Dynamics of Intramolecular Contact Formation in Polypeptides: Distance Dependence of Quenching Rates in a Room-Temperature Glass

Lisa J. Lapidus, William A. Eaton, and James Hofrichter

Laboratory of Chemical Physics, Building 5, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

(Received 11 July 2001; published 30 November 2001)

Quenching of the triplet state of tryptophan by cysteine is an important new tool for measuring the rate of forming a specific contact between amino acids in a polypeptide chain. To determine the length scale associated with this contact, tryptophan was embedded in a room-temperature glass containing a high concentration of cysteine. The decay of the triplet population is extended in time, consistent with a rate coefficient that decreases exponentially with distance. Solving the diffusion equation with this distant-dependent rate reproduces the observed bimolecular rates in water and shows that quenching at low viscosities takes place $\lesssim 1$ Å from van der Waals contact between the tryptophan and cysteine.

DOI: 10.1103/PhysRevLett.87.258101 PACS numbers: 87.15.Cc, 82.35.Pq, 87.15.He

Understanding how proteins fold from the myriad of conformations of the denatured state to the unique conformation of the functioning biological molecule has emerged as a major challenge to physical scientists. A universal feature of theoretical models is the formation of nonlocal contacts during folding, that is, contacts between amino acids that are distant in sequence [1]. In spite of its central role in protein folding mechanisms, there is very little experimental information on the dynamics of contact formation [2]. This is due to the lack of a method that has both structural specificity and high time resolution and exploits properties of naturally occurring amino acids. We have recently introduced a method that satisfies all three criteria [3]. It is based on physical properties of tryptophan and cysteine that are unique among the 20 naturally occurring amino acids. The basic idea is that optical excitation of tryptophan with an intense nanosecond laser pulse populates a long-lived, excited triplet electronic state which returns to the ground state with a high probability upon contact with cysteine. Cysteine is by far the most efficient quencher among the amino acids [4]. Consequently, in a natural or synthetic polypeptide containing a single tryptophan and a single cysteine but otherwise arbitrary sequence, measurement of the lifetime of the tryptophan triplet state should lead to a direct determination of the rate of forming the tryptophan-cysteine contact. In this Letter we show that the length scale associated with this contact is ~ 1 Å at the viscosity of water. This result is based on measurements of the distance dependence of the quenching rate in a room-temperature trehalose glass in which diffusion is almost negligible.

We have already demonstrated the utility of measuring tryptophan-cysteine contact rates by determining important dynamical quantities of a disordered polypeptide, including the end-to-end contact rate, the dependence of this rate on chain length, and, using the simplest theoretical description of polymer dynamics, the apparent end-to-end diffusion coefficient [3]. The measurements were made on the peptide series: cys-(ala-gly-gln)$_j$-trp with observed quenching rates decreasing from $1/(70\ \text{ns})$ for $j = 1$ to $1/(280\ \text{ns})$ for $j = 6$. To obtain contact rates from the measured rates a kinetic model was used in which quenching occurs in a two-step process: diffusion of the polypeptide ends to form a tryptophan-cysteine contact pair with a rate k_{D+}, followed by quenching with a rate q or diffusive separation with a rate k_{D-}. With this model the observed end-to-end contact rate k^{uni} is given by

$$k^{\text{uni}} = \frac{k_{D+} q}{k_{D-} + q}. \tag{1}$$

From measurements in which the cysteine was replaced by a known diffusion-limited quencher ($q \gg k_{D-}$), the contact rates (k_{D+}) were shown to be about twice the observed rates for cysteine.

These contact rates were interpreted in terms of the first-passage-time theory of Szabo *et al.* (SSS) [5] in which the rate is obtained by solving a one-dimensional diffusion equation for motion of one end of the chain relative to the other in a purely entropic harmonic potential. For the idealized case of a Gaussian chain with mean-squared end-to-end distance $\langle r^2 \rangle$, relative diffusion coefficient D, and contact radius a, the SSS rate is given by $k_{D+} = 4\pi D a / (2\pi \langle r^2 \rangle / 3)^{3/2}$ [5]. This analysis assumes that the contact pair is defined by a single or narrow range of tryptophan-cysteine distances. However, the mechanism of quenching (i.e., depopulation of the triplet state) most probably occurs via electron transfer from the indole ring of tryptophan to the cysteine sulfur atom [6,7], and must therefore have a distance-dependent rate [8]. The objective of this paper is to experimentally determine this distance dependence in order to give a more realistic and complete description of the measured rates. The distance dependence, for example, is an essential ingredient in calculating the observed quenching rates from molecular simulations or by solving a diffusion equation using a model for the chain motion [9]. Moreover, with current

computer power the time scale of the observed rates is accessible to all-atom molecular dynamics calculations, which are in need of experimental benchmarks for testing their validity [10].

To determine the rate as a function of distance, tryptophan at low concentration was embedded in a room-temperature trehalose glass containing a high concentration of cysteine. Since diffusion is almost negligible and the cysteine molecules are randomly distributed, the known tryptophan-cysteine distance distribution allows a straightforward determination of the distance dependence of the quenching rate from the measured decay curves [11]. The samples were prepared by heating a solution with an initial composition of 10.7 g trehalose, 5 mL of 500 μM N-acetyl tryptophan amide (NATA), and between 0.2 and 1.1 g cysteine. Trehalose completely dissolves at $\sim$100 °C. The solution was heated to $\sim$130 °C, at which point the viscous liquid was poured into a disposable 1 cm cuvette and allowed to cool to a solid solution (a procedure similar to that used to make hard candy). The concentration of cysteine was determined from the final volume of the sample. The residual water content was 9% by weight, corresponding to a glass transition temperature (in the absence of tryptophan and cysteine) of $\sim$40°C [12]. All experiments were performed at room temperature. The instrument for measuring the decay of the tryptophan triplet state has been described in detail previously [3]. It employs an $\sim$8 ns, $\sim$1 mJ, 290 nm pulse from a Nd:YAG-pumped cerium doped $LiCaAlF_6$ laser (Ce:LiCAF) for excitation and the 457 or 514 nm line of an argon-ion laser to monitor the triplet state population by optical (triplet-triplet) absorption.

Figure 1a shows absorption kinetics at two wavelengths of the argon-ion laser (457 and 514 nm) of excited-state NATA in a trehalose glass containing no cysteine. Previous work on tryptophan in water [3,7] demonstrated that the absorption changes at these wavelengths included kinetic processes involving radical photoproducts in addition to the triplet decay. These studies suggest that the extended, nonexponential phase at short times in the glass results from geminate recombination of electrons and that the exponential relaxation at times longer than the tryptophan lifetime corresponds to bimolecular radical recombination. Both processes are slowed by the high viscosity of the glass. Since the photoproduct and triplet decay have different wavelength dependences, their decays could be separately determined (see Fig. 1 caption).

A subset of the triplet quenching data obtained in a series of experiments in which the cysteine concentration was varied from 0.3M to 1.2M is shown in Fig. 2. The distance dependence of quenching is quite short ranged. The survival probability for the 1.2M sample at 30 ns is the same as the survival probability for the 0.57M sample at 30 μs. Using the formula first derived by Chandrasekhar [13], the average distance between cysteines in a uniform distribution is 6.3 Å for 1.2M and 7.9 Å for 0.57M. This

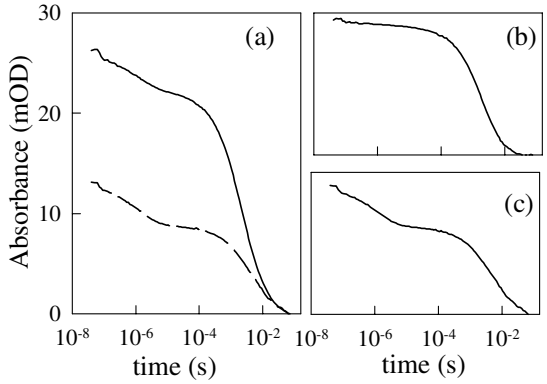

FIG. 1. Decay of the photoproducts of NATA embedded in a trehalose glass. (a) Absorbance changes measured at 457 nm (solid line) and 514 nm (dashed line). Each curve is the average of six experiments of 64 laser shots; the cuvette was repositioned between each experiment to minimize the effect of photodamage. Previous studies [3,7] showed that at 457 nm the absorption changes are dominated (>80%) by the decay of the triplet state, while at 514 nm the disappearance of a radical photoproduct and triplet decay contribute about equally to the absorption changes. Singular value decomposition (SVD) of the data from multiple experiments at the two wavelengths was used as an efficient way to filter the noise and to find linear combinations that represented only triplet decay [panel (b)] and only radical photoproduct decay [panel (c)]. The criterion for these linear combinations was simultaneous minimization of the slope at 30 ms for the triplet decay and maximization of the slope at 30 ms for the radical photoproduct decay. This criterion was based on our previous finding [3] that the radical photoproduct decay continues at least 2 orders of magnitude in time beyond the completion of the triplet decay. The finding of only two significant basis components in the SVD also demonstrates that contamination of the data by photodamage is negligible.

gives a rough estimate of the distance dependence of the quenching rate as $\Delta \ln k/\Delta r \approx -4$ Å^{-1}.

To quantitatively analyze the decay of the triplet population, the survival probability of a uniform initial distribution of quenchers of concentration Q was calculated. The survival probability of the tryptophan triplet can be written as

$$\frac{P(t)}{P(0)} = L(t)e^{-4\pi Q \int_{r_0}^{\infty} r^2[1-S(t\,|\,r)]\,dr}, \qquad (2)$$

where $L(t)$ is the decay of the triplet state in the absence of quencher and $S(t\,|\,r)$ is the time-dependent survival probability of a triplet tryptophan-cysteine pair separated by a distance r. In the absence of diffusion, $S(t|r) = \exp[-k(r)t]$ where $k(r)$ is the distance-dependent quenching rate. The dashed curves in Fig. 2 show the fit assuming a simple exponential distance dependence for the quenching rate, i.e., $k(r) = k_0 \exp[-\beta(r - r_0)]$. r_0, the contact distance, is assumed to be 4 Å. This fit yields $\beta = 3.2$ Å^{-1}.

There are, however, significant differences between the data and the theoretical curve, suggesting that the analysis is oversimplified. One simplification is the assumption of

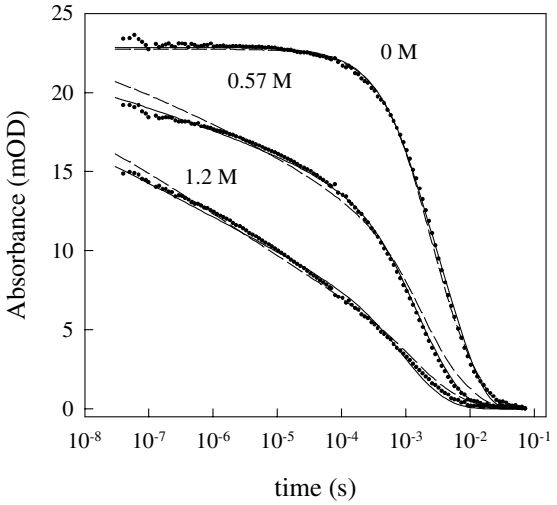

FIG. 2. Decay of the tryptophan triplet state for $0M$, $0.57M$, and $1.2M$ cysteine (separately reconstructed from SVD of the data at 457 and 514 nm) and fits with diffusion (solid lines) and without diffusion (dashed lines). The spontaneous decay of the tryptophan triplet is described by $L(t) = 0.4\exp(-770t) + 0.6\exp(-148t)$. In carrying out the fits, variations in the amplitudes of the calculated decay curves were constrained to be less than 10% relative to the mean amplitude for the three curves. The amplitudes in different fits varied by ± 0.03 and the rates by $\pm 10\%$.

a perfectly rigid glass and therefore no diffusion. Shear relaxation studies on trehalose suggest that even at temperatures close to the glass transition temperature the diffusion constant may not be negligible [12]. Furthermore, at cysteine concentrations greater than $1.3M$ the final solution at room temperature was not hard, indicating that cysteine significantly lowers the glass transition temperature. This prompted us to include diffusion in the analysis of the decay curves.

The survival probability, $S(t\,|\,r)$, satisfies the diffusion equation

$$\frac{\partial S}{\partial t} = \frac{D}{r^2}\frac{\partial}{\partial r}r^2\frac{\partial}{\partial r}S - k(r)S\,, \tag{3}$$

where D is the diffusion coefficient. After substituting the radially weighted function $F(t|x) = r^2S(t|r)$, Eq. (3) was solved with the initial condition that $F(0|x) = x^2$ (a uniform distribution) and the boundary conditions that F is reflecting at $x = r_0$ and $x = \infty$.

$$\frac{\partial F}{\partial t} = D\frac{\partial}{\partial x}x^2\frac{\partial}{\partial x}\frac{F}{x^2} - k(x)F\,. \tag{4}$$

Equation (4) was solved numerically by considering a set of bins of width Δx and generating a rate matrix $\mathbf{R}$ for the probabilities, $\{F\}$, resulting in $\partial\vec{F}/\partial t = \mathbf{R}\vec{F}$, where $\mathbf{R}$ has elements

$$\begin{aligned}
2R_{i+1,i} &= D + Dx_{i+1}^2/x_i^2 \qquad \text{(lower diagonal)}\,,\\
2R_{i,i+1} &= Dx_i^2/x_{i+1}^2 + D \qquad \text{(upper diagonal)}\,,\\
R_{1,1} &= -R_{2,1} - k(x_1) \qquad \text{(diagonal)}\,,\\
R_{i,i} &= -R_{i+1,i} - R_{i-1,i} - k(x_i)\\
R_{n,n} &= -R_{n-1,n} - k(x_n)\,,
\end{aligned} \tag{5}$$

where x_i and $k(x_i)$ were evaluated at the center of each bin. The diagonal elements include quenching of the triplet, which annihilates the population of each bin with a rate $k(x) = k(r)$. This equation can be solved by conventional eigenvalue methods to produce a set of n volume-weighted survival probabilities using k_0, β, D, and the parameters that describe $L(t)$ as free parameters. The triplet survival probability can then be calculated from Eq. (2) for any value of the quencher concentration, Q. As can be seen in Fig. 2 (solid curves), there is a significant improvement in the fit with diffusion (Table I). The fitted diffusion coefficient is about 10^8 times smaller than its value in water, consistent with the expectation that the trehalose glass is not perfectly rigid.

The principal results from this analysis are the values of the quenching rate at van der Waals contact, $k_0 = 4.2 \times 10^9\ \mathrm{s}^{-1}$, and the exponent, $\beta = 4.0\ \text{Å}^{-1}$. Are these values relevant to the quenching rates observed for polypeptides in water [3]? To address this question, Eqs. (4) and (5) were used to calculate the measured bimolecular quenching rates in water using $D = 6.6 \times 10^{-6}\ \mathrm{cm}^2\,\mathrm{s}^{-1}$. The calculated rate is $1.2 \times 10^8\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$, very close to the reaction-limited rate [i.e., $D = \infty$ in Eqs. (4) and (5), or $k_{D-} \gg q$ in Eq. (1)] of $1.4 \times 10^8\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$ obtained from $\int_{r_0}^{\infty} 4\pi r^2 k(r)\,dr$. These calculated rates are in remarkably good agreement with the observed value of $1.3 \times 10^8\ \mathrm{M}^{-1}\,\mathrm{s}^{-1}$ [3], indicating that the distance dependence measured in trehalose is very close to that for water. Because of the large β, quenching occurs over a very narrow distance range of $\sim$1 Å for $D \gtrsim 10^{-7}\ \mathrm{cm}^2\,\mathrm{s}^{-1}$, as shown in Fig. 3. The large value of β compared to the values of 1 to 1.4 often quoted for long-range electron transfer [14,15], arises at least in part from the fact that quenching at distances close to the molecular diameters are being probed. A more complete theory of electron transfer shows that the reorganization energy significantly increases the slope at short distances, resulting in a large apparent β

TABLE I. Parameters and sum of squared residuals (fss) obtained from numerical fits of quenching models to the data in Fig. 2.

β [Å^{-1}]	k_0 [s^{-1}]	D [cm^2 s^{-1}]	fss (mOD^2)
3.23	5.2×10^8	0	64.3
4.0	4.2×10^9	1.5×10^{-13}	16.1

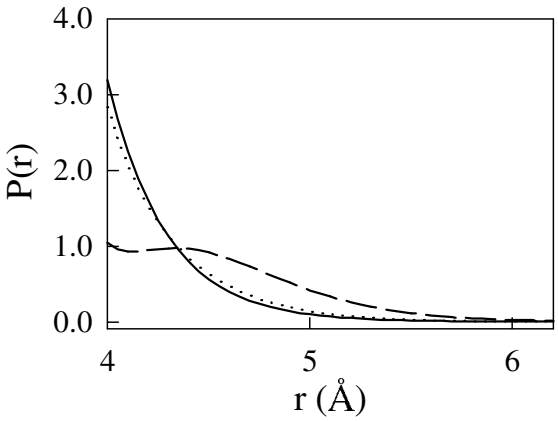

FIG. 3. Probability of quenching as a function of the tryptophan-cysteine separation for various diffusion coefficients: $D = 10^{-6}$ (solid line), $D = 10^{-7}$ (dotted line) and $D = 10^{-8}$ (dashed line) $\mathrm{cm}^2\,\mathrm{s}^{-1}$. The probabilities were calculated as the normalized product of the eigenvector associated with the smallest eigenvalue of the rate matrix **R** [Eq. (5)] and the quenching rates, k. The probabilities for higher diffusion constants are indistinguishable from $D = 10^{-6}\,\mathrm{cm}^2\,\mathrm{s}^{-1}$, and can be calculated from $P(r) = 4\pi r^2 k(r)/\int_{r_0}^{\infty} 4\pi r^2 k(r)\,dr$.

[8,15]. [The fit to the data in Fig. 2 is only marginally improved when using a more complex form of $k(r)$, which includes the reorganization energy [15], and results in the same quenching rate in the 5–7 Å range probed by the experiment.]

The distance dependence determined in the trehalose glass shows that cysteine must come to within ~1 Å of van der Waals contact with the tryptophan for quenching to occur. Triplet state quenching by cysteine is, therefore, probing very short-range interactions, simplifying theoretical models and the analysis of molecular simulations. Specifically, it justifies the comparison of theoretical rates calculated as the mean first passage time to van der Waals contact with experimentally determined diffusion-limited rates [e.g., k_{D+} of Eq. (1)].

We thank Attila Szabo for many helpful discussions.

[1] J. D. Bryngelson and P. G. Wolynes, Proc. Natl. Acad. Sci. U.S.A. **84**, 7524 (1987); J. N. Onuchic, Z. Luthey-Schulten, and P. G. Wolynes, Annu. Rev. Phys. Chem. **48**, 545 (1997); C. M. Dobson, A. Sali, and M. Karplus, Angew. Chem. Int. Ed. Engl. **37**, 868 (1998); A. Fersht, *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding* (Freeman, New York, 1999); V. Muñoz and W. A. Eaton, Proc. Natl. Acad. Sci. U.S.A. **96**, 11 311 (1999); O. V. Galzitskaya and A. V. Finkelstein, Proc. Natl. Acad. Sci. U.S.A. **96**, 11 299 (1999); D. Thirumalai and D. K. Klimov, Curr. Opin. Struct. Biol. **9**, 197 (1999); J. N. Onuchic, H. Nymeyer, A. E. Garcia, J. Chahine, and N. D. Socci, Adv. Protein Chem. **53**, 87 (2000); L. Mirny and E. Shakhnovich, Annu. Rev. Biophys. Biomol. Struct. **30**, 361 (2001); J. J. Portman, S. Takada, and P. G. Wolynes, J. Chem. Phys. **114**, 5069 (2001).

[2] C. M. Jones, E. R. Henry, Y. Hu, C.-K. Chan, S. D. Luck, A. Bhuyan, H. Roder, J. Hofrichter, and W. A. Eaton, Proc. Natl. Acad. Sci. U.S.A. **90**, 11 860 (1993); S. J. Hagen, J. Hofricher, A. Szabo, and W. A. Eaton, Proc. Natl. Acad. Sci. U.S.A. **93**, 11 615 (1996); S. J. Hagen, J. Hofrichter, and W. A. Eaton, J. Phys. Chem. B **101**, 2352 (1996); D. Thirumalai, J. Phys. Chem. B **103**, 608 (1999); O. Bieri, J. Wirz, B. Hellrung, M. Schutkowski, M. Drewello, and T. Kiefhaber, Proc. Natl. Acad. Sci. U.S.A. **96**, 9597 (1999); W. A. Eaton, V. Muñoz, S. J. Hagen, G. Jas, L. J. Lapidus, E. R. Henry, and J. Hofrichter, Annu. Rev. Biophys. Biomol. Struct. **29**, 327 (2000); S. J. Hagen, C. W. Carswell, and E. M. Sjolander, J. Mol. Biol. **305**, 1161 (2001).

[3] L. J. Lapidus, W. A. Eaton, and J. Hofrichter, Proc. Natl. Acad. Sci. U.S.A. **97**, 7220 (2000).

[4] M. Gonnelli and G. B. Strambini, Biochemistry **24**, 13 847 (1995).

[5] A. Szabo, K. Schulten, and Z. Schulten, J. Chem. Phys. **72**, 4350 (1980).

[6] M. Bonifačić and K.-D. Asmus, J. Chem. Soc. Perkin Trans. 2 **11**, 1805 (1986).

[7] D. V. Bent and E. Hayon, J. Am. Chem. Soc. **97**, 2612 (1975).

[8] R. A. Marcus and N. Sutin, Biochim. Biophys. Acta **811**, 265 (1985).

[9] L. J. Lapidus, P. Steinbach, W. A. Eaton, and J. Hofrichter (to be published).

[10] Y. Duan and P. A. Kollman, Science **282**, 740 (1998); M. R. Shirts and V. S. Pande, Phys. Rev. Lett. **86**, 4983 (2001).

[11] Z. Li, W. E. Lee, and W. C. Galley, Biophys. J. **56**, 361 (1989).

[12] S.-P. Ding, J. Fan, J. L. Green, Q. Lu, E. Sanchez, and C. A. Angell, J. Therm. Anal. **47**, 1391 (1996).

[13] S. Chandrasekhar, Rev. Mod. Phys. **15**, 1 (1943).

[14] J. R. Miller, J. V. Beitz, and R. K. Huddleston, J. Am. Chem. Soc. **106**, 5057 (1984); C. C. Moser, J. M. Keske, K. Warncke, R. S. Farid, and P. L. Dutton, Nature (London) **355**, 792 (1992).

[15] H. L. Tavernier and M. D. Fayer, J. Phys. Chem. B **104**, 11 541 (2000).