Ultrafast Dynamics

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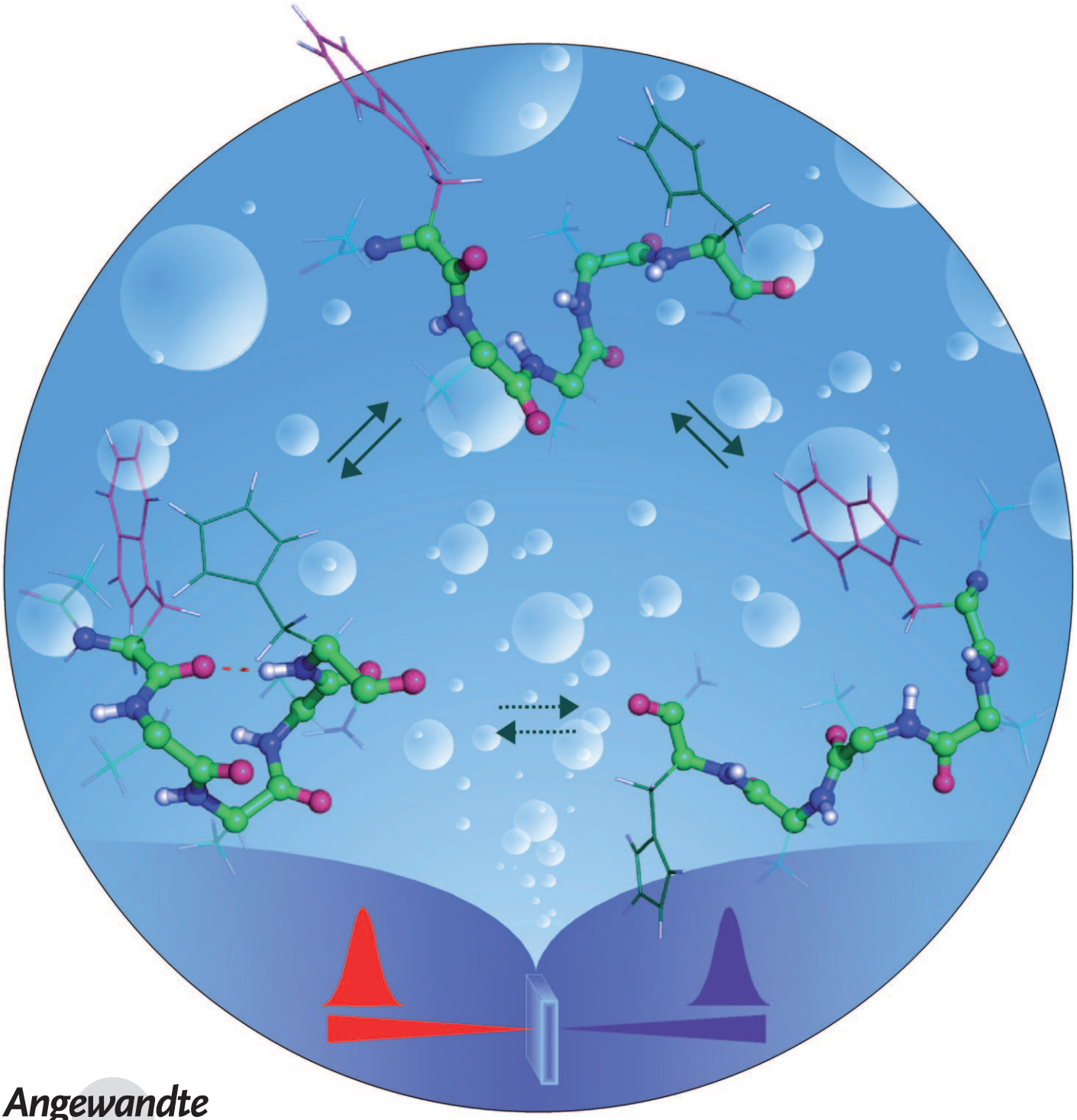
Primary Peptide Folding Dynamics Observed with Ultrafast Temperature Jump**

*Omar F. Mohammed, Gouri S. Jas, Milo M. Lin, and Ahmed H. Zewail**



The folding dynamics of proteins and polypeptides are complex and involve different time and length scales.[1–4] Among secondary structural elements, the α-helix is the most common configuration, with its stability resulting from unique hydrogen bonding; the C=O group of an amino acid at the position *i* forms a hydrogen bond with the N–H group of another amino acid located at the position $i+4$. The thermodynamic properties of the α-helix are understood in the context of the helix–coil transition, but the dynamics, which involve many steps, have a whole range of time scales. The folding rates have been determined using a variety of experimental methods (for recent reviews, see refs. [5–7]), including absorption, NMR, Raman, infrared spectroscopy and circular dichroism (CD). The helix–coil transition was believed to occur on the microsecond timescale, and only recently, by means of fluorescence detection, were the rates measured to be as short as 300 ns,[8–10] prompting the association of the 300 ns results with "ultrafast" dynamics.[9] Theoretical models of helix–coil (polymer-type) transitions and molecular dynamics (MD) simulations (see below) have also provided a range of time scales. For example, using Zimm–Bragg nucleation and elongation parameters,[12] Schwarz estimated the "relaxation time" to be 0.1 μs,[11] whereas the time scale in MD simulations of folding (subnanosecond to microsecond) depends on the length and sequence of the peptide. To resolve the primary processes of folding, the dynamics have to be observed with the shortest time resolution possible.

Herein, we report the ultrafast folding dynamics of the α-helix with time resolution three orders of magnitude shorter than previously reported with fluorescence detection. With the ultrafast temperature-jump (*T*-jump) method,[13] the *in situ* heating time (3.5 ps) is determined by the duration of water relaxation for a solution of the peptide in pure water, as described below. Earlier, a *T*-jump with time resolution of 70 ps was used to study proteins by heating through dye molecules in the solution.[14] Herein, the heating is directly through the water vibrations on the 3.5 ps time scale. We studied the α-helical alanine-based pentapeptide Ac-W-$(A)_3$-H^+-NH_2 (Wh5) in an acetate buffer at pH 4.8. Other alanine-based peptides with varying lengths and compositions and at varying temperatures were also studied (see the Supporting Information, Figure S2), but the focus here is on the small peptide with five residues, as it represents the fundamental nucleus for helix formation without involvement of the elongation process. We also performed all-atom MD simulations on the same system to compare with the experimental results.

The presence of an α-helix for Wh5 (with three alanine residues, as they have the highest propensity to form a helix)[15–17] is evidenced by CD (see below) and NMR spectroscopy studies.[9,18] The peptide was designed to have a single tryptophan residue in position one and a single protonated histidine residue in position five. Tryptophan serves as a fluorescence probe for the local and global structural changes, as its fluorescence is strongly quenched by the nearby protonated histidine in the folded state and is recovered in the unfolded state.[15] Moreover, the spatial interaction between tryptophan and nearby protonated histidine contributes to the stability of this helical structure.[15,16] Figure 1 provides the conformations and chemical sequences of the folded and extended structures of the five- and 21-residue peptides Wh5 and Wh21. These peptides were obtained from California Peptide, CA with greater than 98% purity and characterized as discussed below; all measurements were made in 20 mM acetate buffer at pH 4.8.

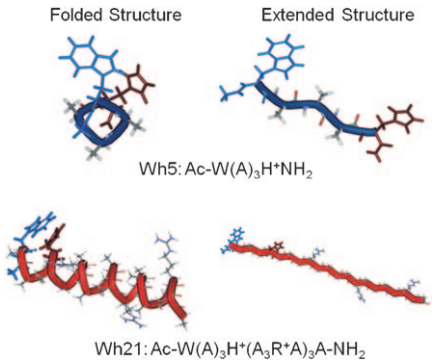


Figure 1. Folded and extended structures of Wh5 and Wh21 and their amino acid sequences. Tryptophan (light blue) and histidine (dark brown) residues are located at the N and C termini of Wh5. Tryptophan and histidine in Wh21 are located at the first and fifth positions, respectively.

Figure 2 displays the time resolution achieved in the *T*-jump together with typical results for characterization of the helical structure using far-UV circular dichroism; also included is the thermal denaturation curve as a function of temperature. Figure 2A shows the observed fluorescence change of free tryptophan in water during the *T*-jump (296→308 K). The measured data are the circles and the fit to the data is the solid line, giving the overall relaxation time of tryptophan in aqueous solution to be (3.2 ± 0.3) ps. The solvation time of tryptophan is known to be approximately 1 ps.[19,20] Because tryptophan is heated by the *T*-jump through absorption of the overtone of the OH stretching vibration of

[*] O. F. Mohammed, M. M. Lin, A. H. Zewail
Physical Biology Center for Ultrafast Science and Technology
Arthur Amos Noyes Laboratory of Chemical Physics
California Institute of Technology, Pasadena, CA 91125 (USA)
Fax: (+1) 626-792-8456
E-mail: zewail@caltech.edu
G. S. Jas
Department of Chemistry, Biochemistry, and Institute of Biomedical Studies, Room: C322 Baylor University
101 Bagby Avenue, Waco, TX 76706 (USA)

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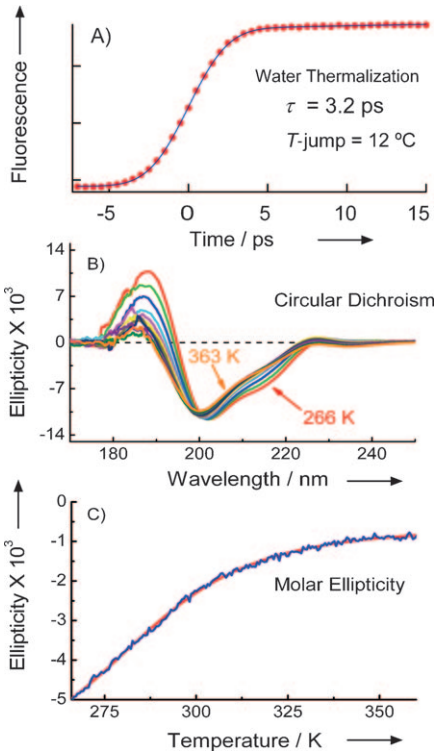

Figure 2. Time resolution of the water *T*-jump and helicity curves of Wh5. A) Time dependence of the fluorescence intensity change of free tryptophan in water observed during the *T*-jump from 296 to 308 K. The probe is an ultrafast UV (280 nm) pulse, and the initial heating is through the excitation of the overtone of the OH stretching vibration of water with a near-IR ultrafast pulse centered at 1.45 μm. This process induces a 10–15 °C temperature jump with a time constant (3.2 ± 0.3) ps. B) Far-UV CD spectra (molar ellipticity/deg $cm^2\,dmol^{-1}$) of Wh5 (170–250 nm) as a function of temperature. C) Temperature denaturation curve (measured at 220 nm) of Wh5 as a function of temperature.

water, the hydrogen-bond dynamics, which occur in about 5 ps,[21,22] control the energy transfer process and are therefore consistent with the observed 3.2 ps response. Figure 2B,C provides the CD spectra and melting curve for the five-residue peptide.

The far-UV CD spectra of Wh5 shows the characteristic signature of an α-helical structure by the presence of double minima around 220 and 210 nm. The decrease in molar ellipticity as a function of temperature (266 to 363 K) indicates that the transition from α-helical to random-coil conformations extends over tens of degrees. As in other studies in the literature, the evidence for the helix formation is clear from the appearance of the band near 220 nm. We have examined the CD spectra of both the five- and 21-residue peptides (Figure 2B and Figure S1 in the Supporting Information), and our spectra are in perfect accord with those reported for short peptides and proteins.[23,24] The fraction of helical structure was obtained from the melting temperature and characteristic spectral features of the folded and unfolded species at equilibrium. From the reported CD, the helix content at 310 K is at least 15% and increases at lower temperatures; more details will be provided in our full account of this work. The stability of the α-helical structure is consistent with NMR spectroscopy results[18] and with the study of small polypeptides containing alanine.[25,26]

The transients describing the folding dynamics of Wh5 are presented in Figure 3. Triggering the peptide system with the ultrafast infrared heating pulse at 1.45 μm shifts the equilibrium toward the unfolded transient structures. The refolding

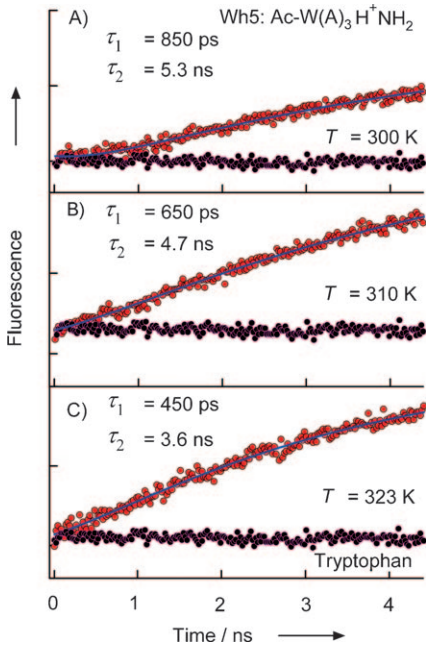

Figure 3. Transient evolution of peptide after *T*-jump. The tryptophan fluorescence of Wh5 (20 mM acetate buffer at pH 4.8) is shown in response to the *T*-jump for final temperatures of A) 300, B) 310, and C) 323 K over a time window from −50 ps to 4.8 ns. Note that the fluorescence signals are shown with negative amplitude in arbitrary units. Tryptophan was excited using an ultrafast UV pulse at 280 nm, and the fluorescence was monitored in the window of 310–500 nm. The circles represent experimental data and the solid curves are the best fits with a double-exponential function. For calibration, the experiments were repeated using free tryptophan in aqueous solution under identical conditions and the obtained data (reference) are the unchanging response shown for each transient.

is then followed in time by monitoring the tryptophan quenching fluorescence. Figure 3A–C gives the temporal behavior observed for the Wh5 peptide after the *T*-jump over the time range of −50 ps to 4.8 ns and for several final temperatures. At all temperatures, the experimental data were fitted to two exponential decays, and the corresponding time constants were $\tau_1 = (850 \pm 300)$ ps and $\tau_2 = (5.3 \pm 1.9)$ ns at the final temperature of 300 K. At higher temperatures, the rates increase (Figure 3): $\tau_1 = (650 \pm 250)$ ps and $\tau_2 = (4.7 \pm 0.6)$ ns at 310 K and $\tau_1 = (450 \pm 150)$ ps and $\tau_2 = (3.6 \pm 0.5)$ ns at 323 K. The fast component contributes about 10–20% depending on the final temperature, and the error in τ_2 is large because of the short time window (4.8 ns). We also performed the experiments at higher temperature (330 K) to identify the asymptotic level of the recovery (Figure S3 in the Supporting Information), and thus determined that the quoted errors are reasonable. The other peptides (four, six, and 21 residues) were similarly studied.

The experimental observations indicate the existence of ultrafast dynamics, the fastest relaxation time observed to date in the folding–unfolding process. The rate of nucleation, stabilized by a single i, $i+4$ hydrogen bond without elongation, in the smallest possible α-helical polypeptide depends on temperature. It changes from 450 ps at 323 K to 850 ps at 300 K, and, similarly, the long decay component shortens as the temperature increases (from 5 to 3 ns). Of significance is the fact that, as the temperature rises, the final amplitude change increases (Figure 3), while the amplitude of the fast component decreases. We note that the CD spectrum indicates that the peptide forms the helix at equilibrium or at long times.

The folding dynamics involve a landscape of different paths. The peptide may search for the correct hydrogen-bonded conformation through intermediate collapsed structures,[27] and different trajectories are involved: those beginning from the unfolded peptides and forming intermediate collapsed structures prior to the helical structure, and those which directly form the helical structure from collapsed structures at shorter length and time scales. Such bifurcation is a general feature of complex molecular reactions with different pathways.[28] To confirm this view, we repeated these experiments for a system of 21 residues which, besides nucleation, also undergoes the helix elongation process. Indeed, when the 21-residue peptide was partially denatured to have, on average, the Wh5 length characteristics, its rates resemble those of Wh5. However, and just as importantly, the rates in buffer solution for the 21-residue peptide are still similar ($\tau \approx 7$ ns) for the subensemble of polypeptides probed in the time window of 4.8 ns and are clearly two orders of magnitude shorter than the 300 ns reported earlier.[9] We note that quenching of the fluorescence may be caused by side-chain motions. However, the disparity in time constants in the experiments with 21- and five-residue peptides (despite the fact that the locations of the tryptophan and histidine residues are the same in both peptides), together with the MD results, do not support this possibility.

A simple theoretical model involving the rotation of peptide bonds for the transformation from helical to non-helical basins of conformation space (as defined by the Ramachandran plot) predicts folding time constants of about 2 ns at room temperature. This time constant was obtained from knowledge of the water viscosity at different temperatures and the effective volume of the peptide. The results, which are consistent with the measured τ_2 values, indicate that the diffusive motion accounts for the rate-limiting step, as τ_1 is much smaller than τ_2. Accordingly, the helix nucleation step is an entropically driven process. However, understanding the complete process, including the enthalpic contribution to helix formation, requires an atomic-scale analysis rather than coarse graining.

We carried out MD simulations of the Wh5 peptide in explicit water with periodic boundary conditions at 311 K, using the CHARMM program (for details, see the Supporting Information).[29] During the simulations, a folding event is deemed to occur when the peptide comes within 0.5 Å root-mean-squared deviation (RMSD) of the native helical structure for a period of four consecutive picoseconds. The results are presented in Figure 4 for 100 trajectories of 50 ns each and for a total simulation time of 5 μs. The percent helicity over the entire trajectory was 20%, consistent with the CD results at the same temperature (310 K). It is found that folding involves a range of rates, but the time scale has two types: fast annealing to the native structure and slow diffusion to a collapsed structure. This situation is illustrated by the graph of the maximum RMSD achieved during folding versus folding time (Figure 4), which shows that the fastest folding events do not reach a completely unfolded structure. Significantly, the frequency histogram of number of folding events versus folding time shows biexponential behavior, and the timescales are in good agreement with those obtained experimentally at the same temperature (5 ns and 370 ps at 310 K). The MD simulations are consistent with those for

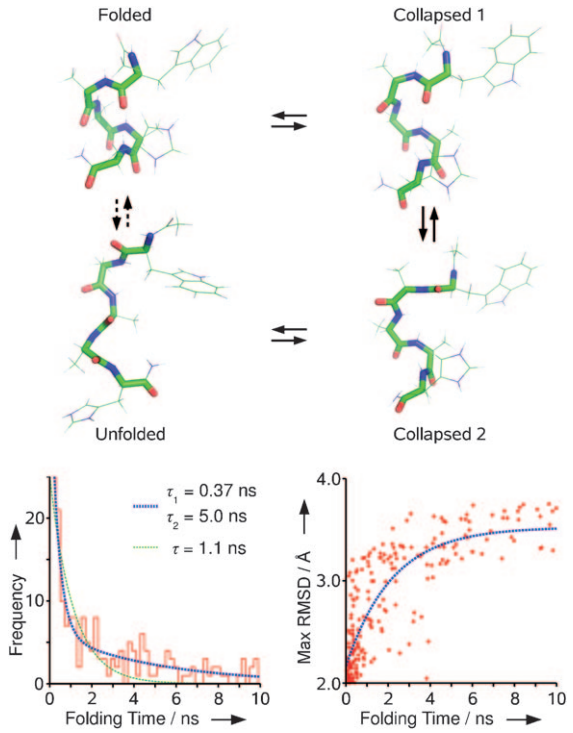

Figure 4. Folding statistics from molecular dynamics trajectories. One hundred separate explicit-solvent MD trajectories of 50 ns each were carried out at 311 K and 1 atm pressure. Top: Snapshots taken from the MD simulations depicting reversible folding and unfolding between the helical, collapsed, and unfolded structures. Many of the fast refolding events involve first the breaking of hydrogen bonds and small deformations of the helical backbone with subsequent relaxation of the structure back to the helical form. Bottom left: folding frequency (histogram counts of folding events) as a function of folding times showing that folding times range from less than one nanosecond to many nanoseconds. The slow decay in the frequency of longer folding times indicates that there is more than a single time scale for folding. When fitted with a double exponential (blue), the mean-squared error was half that of the single-exponential fit (green). Significantly, the values of the two fitted timescales are in good agreement with those measured experimentally at the same temperature. Bottom right: the maximum RMSD between the native helix and the folding peptide as a function of folding time is plotted for each folding event. The shorter folding times correspond to refolding of collapsed or native-like structures with smaller RMSD, while longer timescales correspond to folding from a completely unfolded structure with larger RMSD.

other similar systems. For example, Hummer and co-workers[30,31] examined short Ala–Gly-based peptides and showed that the formation of the first single-helical turn occurs on a time scale ranging from less than one nanosecond to a few nanoseconds depending on sequence and temperature. Additionally, Tobias et al.[32] carried out MD simulations of a turn-forming pentapeptide in water at room temperature and concluded that the helix structures can form and unform on subnanosecond and nanosecond time scales.

In conclusion, using our ultrafast *T*-jump methodology for peptides in water solution, with temporal resolution reaching the water heating time (3.5 ps), we have observed the fastest elementary events in the folding of secondary structures, to date reported to have rates that are orders of magnitude slower. Even in the nucleation of the shortest five-residue structures, the helix–coil transition involves "multistep" dynamics (bifurcation of trajectories), and the two-state concept in which the peptide molecules are either in the fully folded (helical) or fully unfolded (random coil) conformations cannot describe the observed behavior. We have examined the dynamics of both the five- and 21-residue peptides and the dependence of the latter on denaturant. Together with MD simulations we could address issues of folding bifurcation and side-chain motions. These findings suggest future studies of early ultrafast events for other polypeptides and proteins, and, perhaps most significantly, they redefine what is meant by ultrafast dynamics in protein and peptide folding.

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