxation kinetics vary with both the final temperature of the T-jump and the ligand concentration. The kinetic scheme above, Equation 2, was found to adequately explain the results. The higher the final jump temperatures, the higher the rate constant of the single exponential fit to the relaxation kinetics. Also, as the concentration of the ligand increases, the rate constant and the amplitude of the relaxation kinetic profile decrease. At 25°C, the loop opening rate was found to be  $k_{\text{open}} = 2500 \text{ s}^{-1}$ , whereas  $k_{\text{closed}} = 46\,700 \text{ s}^{-1}$ . Enthalpies of activation of the loop motion,  $\Delta H_{\text{close}}^{\ddagger}$  and  $\Delta H_{\text{open}}^{\ddagger}$ , were estimated to be 13.8 and 14.1 kcal/mol. The dynamical characterization of the loop motion by the T-jump approach was found to be in remarkable agreement with previous investigations using solution and solid-state NMR spectroscopy [18,19].

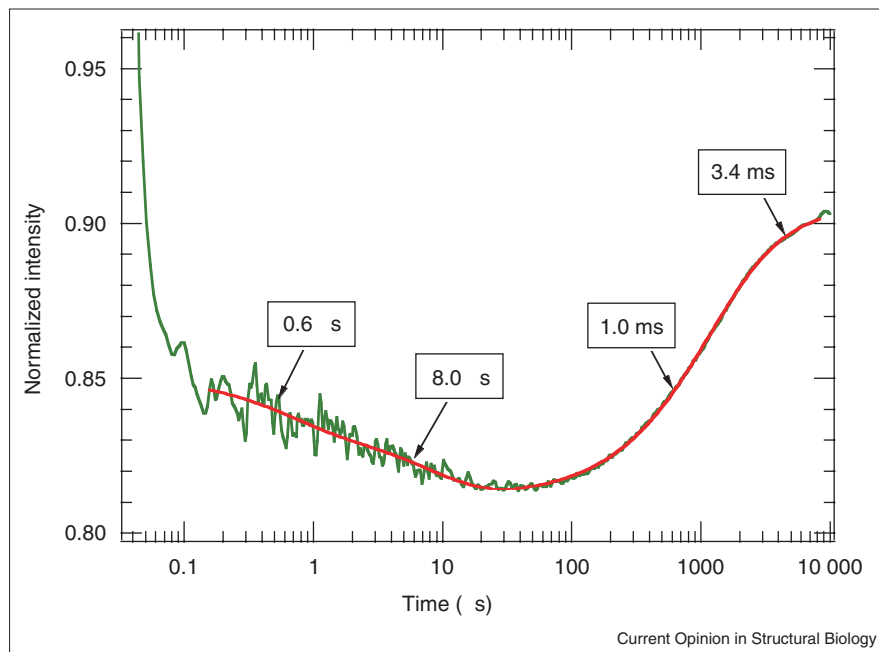
### Fast events in enzymatic catalysis

Figure 3 shows some preliminary data for the chemical reaction catalyzed by LDH, the reversible conversion of pyruvate to lactate. It can be shown that, by mixing LDH,  $\text{NAD}^+$  and lactate under suitable conditions, the following species are formed in the mixture at equilibrium:



The LDH–NADH–pyruvate ternary complex concentration is quite low and the concentration of LDH–NADH + pyruvate equals approximately that of LDH–NAD<sup>+</sup>–lactate.

Figure 3



Time-resolved fluorescence at 450 nm (NADH emission) of a sample initially containing 100  $\mu$ M LDH, 100  $\mu$ M NAD<sup>+</sup> and 10 mM lactate in response to a T-jump of 10 to 23°C.  $\lambda_{\text{ex}} = 290$  nm (see text for further details). Green line, data; red line, exponential fits to data.

A temperature increase tips the equilibrium from right to left in Equation 3. Figure 3 shows the time-resolved fluorescence emission of NADH at 450 nm in response to a T-jump from 10 to 23°C. Some of the emission signal is due to NADH bound to the binary LDH–NADH complex. However, some is also due to low concentrations of various conformers of ternary LDH–NADH–pyruvate complex(es) that are formed transiently as a result of the rapid T-jump. Normally, emission from the ternary complex in the equilibrium mixture is highly quenched; however, transiently produced ternary complexes, whose structures differ from that found at equilibrium, can show substantial emission of the reduced nicotinamide ring. There are two instrument response times. One is near 30 ns, which is the pulse width of the laser irradiation heating the sample. The second is the diffusion of heat from the laser interaction volume, which occurs around 15 ms (the latter response is not shown). Fitting the data (red line) with a function of multiexponentials yielded four rates, as indicated in Figure 3, in addition to these instrument response functions. The data in Figure 3 are remarkable in showing that there exist several fast processes heretofore unresolved in studies of the enzymic dynamics of LDH. The dissociation of pyruvate from LDH–NADH would be observed on the millisecond timescale and, tentatively, we assign the 3.4 ms transient to this event. Such an assignment is reinforced by the increasing signal associated with the 3.4 ms transient because there is a large emission increase as pyruvate dissociates from LDH–NADH. The other three transients are then assigned to unimolecular events. The motion of the mobile loop opening before pyruvate is released is near 1 ms (see above), so the 1 ms transient shown in Figure 3

may result from this event. It is unclear at this time what the faster events, at 0.6 and 8.0 s, are due to. The NADH emission is quenched, so it may be that these events reflect structural changes associated with the ‘tightening’ of the protein pockets around the substrate and cofactor. These may involve motions of the mobile loop or other important protein residues.

Further insights on the dynamics of the LDH active site have been obtained from T-jump experiments on the NAD–pyruvate adduct bound to LDH employing time-resolved, isotope-edited IR spectroscopy [14<sup>\*</sup>]. The bound NAD–pyruvate adduct, whose pyruvate moiety probably interacts with the same residues that interact with pyruvate in its ternary complex with LDH, served as a probe for any relative motions of active site residues against the substrate. No chemistry takes place in the LDH–adduct complex, so that experimental studies can focus on atomic motions that may occur apart from those resulting from catalysis. The frequencies of the pyruvate C=O stretch and –COO<sup>–</sup> antisymmetric stretch are exquisitely sensitive to the motion of polar moieties at the active site (His195, Asp168, Arg109 and Arg171). Apart from the melting of a few residues at the protein surface, however, no kinetics were observed on any timescale (10 ns–1 ms), even for final temperatures close to the unfolding transition of the protein. This unexpected result demonstrates that once a productive protein–substrate complex is formed, the active site is quite rigid, with very little motion apart from the mobile loop.

## Conclusions

Protein dynamics and its relation to function are poorly understood. Several techniques are now being brought to

bear on this issue, including NMR relaxation spectroscopy, computational approaches (i.e. molecular dynamics calculations and so forth) and others. T-jump relaxation spectroscopy, when used with proper probes of structure, can provide outstanding time resolution and dynamic range, and quite good structural specificity in measurements of the dynamical nature of proteins.

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