Real-time NMR studies on a transient folding intermediate of barstar

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Abstract

The refolding of barstar, the intracellular inhibitor of barnase, is dominated by the slow formation of a *cis* peptidyl prolyl bond in the native protein. The triple mutant C40/82A P27A in which two cysteine residues and one *trans* proline were replaced by alanine was used as model system to investigate the kinetics and structural consequences of the *trans/cis* interconversion of Pro48. One- and two-dimensional real-time NMR spectroscopy was used to follow the *trans/cis* interconversion after folding was initiated by rapid dilution of the urea denatured protein. Series of ^1H , ^{15}N HSQC spectra acquired with and without the addition of peptidyl prolyl isomerase unambiguously revealed the accumulation of a transient *trans*-Pro48 intermediate within the dead time of the experiment. Subtle chemical shift differences between the native state and the intermediate spectra indicate that the intermediate is predominantly native-like with a local rearrangement in the Pro48 loop and in the β -sheet region including residues Tyr47, Ala82, Thr85, and Val50.

Keywords: *cis-trans* peptidyl prolyl isomerization; fluorescence spectroscopy; heteronuclear NMR

Our current knowledge of the mechanisms that govern protein folding is based on a careful analysis of all major species along the folding coordinate. Protein engineering studies (Fersht et al., 1992), using site-directed mutagenesis and Φ value analysis, have provided a detailed description of all major species along the folding coordinate such as metastable intermediates (Matouschek et al., 1992) and transition states (Serrano et al., 1992). NMR spectroscopy, which has been primarily involved in the study of the structure of biomolecules in solution, has recently advanced into a powerful technique for the structural and dynamic analysis of the denatured state, the starting point of the folding coordinate. For the study of short-lived intermediates, indirect NMR methods based on hydrogen/deuterium (H/D) exchange were developed to gain information about the solvent accessibility of amide protons in various stages along the folding coordinate (Roder et al., 1988). The advent of efficient heteronuclear labeling has overcome limitations from severe overlap of “random coil” proton signals in partially or fully denatured states by dispersing them into ^{15}N and/or ^{13}C dimensions in multidimensional spectra. Recently, NMR

spectroscopy has been used to the direct measurement of protein folding by rapidly recording spectra as the folding reaction proceeds. These “real-time” NMR studies have been successfully applied to protein folding and unfolding initiated by temperature jump (Akasaka et al., 1991; Kautz & Fox, 1993), denaturant dilution (Balbach et al., 1995; Kiefhaber et al., 1995), and acid/base jump (Killick et al., 1998). The method is based on a rapid change of the conditions in situ, for example, through the injection of small volumes of highly concentrated denaturants, acids, or bases, followed by the acquisition of a series of 1D or 2D experiments. (For a general review, see Dobson & Hore, 1998.) Rapid mixing within the magnet has reduced the dead time to seconds (Koide et al., 1993; Hoeltzli et al., 1994; Balbach et al., 1995). The time dependency of signals from all species present at any time during the folding pathway determines the kinetics and can provide information about potential intermediates. Kinetic and structural information about all species present can be obtained. The time resolution of 1D experiments is in the order of seconds. The highest structural information can be gained from a series of 2D experiments, albeit with a reduced time resolution that is in the order of minutes for a ^1H , ^{15}N HSQC, and, therefore, mainly feasible for slow folding kinetics (Lui et al., 1996). Spectral resolution for faster processes can be obtained by the acquisition of one 2D spectrum over the whole time course of the folding process, the deconvolution of line shapes in the indirect dimension yields folding/unfolding rates for all detectable species (Balbach et al., 1996).

The *cis/trans* interconversion of peptidyl prolyl bonds is a slow process in the order of minutes, well within the time scale of 2D

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Abbreviations: 1D, one-dimensional; 2D, two-dimensional; 3D, three-dimensional; ANS, 1-anilino-8-naphthalene-sulfonate; CD, circular dichroism; HSQC, heteronuclear single quantum coherence; PPI, peptidyl prolyl isomerase; TOCSY, total correlation spectroscopy.

real-time NMR experiments. In particular, for proteins with *cis* proline in the native state, *trans/cis* isomerization causes a kinetic trap leading to the accumulation of a *trans* intermediate. Its slow interconversion rate dominates in most cases the refolding kinetics and necessitates the use of double-jump experiments for the study of faster processes. A number of NMR folding studies of proteins with *cis* prolines have been carried out. The folding of staphylococcal nuclease was studied (Loh et al., 1991; Kautz & Fox, 1993) by following the chemical shift changes of isolated histidine signals in 1D NMR experiments. The peptidyl prolyl bond of Pro117 is predominantly *cis* in the folded state, with only a small *trans* population at equilibrium. The transient *trans* intermediate was also studied by complementary methods such as stopped-flow circular dichroism (CD) (Kuwajima et al., 1991; Sugawara et al., 1991). The folding intermediate of apoplastocyanin was examined by a number of techniques including fluorescence, CD, H/D exchange, and real-time NMR (Koide et al., 1993). During folding there is an accumulation of an unstructured intermediate trapped by the slow *cis/trans* isomerization of two prolines. In the present study, we have used 1D and 2D real-time NMR experiments to follow the *trans/cis* isomerization of Pro48 in the barstar C40/82A P27A triple mutant.

The folding pathway of barstar has been studied extensively (Shastry & Udgaonkar, 1995). The heterogeneity of the oxidation state and the tendency of the two cysteine residues to aggregate hampered attempts to crystallize and biophysical studies of wild-type barstar. For this reason, the "pseudo" wild-type barstar C40/82A was used to solve the crystal structure of the barstar/barnase complex (Guillet et al., 1993; Buckle et al., 1994), for extensive protein folding studies (Schreiber & Fersht, 1993; Martinez et al., 1995; Wintrod et al., 1995) and for the analysis of residual structure in the cold-denatured state (Wong et al., 1996). An NMR solution structure was obtained for wild-type barstar (Lubienski et al., 1994) and the C40/82A mutant (Wong et al., 1997). The latter included a detailed analysis of backbone dynamics based on ^{15}N relaxation time measurements. The triple mutant barstar C40/82A P27A was specifically designed to investigate the *trans/cis* interconversion of the peptidyl prolyl bond of Pro48 by a range of biophysical methods (R. Golbik, unpubl. obs.).

The early stages of barstar refolding involve rapid formation of transient intermediates, on a time scale inaccessible to NMR spectroscopy (Nölting et al., 1997). Here, we have investigated the later stages of barstar folding, which occur on a time scale of minutes. Not accounting for initial rapid processes within the dead time of the real-time study, the basic two state model for protein folding $D \rightleftharpoons N$ is replaced by $D_T \rightleftharpoons N_T \rightleftharpoons N_C$, where D_T represents the denatured state with the peptidyl prolyl bond of Pro48 in predominantly *trans* conformation and N_T represents the partially folded protein with the peptidyl prolyl bond in the non-native *trans* conformation. N_C is the folded state with a *cis* peptidyl prolyl Pro48 bond.

Earlier studies (Schreiber & Fersht, 1993) have shown that the *trans/cis* proline interconversion is invisible to CD or fluorescence spectroscopy. The native-like *trans* intermediate is able to inhibit barnase, but curiously binds ANS (Shastry & Udgaonkar, 1995). Quench flow NMR studies (Roder et al., 1988), which trap intermediates by local H/D exchange, are hampered by the high aggregation tendency of barstar under acidic conditions. Based on the assignments available from earlier NMR studies for the denatured and native states of barstar C40/82A, we have studied structural changes and the kinetics of the *trans/cis* interconversion of Pro48

by real-time NMR refolding of the urea denatured barstar C40/82A P27A.

Results

Backbone ^1H , ^{15}N chemical shift assignments of barstar C40/82A P27A

^1H , ^{15}N backbone assignments of native ^{15}N -labeled barstar C40/82A P27A under the final conditions of the real-time experiment (0.8 M urea, 288 K) are based on the complete ^1H , ^{15}N , ^{13}C assignment of barstar C40/82A (Wong et al., 1997). A ^{15}N -edited TOCSY was used to confirm these tentative assignments based on residue specific side-chain assignments. As already observed for cold-denatured barstar C40/82A (Wong et al., 1997), some otherwise completely exchange broadened C-terminal residues (Ile86, Leu88, Ser89) became observable under the conditions of the real-time experiments. A possible explanation for this observation is that the addition of low concentrations of urea increases the interconversion rate between multiple conformers present. A detailed titration study for barstar C40/82A had already established that under these low urea concentrations (0–1 M urea) only signals for the native, folded species are present (Wong et al., 1996), and urea concentration-dependent chemical shift changes are marginal.

Real-time NMR with and without addition of prolyl isomerase

Indirect results from fluorescence measurements indicated that the kinetics of the *trans/cis* isomerization is in the order of minutes under the conditions chosen. The experimental setup was optimized based on a dummy sample with the same volume and concentrations of protein and denaturant as used in the real-time experiments. Folding was initiated by rapid dilution of small volumes of 4 M urea-denatured barstar C40/82A P27A outside the magnet. The estimated dead time in the order of 100 s mainly arises from the slow mixing of the viscous urea solution, which affects the magnetic field homogeneity. All real-time experiments observe only the interconversion of the *trans* intermediate into the native state. No signals from the denatured state should be observed (R. Golbik, unpubl. obs.) because the initial folding of urea-denatured barstar C40/82A P27A to the *trans* intermediate is completed in the order of seconds. Real-time experiments were acquired as series of 2D ^1H , ^{15}N HSQC spectra with and without addition of peptidyl prolyl isomerase (PPI) over a time range of 30 min; each 2D experiment was completed in 100 s. Figure 1 illustrates the quality of the obtained ^1H , ^{15}N HSQC spectra: A1 (B1) are the first and A16 (B16) the last time points of a series of experiments without and with addition of PPI, respectively. Superficially most of the signals in A1–A16, B1–B16 appear to be almost identical to signals in the ^1H , ^{15}N HSQC spectrum of native barstar C40/82A P27A. For most of the resonances no overall changes in intensity or line broadening over the time course of the experiment were observed. This finding agrees with the conclusion from fluorescence studies that indicated that the folding intermediate is largely native-like and adopts the same global fold as the native state.

Native-like intermediate

A closer inspection of the first time points in the series without PPI revealed a subset of signals that are not present in the final ^1H , ^{15}N

Fig. 1. ^{15}N , ^1H HSQC spectra of barstar C40/82A P27A acquired during the time course of *trans/cis* interconversion of Pro48. A1 (A16) are the first (last) time points of a real-time experiment. B1 (B16) are the first (last) time points of an identical experiment acquired in the presence of peptidyl prolyl isomerase. Signals of the intermediate N_i that decay during the time course of Experiment A are marked in A1. They were not observed in B.

HSQC spectra and decay during the time course of the experiment. The line shapes of these signals are comparable to the native protein, indicating that both species have similar compactness. This emphasizes that the *trans* intermediate and the native *cis* protein differ only in a localized region around Pro48. Figure 2 illustrates how decay curves for signals at a specific ^{15}N chemical shift position were extracted. An example for one of the obtained decay curves is shown in Figure 3. Altogether five isolated signals were unambiguously assigned for the *trans* intermediate, they decayed with rate constants of $0.002 \pm 0.0005 \text{ s}^{-1}$, which are of the correct order of magnitude for proline isomerization. Addition of PPI leads to an increase of the interconversion rate; the lack of the aforementioned additional signals in B1 indicates that the *trans/cis* interconversion is essentially complete within the dead time of the experiments.

To elucidate overall changes during the time course of the experiment, difference spectra A16–A1 and B16–B1 were calculated (Fig. 4). The B16–B1 difference spectrum was used as an internal control to eliminate all effects not due to the prolyl isomerization. The double-difference spectrum (A16–A1)–(B16–B1) revealed signals with negative intensity corresponding to those already ob-

served in the first time points without PPI. They were attributed to the *trans* intermediate. Signals with positive intensity correspond to resonances of the native protein that exhibit the greatest changes during the time course of the experiment. Assignments for some of these peaks (Tyr47, Ala82, Thr85, Val52) were possible by comparison with the ^{15}N , ^1H HSQC of the native form.

Discussion

Real-time NMR studies can provide detailed structural information about the nature of transient species along the folding coordinate. We have studied the slow interconversion of a native-like intermediate in the folding of barstar, which has a *trans* peptidyl prolyl Pro48 bond. Amide proton chemical shifts were used as reporters of localized structural changes. For barstar C40/82A P27A, the majority of amide signals adopt native-like chemical shift positions within the experimental dead time of 100 s. This implies that the 3D fold of the *trans* intermediate closely resembles the native form, in agreement with folding studies that show that there is only a small energy difference $\Delta G_{N_T-N_C} = -1.56 \text{ kcal/mol}$ between the *trans*-intermediate N_T and the native form N_C (R. Golbik,

Fig. 2. Time dependency of signals in the ^{15}N , ^1H HSQC spectra of barstar C40/82A P27A. Sorting amide proton (H_N) signals according to the chemical shifts of their directly attached ^{15}N nuclei facilitates the analysis of intermediate signals. The stack plot illustrates the time dependency of H_N signals at $\delta(^{15}\text{N}) = 125.8$ ppm. The decaying signal at $\delta(^1\text{H}) = 8$ ppm is attributed to a residue in the *trans* intermediate.

unpubl. obs.). PPI catalysis of the *trans/cis* peptidyl prolyl isomerization results in a single set of signals corresponding to native barstar C40/82A P27A. This indicates that N_T interconverts rapidly to the native form within the dead time of the NMR experi-

ment as expected from fluorescence and CD studies (R. Golbik, unpubl. obs.).

The structural changes during the *trans/cis* isomerization are illustrated in Figure 5. Residues that undergo the largest conformational changes are highlighted, based on chemical shift changes of their amide signals. The changes are localized, in space but not in sequence, around Pro48. Only a small part of the protein must be disrupted by the *trans/cis* isomerization, and the disruption is localized in the loop and does not significantly affect the hydrophobic core or the overall tertiary or secondary structure of the protein.

Pro48 forms part of the loop interconnecting helix 2 with the central strand of the β -sheet. Residues with chemical shift differences are mainly located in the loop region, in the last turn of helix 2 and at the beginning of the β -sheet. Residues buried in the hydrophobic core are not affected by the *trans/cis* isomerization. This finding accounts for the fluorescence studies that show no changes for the environment of Trp44, Trp38, and Trp53. This finding is confirmed by the observation that the chemical shift positions of all Trp $H_N\epsilon 1$ signals are invariant during the *trans/cis* interconversion. Interestingly, the chemical shift position of Leu49 is also not affected, even though it directly follows Pro48. This implies that the structural changes to accommodate a *trans* peptidyl prolyl bond of Pro48 must be relatively minor and do not involve a major reorientation of the hydrophobic core. A local rearrangement of the residues in the loops may expose some hydrophobic side chains, which would explain the observed ANS binding of the intermediate (Shastry & Udgaonkar, 1995). One of the potential candidates is the aromatic ring of Tyr47, which is buried inside the protein in the native form. A basic modeling study has indicated that this aromatic ring protrudes into the solution for the *trans* Pro48 intermediate.

Fig. 3. Decay curve extracted from a time dependent set of ^{15}N , ^1H HSQC spectra for an amide signal of the *trans* intermediate. The observed rate of $0.002 \pm 5 \times 10^{-4} \text{ s}^{-1}$ is of the correct order of magnitude for proline isomerization under the experimental conditions.

Fig. 4. Double difference spectrum (A16–A1)–(B16–B1) illustrating signals that undergo the largest intensity changes during the time course of the *trans/cis* interconversion. Black signals are negative peaks that attributed to the native-like intermediate *N_i*, whereas gray signals correspond to peaks of positive intensity and are attributed to native state signals that interconvert during the time course of the experiment. The signal at $\delta(^1\text{H}) = 10$ ppm and $\delta(^{15}\text{N}) = 120$ ppm is an artefact at the on-resonance ^{15}N chemical shift position.

Conclusion

The NMR data are consistent with the folding pathway previously presented. They confirm that an intermediate exists, and that overall, this folding intermediate is of a native-like structure. Changes are observed in signals of residues in the proximity of Pro48. These changes are localized in space and indicate subtle structural rearrangements. Signals attributed to the folding intermediate decay rapidly during the course of the experiment and are, therefore, difficult to assign. However, based on their proximity to native signals tentative assignments can be attempted. Overall, the picture emerges of a protein that can rapidly adopt its global fold, regardless of the conformation of the peptidyl prolyl bond. Barstar folds with the peptidyl prolyl bond in the incorrect (*trans*) orientation and, consequently, must undergo a rearrangement. Even though the native and intermediate states are very close in structure, the energy barrier for this rearrangement is high enough to trap the intermediate for a significant length of time. These real-time NMR studies demonstrate their utility in addressing genuine biological problems on a time scale of minutes. Subtle localized effects can be observed transiently along the folding coordinate, which are difficult or impossible to observe by optical techniques.

Materials and methods

^{15}N -labeled barstar C40/82A P27A mutant was prepared according to established procedures (Schreiber & Fersht, 1993). NMR experiments were acquired on a Bruker (Karlsruhe, Germany) AMX500 spectrometer equipped with a triple resonance probe and single axis gradients. The samples contained 1.5 mM ^{15}N barstar C40/82A P27A in 500 μL of 10 mM potassium buffer at pH 6.7. Two-dimensional ^1H , ^{15}N HSQC spectra were acquired with spectral widths of 7,400 Hz (1,800 Hz) and 1,024 (128) complex points

Fig. 5. MOLSCRIPT (Kraulis, 1991) representation of barstar C40/82A P27A. *cis* Pro48 and Tyr47 are shown with side chains. The amide signals of Ala82, Val50, and Thr85 (shaded black) undergo significant intensity changes during the time course of the *trans/cis* isomerization. A possible explanation for the ANS binding of the *trans*-intermediate is the rotation of the buried Tyr47 aromatic side chain into a solvent exposed position.

in the t_1 (t_2) dimensions, respectively. 2D ^{15}N edited TOCSY experiments were obtained with sweep widths of 7,400 Hz in both ^1H dimensions and $1,024 \times 256$ complex points. All experiments included gradient selection and sensitivity enhancement schemes. For the real-time NMR studies, barstar C40/82A P27A was dialyzed overnight into 25 mM sodium phosphate buffer. Samples were prepared with and without the addition of 5 mM peptidyl prolyl isomerase (PPI, recombinant human cytosolic cyclophilin, M_r 18,000). In each kinetic experiment 100 μL of 4 mM barstar in 4 M urea, 25 mM sodium phosphate buffer pH 6.7, 10% D_2O was diluted into 400 μL of 25 mM sodium phosphate buffer pH 6.7, 10% D_2O outside the magnet. The dead time was estimated at 100 s. A series of 16 HSQC experiments, with 2,048 points in the t_1 dimension and 32 points in the t_2 dimension, was recorded at 288 K. Each experiment took 100 s to acquire. The data were processed in FELIX 2.30 (Biosym Technologies, San Diego, California) and fitted to single exponential decay curves with Kaleidagraph (Abelbeck Software, Reading, Pennsylvania).

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