Review

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Triplet–triplet energy transfer studies on conformational dynamics in peptides and a protein

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Peptides and proteins are highly dynamic systems, which can adopt more or less stable conformations. The dynamics of these molecules, particularly those on the nanosecond to tens of microsecond time scale, are difficult to assess with conventional techniques. This review summarizes experiments using TTET, a technique that reports on van der Waals contact formation between a triplet donor and acceptor group, and which is sensitive in this time range. TTET allows to directly measure the chain dynamics of unstructured model peptides, i.e. large-amplitude fluctuations on the nanosecond time scale. Furthermore, contact formation can be used as irreversible probing reaction to study the kinetics of conformational equilibria. This approach enabled us to measure local α-helix folding and unfolding in helical peptides, which gave new insight into the equilibrium dynamics of this fundamental secondary structure element. TTET has also been applied to study the dynamics both in the native and unfolded state of a protein, the villin headpiece subdomain. The contact formation kinetics between different positions revealed an unlocking and local unfolding reaction in the native state of this model protein, and gave information about the chain dynamics in the unfolded state ensemble.

Keywords: conformational dynamics; triplet-triplet energy transfer; contact formation; loop closure; protein folding; α-helix; helix–coil transition; villin headpiece subdomain

Background

Conformational dynamics and structure formation in peptides and proteins are poorly understood phenomena. The building blocks, amino acids linked by amide bonds, are relatively simple entities, but the conformational degrees of freedom in their backbone and side chains give rise to large ensembles of conformations [1,2]. Non-covalent, weak and competing interactions determine the free energies of these conformations. However, even in short peptides, cooperative effects can support the formation of local secondary structure elements and drive the folding of proteins into their native state.

The interconversion between different conformations, especially those which are only marginally stable, is usually fast and difficult to capture with conventional techniques. For example, unstructured peptide chains undergo large-amplitude motions on the nanosecond time scale. These chain dynamics determine how fast a chain can sample its conformational space and how fast interactions between different sites can form [3]. Neither NMR nor spectroscopic techniques like FRET are generally suited to study such dynamics on this time scale. Also the formation of secondary structure elements falls into the nanosecond to microsecond time range, and only limited kinetic data on these fundamental processes are available from relaxation experiments. For the same reasons, it is also difficult to address the role of nanosecond to microsecond fluctuations in the native state of proteins.

TTET, a spectroscopic technique probing formation of van der Waals contact between two specific amino acid side chain groups, gives access to this time regime. With this technique it has now become possible to study chain dynamics in unfolded peptide chains, as well as secondary structure formation and fluctuations in the native state of proteins on the tens of picosecond up to tens of microsecond time scale.

TTET Allows to Measure Contact Formation between Side Chain Groups

TTET between a triplet donor and acceptor group provides a method to measure contact formation between specific sites of a polypeptide chain [4,5]. Unlike FRET (singlet–singlet energy transfer), which is mediated by a dipolar coupling mechanism and occurs through space with a $1/r^6$ dependence [6], TTET requires the exchange of two electrons and is therefore restricted to small distances with significant orbital overlap of donor and acceptor (Dexter mechanism) [7,8].

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Abbreviations used: *FRET, Förster resonance energy transfer; GdmCl, guanidinium chloride; HP35, villin headpiece subdomain; k_c, rate constant of contact formation; $k_{f/u}$ rate constants of folding/unfolding; Nal, L-1-naphthylalanine; TTET, triplet–triplet energy transfer; Xan, amide of 9-oxoxanthene-2-carboxylic acid.*

Biography

Andreas Reiner grew up in Munich and studied Biochemistry at the University of Bayreuth, Germany. There he became attracted to protein biochemistry in his undergraduate work with Franz X. Schmid. For his graduate studies he then joined the lab of Thomas Kiefhaber at the Biozentrum Basel, Switzerland, with whom he moved to the Technische Universität München, Germany in 2008. His PhD work on protein folding, which also involved TTET measurements, focused on conformational dynamics in structured peptides and small proteins. Following his interests in the molecular, biophysical and functional properties of proteins he continues to study conformational transitions and protein dynamics. Currently, he works as postdoctoral researcher at the University of California, Berkeley, USA.

A triplet donor–acceptor pair well suited to measure TTET in aqueous solution is xanthone and naphthalene (Figure 1(A)). Both groups can be specifically incorporated during solid-phase peptide synthesis. We use a carboxylic acid derivative of xanthone (Xan) for attachment to an amino functionality, e.g. to the *N*-terminus or to the side chain of α,β-diaminopropionic acid. Naphthalene is incorporated as 1-naphthylalanine (Nal), which is structurally similar to tryptophan. The principle of TTET is depicted in Figure 1(A). A short laser pulse at 355 nm is used to excite Xan to its singlet state, from where it undergoes fast (~2 ps) and efficient (>95%) intersystem crossing (ISC) to the triplet manifold [9,10]. The triplet state is relatively long-living (~30 µs), since relaxation to the ground state is a spin forbidden process. Contact formation with the acceptor Nal leads to fast (~1 ps) and irreversible transfer of the triplet state, because the Nal triplet state is lower in energy. TTET can be directly followed by time-resolved absorption spectroscopy using the characteristic absorption bands of the Xan and Nal triplet states at 590 nm and 420 nm, respectively (Figure 1(B)).

Introducing Xan and Nal side chains at specific positions in polypeptides allows to directly follow intramolecular contact formation (or loop closure) between these sites. It is important to note that both triplet generation and the actual transfer step occur within picoseconds, which is much faster than the time the two side chains need to come close enough for TTET to occur. This means that forming a conformation with donor and acceptor in contact is the rate limiting step, i.e. the system is determined by intrachain diffusion. In other words, the observed time course of TTET gives the absolute rate constants of contact formation, k_c, without further assumptions. Other techniques to probe contact formation like triplet quenching are usually reaction-limited and do therefore not directly yield absolute rate constants. The available methods and early applications to unstructured model peptides have been reviewed in detail elsewhere [11].

Chain Dynamics in Unstructured Peptides

TTET measurements provided for the first time comprehensive data on the chain dynamics, i.e. large-amplitude fluctuations, in unstructured model peptides [4,5,11]. Theoretical predictions had suggested that contact formation (or loop closure) between two sites is a single exponential process, as long as only a minor fraction of molecules forms contact at a given time point, and as long as the equilibration between all conformations is fast compared to contact formation [12]. Experiments on poly(GlySer) and poly(Ser) peptides indeed showed that contact formation at ambient temperatures is a single exponential process occurring on the time scale of ten to hundreds of nanoseconds [4,5].

Contact formation between two sites depends on the length of the linking peptide segment [5]. For long chains contact formation between the ends of the chain obeys a scaling law in accordance with polymer theory. As predicted for Gaussian chains with excluded volume effects, the rate constant of contact formation, k_c, scales with $k_c \propto N^{-1.7}$, N being the number of peptide bonds [5,12]. In peptides too short to represent ideal chains, contact formation becomes length independent. Contact formation involving internal chain positions is additionally influenced by the size of the adjacent chain segments [13]. In the

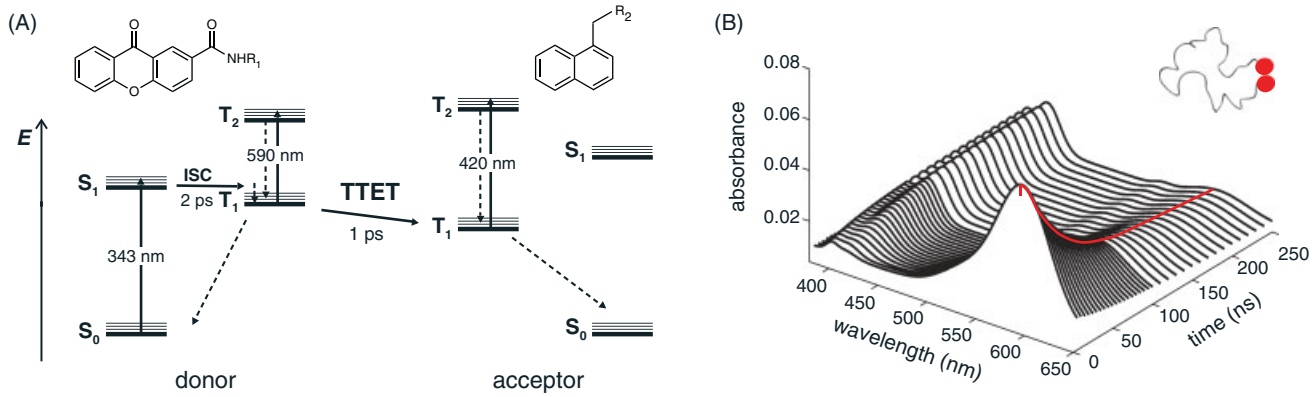

Figure 1. (A) TTET between xanthone (donor) and naphthalene (acceptor). Xan is excited to the singlet state S_1 from where it undergoes fast ISC to its triplet state T_1 (actually two states in Xan, $^3n\pi^*$ and $^3\pi\pi^*$, not shown) [9]. Van der Waals contact with Nal leads to fast and irreversible transfer of the triplet state. This process can be monitored by the absorption bands arising from the $T_1 - T_2$ transitions of Xan and Nal. Dashed arrows indicate slow internal conversion. (B) End-to-end contact formation between Xan and Nal in an unstructured $(GlySer)_{14}$ model peptide. The time-resolved absorption spectra show the Xan triplet state absorbance at 590 nm after excitation with a short laser pulse. Contact formation leads to TTET and a single exponential decay of the Xan triplet state (line), which is accompanied by a rise of the Nal triplet state absorbance at 420 nm. Panel (B) reprinted from Ref. 5, with permission from Elsevier.

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case of very large extensions this effect can reduce k_c down by a limiting factor of 2.5. Internal segments of a peptide chain are hence intrinsically less flexible than the ends of the chain. Furthermore, the effect of different solvents, such as solutions of the common denaturants urea and GdmCl, has been studied [14].

Besides the dynamics of poly(GlySer) and poly(Ser) peptides, the influence of different amino acid side chains has been investigated [5,15]. Host–guest studies revealed that glycine leads to significantly faster contact formation than all other amino acids, whereas proline gives double exponential contact formation kinetics. The faster phase could be assigned to peptides with a *cis* conformation of the amide bond preceding proline, causing a drastic restriction of the conformational space [15]. Proline in the *trans* conformation leads to significantly slower contact formation. Contact formation was also studied in some natural, but unstructured sequences, derived from carp parvalbumin or the GB1 hairpin [16]. Another study addressed fast contact formation in short loop sequences using femtosecond-laserflash spectroscopy, which revealed a hierarchy of motions [17]. There, in addition to the diffusional processes on the 10 ns time scale, a subfraction of peptides showed complex contact formation kinetics on the time scale of 50–500 ps, probably representing motions within a local well of the free energy landscape. Only in a small fraction of molecules the labels had formed contact during excitation. Further TTET measurements on model peptide chains will provide more details on how peptides can sample their conformational space and how fast single interactions can form that might be the first step of structure formation [3]. Of particular interest is also the question, how the chain dynamics relate to the dimensions of the sampled conformational space [18].

TTET Coupled to Conformational Equilibria

TTET, being a fast and irreversible probing reaction that reports on a structurally well-defined event, namely the contact formation between two groups, can also be utilized to study the dynamics of conformational transitions in equilibrium. The basic idea is depicted in Scheme 1. Some conformations, e.g. structured states (N), might separate the labels and place them at distant sites, thereby preventing contact formation. However, these conformations are in a dynamic equilibrium with more flexible states, e.g. locally or globally unfolded conformations (U) that allow contact formation and TTET (U*) with a characteristic rate constant k_c.

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} U \xrightarrow{k_c} U^*$$

Scheme 1. Contact formation coupled to a folding/unfolding equilibrium.

Linking an irreversible process, in this case contact formation, to a reversible transition is a general and powerful approach to study the thermodynamics and kinetics of chemical equilibria. Similarly, hydrogen/deuterium exchange is used to probe the opening and closing of individual hydrogen bonds in proteins [19–21]. However, choosing contact formation as probing reaction, which occurs on the ten to hundreds of nanosecond time scale, depending on the loop length, the amino acid composition and the positions within the chain (see above), allows to monitor dynamics that are 4–5 orders of magnitude faster than those accessible in hydrogen/deuterium exchange experiments.

The kinetic mechanism depicted in Scheme 1 encompasses three states. The observed time course of TTET, which reflects the formation of U*, depends on how fast the reactions proceed relative to each other. In extreme cases, one might encounter limiting regimes with virtually single exponential kinetics. If, for example, k_c is much faster than k_u and $k_u < k_f$ the observed rate constant λ will mainly reflect k_u, also known as the EX1 limit in hydrogen/deuterium exchange experiments. In the other extreme of the probing reaction, k_c, being slow compared to the pre-equilibrium ($k_c \ll k_u < k_f$), the apparent rate constant λ equals $(k_u/k_f) \times k_c$, which might allow to extract the equilibrium constant $K = k_u/k_f$ (EX2 limit). However, if the rate constants are within a similar range, double exponential kinetics will be observed. In this favorable case, the analytic solution of the three-state mechanism in Scheme 1 can be used to calculate all three microscopic rate constants, k_u, k_f and k_c, from the measured rate constants λ_1 and λ_2 and their relative amplitudes [22]. We successfully applied this approach to measure the kinetics of local folding and unfolding in α-helical peptides, which gave new insight into the equilibrium dynamics of this important secondary structure element.

Local Folding and Unfolding in α-Helical Peptides

α-Helices can form in the absence of tertiary interactions, which explains why they can be observed in relatively short peptides, as well as folding intermediates. Isolated α-helices are only marginally stable structures, which are weakly cooperative and highly dynamic. Their thermodynamic properties have been studied in detail and are well described by formalisms, which were originally derived for the helix–coil transition in long homopolymers [23,24]. However, little is known about the kinetics of helix formation. Only few methods, usually probing rather global processes, provide the necessary time resolution. Moreover, relaxation kinetics, e.g. obtained with temperature jump techniques [25–28], are difficult to interpret when multiple states are present [29].

We set out to use TTET to study local helix folding and unfolding in α-helical peptides. For this, we chose peptides based on alanine, which are devoid of complex side chain interactions, but form relatively stable helices in water, as discovered by Baldwin and coworkers [30–32]. Arginine residues are included at every fifth position to prevent oligomerization, and the *N*- and *C*-termini are acetylated and amidated, respectively, to avoid unfavorable electrostatic interactions with the helix dipole (Figure 2(A)). To probe the local dynamics along the helix, we prepared a series of 21 amino acid peptides carrying triplet donor and acceptor groups at different positions [22]. An i, $i+6$ spacing of the labels places Xan and Nal on opposite sides of the helix, thereby preventing contact formation as long as the helix is folded (Figure 2B)). Upon local or global unfolding with k_u the labels gain enough freedom to form contact with k_c, or, alternatively, the helix can refold with k_f. Circular dichroism spectroscopy confirmed that all labeled peptides formed α-helices with an average helix content of ~65% [22].

The TTET measurements revealed different kinetics at all positions, indicating that helix folding/unfolding is not a fully cooperative two-state process, but involves many conformations. All kinetics were double exponential, which is expected for the three-state mechanism depicted in Scheme 1 and which allowed

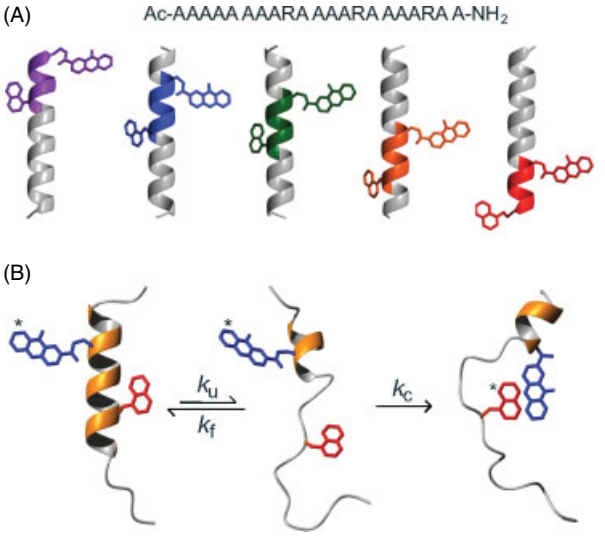

Figure 2. Probing local dynamics and stability in α-helical peptides. (A) Series of alanine-based peptides carrying i, $i + 6$ spaced donor and acceptor groups. (B) Local (or global) helix unfolding (k_u) can be followed by contact formation (k_c), or refolding of the helical structure (k_f). Adapted from Ref. 22.

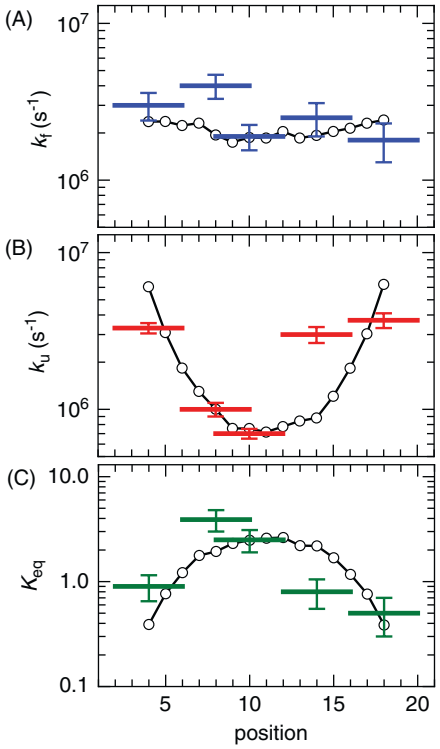

Figure 3. (A) Rate constants for local helix folding, k_f, and (B) unfolding, k_u, obtained from TTET measurements. (C) Local helix stability given as equilibrium constant $K_{eq} = k_f/k_u$. The bars represent the experimental data from the peptides shown in Figure 2(A). The connected points results from the simulations with a kinetic Ising model. Adapted from Ref. 22.

us to determine the microscopic rate constants of helix unfolding, k_u, folding, k_f, and contact formation, k_c, without any further assumptions. Additional data were obtained by shifting the helix–coil equilibrium with urea, which destabilizes the helical state [33]. The experiments showed that contact formation ($1/k_c$) occurs on the time scale of ~100 ns (at 5 °C), similar to contact formation in unstructured peptides [5,13]. Local helix formation ($1/k_f$) shows no systematic position dependence and is similarly fast at all positions (~400 ns) (Figure 3). In contrast, helix unfolding is fast at the termini (~250 ns) and slow in the helix center (~1.4 µs).

Having determined k_f and k_u, we can also calculate the local helix stabilities. The differences in the kinetics of helix unfolding lead to a smaller amount of helical structure toward the ends (Figure 3(C)). This phenomenon, also known as helix fraying, is predicted by helix–coil theories and has been observed with various other techniques probing helix stability [32,34–38].

What does our main finding, namely, that local helix folding proceeds similarly fast at all positions, but helix unfolding gets faster toward the termini, implicate for the mechanism of helix formation? To understand the kinetically complex behavior of α-helical peptides, we performed Monte-Carlo simulations with an Ising model that gives a simple, but adequate description of this multi-state system [22,29]. These simulations, parameterized with well-established thermodynamic data, reproduce the observed position dependence (Figure 3), and suggest that the kinetics are dominated by a diffusion-of-boundary mechanism. In peptides of this length, folding and unfolding mainly occur at the ends of a single helical structure, whereas internal breaks only play a minor role. By quantitative comparison we can further estimate that elongation of an existing helix by one residue occurs with ~50 ns. In summary, coupling TTET to a conformational equilibrium allowed us to measure local dynamics on the nanosecond to microsecond time scale and gave unprecedented insight into the dynamics of α-helix formation.

Fluctuations in the Native and Unfolded State of a Small Protein

Contact formation between side chains can also be used to study the dynamics in proteins. Like in peptides, TTET allows to measure contact formation in the unfolded state and to see, whether residual structure is present. In addition, it gives the possibility to capture conformational fluctuations in the native state ensemble. One advantage is that the dynamics in the folded, native state (N) and the unfolded state (U) can be probed separately, if global unfolding is too slow to occur within the triplet lifetime (Scheme 2). In particular, this gives access to the contact formation dynamics (k_c) in the unfolded state even when it is only slightly populated (≥5%), i.e. under conditions that highly favor the native state. However, TTET in the native fraction of molecules can be used to detect dynamics in the native state ensemble.

$$N^* \longleftarrow N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} U \xrightarrow{k_c} U^*$$

slow

Scheme 2. Slow folding/unfolding separates contact formation processes occurring in N and U.

We used TTET to probe the dynamics in the villin headpiece subdomain (HP35). This naturally occurring subdomain consists of 35 amino acids and folds into a well-defined structure with three short helices packed around a hydrophobic core [39–41]. Its structure, stability and folding have been studied by various

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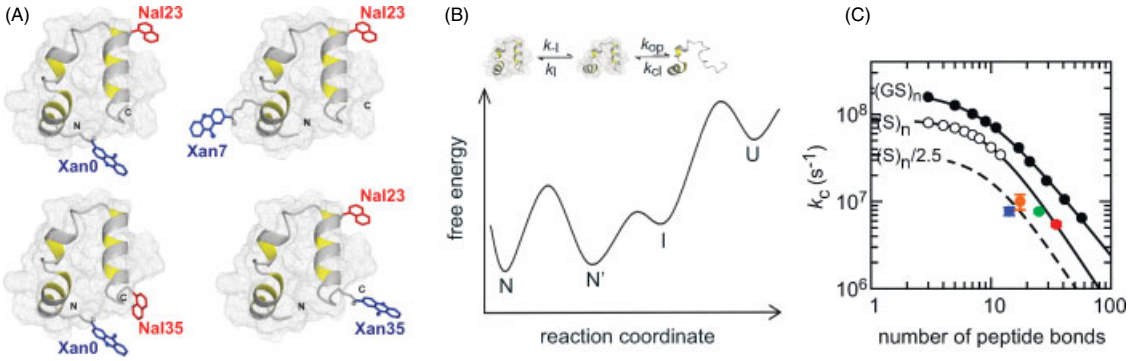

Figure 4. TTET experiments in HP35. (A) Variants with different donor and acceptor positions. (B) TTET experiments indicate that the native state N is in a reversible locking/unlocking equilibrium with a somewhat loosened state N′, from which the *C*-terminal helix can detach and unfold to give an intermediate I. (C) Comparison of contact formation measured in unfolded HP35 (Xan0–Nal23, green; Xan7–Nal23, orange; Xan0–Nal35, red; Nal23–Xan35, blue) with end-to-end contact formation in poly(GlySer) and poly(Ser) peptides [5]. Additional tails can slow down k_c maximally 2.5-fold, as indicated by the dashed line [13]. Adapted from Ref. 51.

experimental approaches, e.g. [42–47], and due to its small size and fast folding on the microsecond time scale it became an important model system for computational studies [48–50]. To probe various dynamic aspects, we prepared HP35 variants with triplet donor and acceptor groups at four different positions using Fmoc solid-phase peptide synthesis (Figure 4(A)). Circular dichroism and NMR spectroscopy confirmed that all variants fold into the native state with similar thermodynamic stabilities as wild type HP35.

As expected, no TTET was observed for the native state population in the variants Xan0–Nal23 and Xan7–Nal23 [51]. In both variants the labels are well separated from each other in the folded state (Figure 4(A)) and global (or local) unfolding events do not occur within the Xan triplet lifetime (Scheme 2). Hence TTET only occurs in the fraction of unfolded HP35, which is increased by adding GdmCl, and the GdmCl-induced equilibrium unfolding transition monitored by TTET agrees well with unfolding probed by circular dichroism spectroscopy.

In contrast, in folded Xan0–Nal35 the labels are close to each other and the major fraction of the native molecules (70%) forms contact within the 10 ns dead time of the experiment [51]. This is in agreement with a process that involves only a few bond rotations to establish contact, which typically occurs on the subnanosecond time scale [17]. A minor fraction of the native molecules (30%), however, forms contact with a time constant of ~30 ns, revealing a second conformation in which the labels are further apart. Evidence for heterogeneity in the native state came also from the variant Nal23–Xan35, which we had prepared to probe the dynamics along the *C*-terminal helix (Figure 4(A)). The labels should remain separated if this region is well folded, but efficient contact formation was observed. TTET in the native fraction of Nal23–Xan35 molecules showed two kinetic phases with similar amplitudes as the heterogeneity detected in Xan0–Nal35 [51]. The minor fast contact formation reaction (20%) with a time constant of ~170 ns is strongly accelerated by the addition of GdmCl. This behavior indicates that a considerable amount of surface gets exposed, a process, which is favored by higher denaturant concentrations. Quantitative estimates show that the strong denaturant dependence can only be explained by detachment of the *C*-terminal helix from the hydrophobic core and subsequent unfolding. The major contact formation process (80%) in Nal23–Xan35 occurs with a time constant of ~1 μs and most likely reflects a weakening or unlocking of the native state, preceding local unfolding. Indeed, this step does not involve a significant change in accessible surface area but further experiments showed that it encounters a high enthalpic barrier. These data clearly demonstrate the presence of alternative conformations in native HP35 (Figure 4(B)). Reversible locking/unlocking leads to a state resembling the properties of a dry molten globule state [52], from which local unfolding might occur.

The experiments also yielded a wealth of information on the dynamics in unfolded HP35 [51]. In all variants and under all conditions contact formation in the unfolded state was found to be a single exponential process that occurs with comparable rate constants k_c as contact formation in unfolded model peptides (Figure 4(C)). Denaturants exert a similarly strong effect on the dynamics, as in unstructured poly(Ser) peptides [14]. Even at low denaturant concentrations, in which the native state is highly favored and transient structures might form, the relationships between ln(k_c) and denaturant concentration remain linear [14]. Hence, this study gives no evidence for a structural organization in the unfolded state of HP35, which would likely affect the measured chain dynamics. Previously, the presence of residual structure had been discussed as an explanation for the fast folding of this protein domain. Furthermore, the first absolute rate constants determined for contact formation in the unfolded state of a protein are valuable data for direct comparisons with molecular dynamic simulations.

Summary

TTET is a method that reports on van der Waals contact formation between a triplet donor and acceptor group on the picosecond to tens of microsecond time scale. It allows to directly measure how fast two sites on a peptide chain can form contact, which has enabled detailed studies of the chain dynamics in unstructured model peptides. Moreover, TTET experiments have been used to measure conformational dynamics in structured peptides and in a small protein domain. We coupled contact formation to local folding and unfolding equilibria in α-helical peptides, which gave rate constants for these processes and new insight into the mechanism of how this elementary secondary structure forms. In another study, we used TTET to characterize the chain dynamics in an unfolded protein, HP35, and detected fluctuations in its native state, which can be described as a reversible locking/unlocking reaction. These studies demonstrate the enormous potential of TTET measurements to study dynamics that have been difficult

to measure with other biophysical techniques. This makes TTET a useful tool to probe and understand the dynamics that direct folding in peptides and proteins, or are important for their function.

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References

1 Seewald N, Jakubke H.-D. *Peptides: Chemistry and Biology*. Wiley-VCH: Weinheim, 2009.
2 Creighton TE. *Proteins: Structures and Molecular Properties*. Freeman, W.H.: New York, 1992.
3 Bieri O, Kiefhaber T. Elementary steps in protein folding. *Biol. Chem.* 1999; **380**: 923–929.
4 Bieri O, Wirz J, Hellrung B, Schutkowski M, Drewello M, Kiefhaber T. The speed limit for protein folding measured by triplet–triplet energy transfer. *Proc. Natl. Acad. Sci. U.S.A.* 1999; **96**: 9597–9601.
5 Krieger F, Fierz B, Bieri O, Drewello M, Kiefhaber T. Dynamics of unfolded polypeptide chains as model for the earliest steps in protein folding. *J. Mol. Biol.* 2003; **332**: 265–274.
6 Förster T. Zwischenmolekulare Energiewanderung und Fluoreszenz. *Ann. Phys.* 1948; **2**: 55–75.
7 Dexter DL. A theory of sensitized luminescence in solids. *J. Chem. Phys.* 1953; **21**: 836–850.
8 Klán P, Wirz J. *Photochemistry of Organic Compounds: From Concepts to Practice*. Wiley-Blackwell: Chichester, 2009.
9 Heinz B, Schmidt B, Root C, Satzger H, Milota F, Fierz B, Kiefhaber T, Zinth W, Gilch P. On the unusual fluorescence properties of xanthone in water. *Phys. Chem. Chem. Phys.* 2006; **8**: 3432–3439.
10 Satzger H, Schmidt B, Root C, Zinth W, Fierz B, Krieger F, Kiefhaber T, Gilch P. Ultrafast quenching of the xanthone triplet by energy transfer: new insight into the intersystem crossing kinetics. *J. Phys. Chem. A* 2004; **108**: 10072–10079.
11 Fierz B, Kiefhaber T. Dynamics of unfolded polypeptide chains. In *Protein Folding Handbook*, Buchner J, Kiefhaber T (eds). Wiley: Weinheim, 2005; 805–851.
12 Szabo A, Schulten K, Schulten Z. First passage time approach to diffusion controlled reactions. *J. Chem. Phys.* 1980; **72**: 4350–4357.
13 Fierz B, Kiefhaber T. End-to-end *vs.* interior loop formation kinetics in unfolded polypeptide chains. *J. Am. Chem. Soc.* 2007; **129**: 672–679.
14 Möglich A, Krieger F, Kiefhaber T. Molecular basis for the effect of urea and guanidinium chloride on the dynamics of unfolded polypeptide chains. *J. Mol. Biol.* 2005; **345**: 153–162.
15 Krieger F, Möglich A, Kiefhaber T. Effect of proline and glycine residues on dynamics and barriers of loop formation in polypeptide chains. *J. Am. Chem. Soc.* 2005; **127**: 3346–3352.
16 Krieger F, Fierz B, Axthelm F, Joder K, Meyer D, Kiefhaber T. Intrachain diffusion in a protein loop fragment from carp parvalbumin. *Chem. Phys.* 2004; **307**: 209–215.
17 Fierz B, Satzger H, Root C, Gilch P, Zinth W, Kiefhaber T. Loop formation in unfolded polypeptide chains on the picoseconds to microseconds time scale. *Proc. Natl. Acad. Sci. U.S.A.* 2007; **104**: 2163–2168.
18 Möglich A, Joder K, Kiefhaber T. End-to-end distance distributions and intrachain diffusion constants in unfolded polypeptide chains indicate intramolecular hydrogen bond formation. *Proc. Natl. Acad. Sci. U.S.A.* 2006; **103**: 12394–12399.
19 Schellman JA. The stability of hydrogen-bonded peptide structures in aqueous solution. *C.R. Trav. Lab Carlsberg Ser. Chim.* 1955; **29**: 230–259.
20 Linderstrøm-Lang K. Deuterium exchange between peptides and water. *Chem. Soc. Spec. Publ.* 1955; **2**: 1–20.
21 Hvidt A, Nielsen SO. Hydrogen exchange in proteins. *Adv. Protein Chem.* 1966; **21**: 287–386.
22 Fierz B, Reiner A, Kiefhaber T. Local conformational dynamics in α-helices measured by fast triplet transfer. *Proc. Natl. Acad. Sci. U.S.A.* 2009; **106**: 1057–1062.
23 Zimm BH, Bragg JK. Theory of the phase transition between helix and random coil in polypeptide chains. *J. Chem. Phys.* 1959; **31**: 526–535.
24 Lifson S, Roig A. On the theory of helix-coil transition in polypeptides. *J. Chem. Phys.* 1961; **34**: 1963–1974.
25 Williams S, Causgrove TP, Gilmanshin R, Fang KS, Callender RH, Woodruff WH, Dyer RB. Fast events in protein folding: helix melting and formation in a small peptide. *Biochemistry* 1996; **35**: 691–697.
26 Thompson PA, Eaton WA, Hofrichter J. Laser temperature jump study of the helix-coil kinetics of an alanine peptide interpreted with a 'kinetic zipper' model. *Biochemistry* 1997; **36**: 9200–9210.
27 Huang CY, Getahun Z, Zhu Y, Klemke JW, DeGrado WF, Gai F. Helix formation via conformation diffusion search. *Proc. Natl. Acad. Sci. U.S.A.* 2002; **99**: 2788–2793.
28 Mikhonin AV, Asher SA, Bykov SV, Murza A. UV Raman spatially resolved melting dynamics of isotopically labeled polyalanyl peptide: slow α-helix melting follows 3_{10}-helices and π-bulges premelting. *J. Phys. Chem. B* 2007; **111**: 3280–3292.
29 Schwarz G. On the kinetics of the helix-coil transition of polypeptides in solution. *J. Mol. Biol.* 1965; **11**: 64–77.
30 Marqusee S, Robbins VH, Baldwin RL. Unusually stable helix formation in short alanine-based peptides. *Proc. Natl. Acad. Sci. U.S.A.* 1989; **86**: 5286–5290.
31 Scholtz JM, Qian H, York EJ, Stewart JM, Baldwin RL. Parameters of helix-coil transition theory for alanine-based peptides of varying chain lengths in water. *Biopolymers* 1991; **31**: 1463–1470.
32 Doig AJ, Errington N, Iqbalsyah TM. Stability and design of α-helices. In *Protein Folding Handbook*, Buchner J, Kiefhaber T (eds). Wiley-VCH: Weinheim, 2005; 247–313.
33 Scholtz JM, Barrick D, York EJ, Stewart JM, Baldwin RL. Urea unfolding of peptide helices as a model for interpreting protein unfolding. *Proc. Natl. Acad. Sci. U.S.A.* 1995; **92**: 185–189.
34 Chakrabartty A, Schellman JA, Baldwin RL. Large differences in the helix propensities of alanine and glycine. *Nature* 1991; **351**: 586–588.
35 Rohl CA, Baldwin RL. Exchange kinetics of individual amide protons in 15N-labeled helical peptides measured by isotope-edited NMR. *Biochemistry* 1994; **33**: 7760–7767.
36 Reiner A, Wildemann D, Fischer G, Kiefhaber T. Effect of thioxopeptide bonds on α-helix structure and stability. *J. Am. Chem. Soc.* 2008; **130**: 8079–8084.
37 Miick SM, Casteel KM, Millhauser GL. Experimental molecular dynamics of an alanine-based helical peptide determined by spin label electron spin resonance. *Biochemistry* 1993; **32**: 8014–8021.
38 Jaravine VA, Alexandrescu AT, Grzesiek S. Observation of the closing of individual hydrogen bonds during TFE-induced helix formation in a peptide. *Protein Sci.* 2001; **10**: 943–950.
39 McKnight CJ, Doering DS, Matsudaira PT, Kim PS. A thermostable 35-residue subdomain within villin headpiece. *J. Mol. Biol.* 1996; **260**: 126–134.
40 McKnight CJ, Matsudaira PT, Kim PS. NMR structure of the 35-residue villin headpiece subdomain. *Nat. Struct. Biol.* 1997; **4**: 180–184.
41 Chiu TK, Kubelka J, Herbst-Irmer R, Eaton WA, Hofrichter J, Davies DR. High-resolution X-ray crystal structures of the villin headpiece subdomain, an ultrafast folding protein. *Proc. Natl. Acad. Sci. U.S.A.* 2005; **102**: 7517–7522.
42 Kubelka J, Eaton WA, Hofrichter J. Experimental tests of villin subdomain folding simulations. *J. Mol. Biol.* 2003; **329**: 625–630.
43 Xiao S, Bi Y, Shan B, Raleigh DP. Analysis of core packing in a cooperatively folded miniature protein: the ultrafast folding villin headpiece helical subdomain. *Biochemistry* 2009; **48**: 4607–4616.
44 Kubelka J, Henry ER, Cellmer T, Hofrichter J, Eaton WA. Chemical, physical, and theoretical kinetics of an ultrafast folding protein. *Proc. Natl. Acad. Sci. U.S.A.* 2008; **105**: 18655–18662.

45 Wang M, Tang Y, Sato S, Vugmeyster L, McKnight CJ, Raleigh DP. Dynamic NMR line-shape analysis demonstrates that the villin headpiece subdomain folds on the microsecond time scale. *J. Am. Chem. Soc.* 2003; **125**: 6032–6033.
46 Brewer SH, Vu DM, Tang Y, Li Y, Franzen S, Raleigh DP, Dyer RB. Effect of modulating unfolded state structure on the folding kinetics of the villin headpiece subdomain. *Proc. Natl. Acad. Sci. U.S.A.* 2005; **102**: 16662–16667.
47 Buscaglia M, Kubelka J, Eaton WA, Hofrichter J. Determination of ultrafast protein folding rates from loop formation dynamics. *J. Mol. Biol.* 2005; **347**: 657–664.
48 Duan Y, Kollman PA. Pathways to a protein folding intermediate observed in a 1-microsecond simulation in aqueous solution. *Science* 1998; **282**: 740–744.
49 Ensign DL, Kasson PM, Pande VS. Heterogeneity even at the speed limit of folding: large-scale molecular dynamics study of a fast-folding variant of the villin headpiece. *J. Mol. Biol.* 2007; **374**: 806–816.
50 Lei H, Wu C, Liu H, Duan Y. Folding free-energy landscape of villin headpiece subdomain from molecular dynamics simulations. *Proc. Natl. Acad. Sci. U.S.A.* 2007; **104**: 4925–4930.
51 Reiner A, Henklein P, Kiefhaber T. An unlocking/relocking barrier in conformational fluctuations of villin headpiece subdomain. *Proc. Natl. Acad. Sci. U.S.A.* 2010; **107**: 4955–4960.
52 Baldwin RL, Frieden C, Rose GD. Dry molten globule intermediates and the mechanism of protein unfolding. *Proteins* 2010; **78**: 2725–2737.

