COMMUNICATION

Kinetics of Intramolecular Contact Formation in a Denatured Protein

Marco Buscaglia¹, Benjamin Schuler¹,², Lisa J. Lapidus¹
William A. Eaton¹* and James Hofrichter¹*

¹Laboratory of Chemical Physics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 5
Bethesda, MD 20892-0520 USA
²Physikalische Biochemie
Universität, Potsdam, Germany

Quenching of the triplet state of tryptophan by cysteine has provided a new tool for measuring the rate of forming a specific intramolecular contact in disordered polypeptides. Here, we use this technique to investigate contact formation in the denatured state of CspTm, a small cold-shock protein from Thermotoga maritima, engineered to contain a single tryptophan residue (W29) and a single cysteine residue at the C terminus (C67). At all concentrations of denaturant, the decay rate of the W29 triplet of the unfolded protein is more than tenfold faster than the rate observed for the native protein (~10⁴ s⁻¹). Experiments on the unfolded protein without the added C-terminal cysteine residue show that this faster rate results entirely from contact quenching by C67. The quenching rate in the unfolded state by C67 increases at concentrations of denaturant that favor folding, indicating a compaction of the unfolded protein as observed previously in single-molecule Förster resonance energy transfer (FRET) experiments.

Keywords: triplet state quenching; unfolded state; collapse; cold shock protein

A considerable body of structural information regarding the denatured state of proteins has been accumulating from nuclear magnetic resonance spectroscopy,¹–³ neutron⁴ and small-angle X-ray scattering experiments,⁵,⁶ and diffusion experiments.⁷ Much less is known about the dynamics. Of particular interest are the kinetics of contact formation between residues distant in the amino acid sequence, since this process is believed to be an important step in the mechanism of folding of many proteins. Contact formation in disordered peptides has been studied from measurements of triplet–triplet energy transfer⁸ and fluorescence quenching⁹,¹⁰ using extrinsic probes. We have demonstrated that contact formation can be studied in peptides using naturally occurring amino acids from the quenching of the long-lived triplet state of tryptophan by cysteine.¹¹–¹⁴ Since cysteine is the only naturally occurring amino acid that efficiently quenches the tryptophan triplet state,¹⁵ it should be possible to use this method to study the dynamic properties of a large number of peptide and protein sequences. Here, we report the first application of the technique to measure contact formation in the denatured state of a small protein (Figure 1).

CspTm, the 66 residue cold-shock protein from the hyperthermophilic bacterium Thermotoga maritima, was engineered to contain a single tryptophan residue at position 29, by changing the tryptophan residue at position 7 to tyrosine, and a single cysteine residue, which was added to the C terminus (CspTm-C; see the legend to Figure 1). CspTm was selected because it is robust to mutations and contains only two (slowly exchanging) populations of molecules under partially denaturing conditions, folded and unfolded, as determined in both ensemble and single-molecule measurements.¹⁶–¹⁸ As controls, we studied the modified protein sequence without the added cysteine residue (CspTm) as well as the peptides corresponding to residues 24–35 and 28–67.

Figure 2(a) shows representative triplet decay curves of the cysteine-containing CspTm-C at different concentrations of guanidine chloride (GdmCl). In the absence of denaturant, where the
protein is completely in the folded state, the decay curve cannot be described by a single-exponential function. For this work, we have chosen to analyze the decay curves using a (slightly) stretched exponential function: 
\[ p(t) = \exp(-t/\tau^b) \]
with \( b = 0.8 \) and \( \tau = 90 \mu s \). At 6 M GdmCl, where the protein is completely in the unfolded state, the time-course is not exponential, and can be described by a stretched exponential function (\( b = 0.7, \tau = 10 \mu s \)) with an average rate (i.e. the integral under the decay curve) that is more than tenfold faster than that for the folded protein.\(^\dagger\) When the C-terminal cysteine residue is removed, the triplet lifetime for the unfolded protein is much longer (~40 \mu s), and is very similar to that for free tryptophan and for the 12 residue fragment containing W29 and no cysteine (Figure 2(b)). These results demonstrate that the natural lifetime of the tryptophan triplet in a disordered structure is ~40 \mu s (in the absence of oxygen), and that the shortened lifetime of 10 \mu s is due entirely to quenching by contact with the C-terminal cysteine residue (C67).

At intermediate concentrations of denaturant, the decay curves for the protein (Figure 2(a)) can be described by a linear combination of the decay curves for the completely folded and completely unfolded states, as expected for a two-state protein. Figure 3 compares the populations of the folded and unfolded states as a function of GdmCl concentration, calculated from the relative amplitudes of the two processes, with those derived from tryptophan fluorescence intensity measurements. The agreement of the curves determined by the two techniques is within the uncertainties of the two-state analysis. This result shows that accurate decay rates for the unfolded protein can be extracted from the complex decay kinetics at concentrations of denaturant where the folded protein is also present. The resulting rates are shown in Figure 4.

The decay rate for the folded state exhibits no significant dependence on the concentration of denaturant. The interesting result is that the decay rate for the unfolded state increases significantly at low concentrations of denaturant. According to the simplest model for triplet quenching, the process takes place in two steps. In the first, tryptophan in its excited triplet state and cysteine diffuse together to form a contact pair (Figure 1). This diffusion-limited process is followed either by quenching of the triplet or dissociation without quenching. For cysteine, the quenching rate is much closer to reaction-limited than to diffusion-limited. That is, the probability of quenching in the contact pair is relatively small, so the overall rate depends almost entirely on the distribution of tryptophan–cysteine distances and the rate of quenching at contact.\(^{11,12,14}\) Since there is little or no change in the quenching rate for the free amino acids with concentration of denaturant (M.B., unpublished results), the increase in rate reflects a decrease in the average distance between the tryptophan and cysteine residues. A decrease in chain dimensions, presumably resulting from an increase in attractive inter-residue interactions at lower concentrations of GdmCl, is consistent with recent measurements of Förster resonance energy transfer (FRET) between dye molecules attached to the N and C termini of CspTm.\(^2\) These single-molecule experiments separately resolved the

\(^\dagger\) The origin of the complexity in the decays is not yet clear. It should be noted that both the 24–35 fragment, which does not contain a cysteine residue, and CspTm exhibit a more nearly exponential decay than that of CspTm-C. The 28–67 fragment, a non-folding sequence, also shows a single-exponential decay (\( b = 0.98, \tau = 10 \mu s \)) at 6 M GdmCl, with a mean lifetime that is about 30\% shorter than that observed for the full 67 residue protein.
FRET efficiencies for the unfolded and folded protein, which increased below the mid-transition concentration of denaturant, indicating chain compaction as we have observed in the present experiments. A quantitative comparison will require dissection of the measured rates into reaction-limited and diffusion-limited components, which can be done from studies of the viscosity dependence.
of the quenching rate, as has been done on small peptides (our unpublished results, and see Lapidus et al.14), as well as more detailed modeling.

References


Edited by C. R. Matthews

(Received 13 February 2003; received in revised form 8 July 2003; accepted 8 July 2003)