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The rate-limiting steps for the folding of an antibody scFv fragment

Marcus Jäger, Andreas Plückthun*

Biochemisches Institut der Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland

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Abstract The refolding kinetics of a single-chain Fv (scFv) fragment, derived from the phosphorylcholine binding antibody McPC603, was investigated. Both prolyl-peptide bonds which are *cis* in the native state affect the refolding kinetics of long-term denatured protein. The rate-limiting step is the *trans*→*cis* isomerization at the ProL95-peptide bond, which is catalyzed by peptidyl-prolyl-*cis*/*trans*-isomerase (PPIase), and is the prerequisite for correct V_H/V_L domain association. Refolding of short-term denatured protein resulted in complex refolding kinetics, too. This kinetic heterogeneity could be ascribed to *cis*→*trans* re-isomerization at the ProL95-peptide bond to the wrong conformation in a folding intermediate. PPIase was shown to increase the fraction of slowly folding species, thereby competing with the fast folding of short-term denatured scFv, having native proline conformations. A trapped intermediate is rapidly populated, and the return from this state becomes rate-limiting.

Key words: Protein folding; Proline *cis*/*trans* isomerization; Folding intermediate; scFv fragment; Antibody engineering

1. Introduction

While scFv fragments derived from antibodies are beginning to be used in numerous biotechnological and biomedical applications [1], little is known about the folding pathway of this type of proteins. Further engineering and production of scFv-based molecules will benefit from an understanding of the crucial intermediates and rate-determining steps. In order to investigate these aspects in more detail, we have used the scFv fragment derived from the well characterized phosphorylcholine binding antibody McPC603 as a model system [2–5].

Previous studies have addressed several aspects of the refolding pathway of the denatured, oxidized scFv fragment, which carries intact disulfide bonds in both V_H and V_L [6,7]. Using nuclear magnetic resonance (NMR) and mass spectroscopic (MS) techniques, an early folding intermediate could be identified. This intermediate is formed in the millisecond time scale and comprised several exchange-resistant amide protons, most of which are positioned in the inner β-sheet of the V_L domain. Subsequent formation of the native state was proposed to be a cooperative, but very slow, process, involving hydrogen bond formation in both domains with the same rate constant.

In this paper, we address the nature of this rate-determining step in more detail. Fluorescence spectroscopy and site-directed mutagenesis allowed us to show that the rate-limiting step for refolding of long-term denatured scFv fragment is the *trans*→*cis* isomerization at the ProL95-peptide bond without which the correct V_H/V_L interface cannot form. PPIase accelerates this step. Refolding of short-term denatured protein was shown to be intrinsically fast. Nevertheless, kinetic heterogeneity is introduced due to spontaneous re-isomerization of proline-peptide bonds in an early folding intermediate, again giving rise to a substantial fraction of slowly folding species. PPIase competes with the intrinsically fast refolding reaction of short-term denatured protein, by effectively catalyzing this re-isomerization, thereby increasing the fraction of slowly folding species.

2. Materials and methods

The scFv fragments are of the orientation V_H-$(Gly_4Ser)_3$-V_L. The V_L domain carries the mutations A(L15)L, S(L56)P, N(L90)Q, all of which stabilize the isolated V_L domain [8,9]. The V_H domain carries the mutations P(H40)A, S(H60)A, A(H61)D, S(H76)N, I(H77)T, which prevent aggregation ([10]; H. Bothmann and A. Plückthun, unpublished data). All numbering is according to Kabat [11], with H denoting heavy chain and L denoting light chain residues. The scFv fragments were expressed in the *Escherichiacoli* strain JM83 and purified by affinity chromatography [12]. Pure and functional protein was stored in buffer A (50 mM sodium borate, pH 7.6; 50 mM NaCl; 5 mM phosphorylcholine (PC)) at 4°C until use. *E. coli* rotamase was purified as described [13].

The unfolding kinetics of the scFv fragment was determined with an Applied Photophysics model SX-17MV spectrofluorimeter. Native scFv fragment (25 μM, in buffer A) was diluted 1:25 to reach final conditions of 4 M GdmCl (in buffer A) at 10°C. Excitation was at 295 nm (2.5 nm bandpass), and the change in fluorescence emission intensity at >360 nm was integrated, using a cut-off filter (2.5 nm bandpass).

Refolding experiments were performed in buffer A at 10°C on a PTI Alpha Scan spectrofluorimeter (Photon Technologies Inc.) and were started by a 1:100 dilution of unfolded protein (40 μM in 4 M GdmCl, in buffer A) into buffer A, while monitoring the change in fluorescence emission intensity at 328 nm (5 nm slit width) after excitation at 295 nm (1.5 nm slit width). The presence of PC in the refolding buffer ensured stabilization of the native protein and thus unidirectionality of folding. When excitation is at 295 nm, binding of PC to the scFv fragment does not change the intrinsic protein fluorescence, and control experiments established that PC has no influence on any folding reaction itself.

Two different types of refolding experiments are described in this paper. In the first type of experiments, the protein was denatured in 4 M GdmCl for at least 12 h at 10°C to produce denatured protein with all prolyl-peptide bonds in thermodynamic equilibrium (long-term denatured protein). In the second type of experiments, native protein was denatured by a 1:1 dilution into 4 M GdmCl (final concentration) at 10°C for 10 s in order to prevent isomerization around prolyl-peptide bonds in the denatured state (short-term denatured protein) and immediately diluted 1:100 into buffer A to initiate refolding (final protein concentration: 0.4 μM). PPIase catalyzed refolding reactions were performed by having PPIase present in the refolding buffer at final concentrations specified in the text. All kinetic traces were evaluated using the software Kaleidagraph (Synergy software, Reading, UK).

*Corresponding author. Fax: (41) (1) 635 5712.
E-mail: plueckthun@biocfebs.unizh.ch

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3. Results

3.1. Description of the scFv fragments

The scFv fragments used in this study carry additional mutations to increase stability and to decrease aggregation during folding in vitro and in vivo (see Section 2). In the following we use the term 'wt' to refer to the presence of two *cis*-prolines in the native state (ProL8, ProL95) (Fig. 1), always in the context of these additional mutations. ProL8 is absolutely conserved in human kappa light chain sequences, but in 16% of all known mouse kappa light chains, it is replaced by a non-proline residue. ProL95 is, despite being part of the CDR3 of the V_L domain, also well conserved in kappa light chains (100% in mouse kappa light chains, 81% in human kappa light chains, 38 of 40 of human germline sequences; A. Honegger, personal communication). ProL95 is further thought to indirectly determine the interface stability of the heterodimer, as two neighboring residues, TyrL94 and LeuL96 as well as AspL91 make contact to the heavy chain in McPC603, but only if they are correctly positioned by the peptide bond of ProL95 being in the *cis*-conformation [3,4].

In order to investigate the role of the two *cis*-prolines on the refolding kinetics of the scFv fragment, we mutated ProL8 into Ala, while leaving ProL95 intact (thereby avoiding complications such as unstable heterodimers). The resulting scFv P(L8)A mutant was fully functional and comparable to scFv 'wt' in both yield and stability (data not shown). This indicates that ProL8, despite being conserved, is not absolutely required at this position for folding into a stable, native conformation.

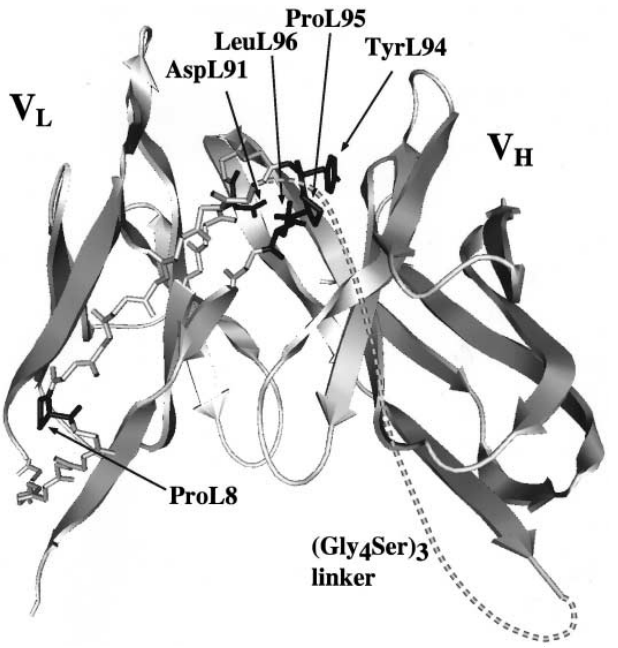

Fig. 1. *Cis*-prolines in the scFv fragment. Only the region L1–L15 and L89–L97 (CDR-L3) is shown as backbone, while the rest of the structure is shown as ribbon diagram. The *cis*-prolines are shown with the side chains in black and are labeled, as are those residues in CDR-L3 which protrude into the interface to V_H. The linker is indicated by a dotted line.

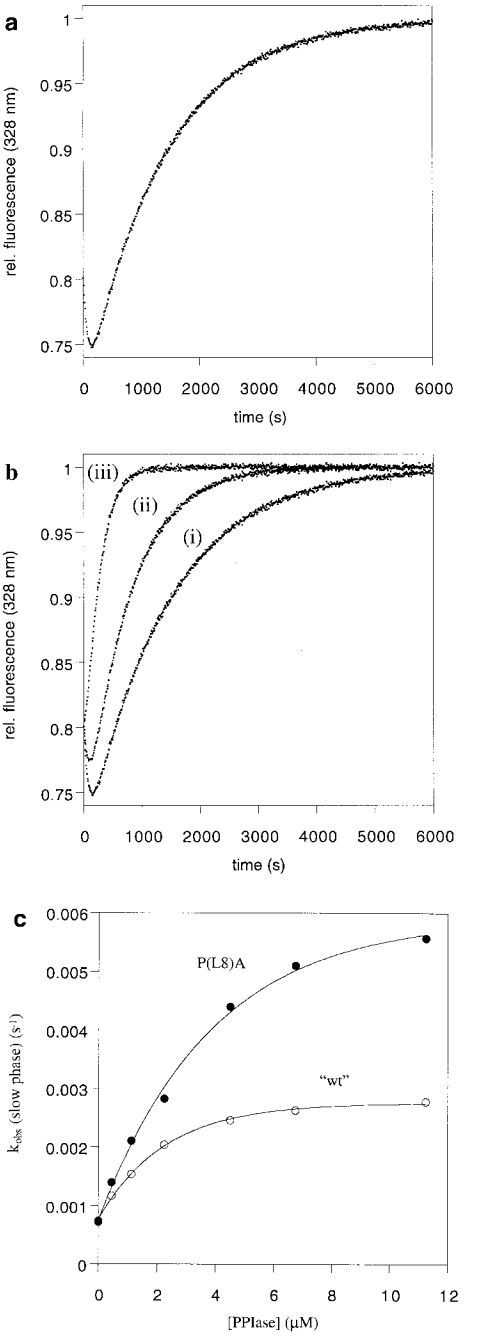

Fig. 2. Fluorescence emission trace obtained after refolding of (a) long-term denatured scFv 'wt' (0.40 μM), (b) long-term denatured P(L8)A mutant (0.40 μM) in (i) the absence of PPIase, (ii) the presence of 0.45 μM PPIase, (iii) the presence of 4.5 μM PPIase. c: Plot of the apparent kinetic rate constant of the slow phase of long-term denatured scFv fragment vs. [PPIase] in the refolding buffer. Closed circles: scFv P(L8)A mutant. Open circles: scFv 'wt'.

3.2. The refolding kinetics of long-term denatured scFv fragment

The refolding kinetics of the scFv fragments was monitored by recording the change in fluorescence emission intensity at 328 nm, the maximum of the difference spectra of native and unfolded protein [10]. The fluorescence trace obtained for the scFv 'wt' was biphasic, with an initial decrease in fluorescence followed by a large fluorescence increase. Kinetic rate constants of 0.013 s^{-1} (fast phase, intensity decrease) and

0.00074 s^{-1} (slow phase, intensity increase) were determined (Fig. 2a). The scFv P(L8)A mutant gave an almost identical kinetic trace (Fig. 2b), with kinetic rate constants of 0.012 s^{-1} (fast phase) and 0.00078 s^{-1} (slow phase). The similarity in the kinetic rate constants between 'wt' and P(L8)A mutant indicates that substitution of ProL8 by Ala does not affect the rate-determining step for refolding of long-term denatured protein. From the fact that only the slower of the manual resolvable phases displayed a fluorescence change going in the direction from unfolded to native protein (intensity increase) [10], we conclude that native protein is formed only in the slow phase, whereas the fast phase (intensity decrease) represents formation of a folding intermediate. This view is also supported by an earlier NMR study, which showed that stable secondary structure in both domains is only formed with kinetics comparable to the slow fluorescence phase of this study [6].

3.3. *Isomerization at ProL95 is rate-limiting for refolding of long-term denatured scFv fragment*

In order to characterize the molecular origin of the two fluorescence phases in more detail, we repeated the refolding experiment in the presence of *E. coli* PPIase. The result obtained for long-term denatured scFv P(L8)A is shown (Fig. 2b). The fast fluorescence decrease (kinetic rate constant: 0.012 s^{-1}) was not catalyzed by PPIase at low enzyme concentration (data not shown). At high PPIase concentration, however, this phase seemed to disappear. The slow phase is clearly catalyzed by PPIase, indicating that proline *cis/trans* isomerization is rate limiting for refolding of long-term denatured scFv fragment. Since the P(L8)A mutation did not affect the refolding kinetics of long-term denatured protein (see above), we believe that the slow acquisition of the native structure is due to an isomerization at the ProL95-peptide bond. PPIase accelerated this re-isomerization in a concentration-dependent fashion (Fig. 2c, closed circles), and a maximal refolding rate of 0.0057 s^{-1} (the kinetic rate constant expected at infinitesimally high enzyme concentration) was estimated for the slow phase from Fig. 2c. This indicates that almost maximal acceleration was obtained already at the highest PPIase concentration (11.25 μM; kinetic rate constant: 0.0055 s^{-1}) used in this study.

3.4. *Refolding of scFv 'wt' is less well catalyzed by PPIase than scFv P(L8)A*

For long-term denatured scFv 'wt', the fast fluorescence decrease also disappeared at high PPIase concentration (data not shown). Differences were, however, found for the catalysis of the slow fluorescence increase. As shown in Fig. 2c (open circles), scFv 'wt' is less effectively catalyzed than the P(L8)A mutant at all PPIase concentrations tested, and the maximal achievable acceleration was reduced from 0.0057 s^{-1} to 0.0028 s^{-1}. This phase, giving rise to a fluorescence increase, remains mono-exponential under all conditions. The presence of a proline residue at position L8 slows down the rate of folding into the native state in the presence of high amounts of PPIase. In contrast, in the absence of PPIase, the isomerization of ProL95 is rate-limiting, obscuring the effect of ProL8 (the two curves in Fig. 2c intersect the *y*-axis at the same point). This observation requires that the nature of the residue at position L8 has an influence how fast the native state can be reached, provided that the rate-limiting isomerization of ProL95 is sufficiently well catalyzed to see this phenomenon. As all crystallized kappa light chains that carry ProL8 have a *cis*-peptide bond, and all crystallized kappa light chains that carry a different residue have a *trans*-peptide bond, we reason that ProL8 has to undergo a *cis*→*trans* isomerization, while AlaL8 does not. It is thus possible that the plateau value of the 'wt' (Fig. 2c) defines the rate of this isomerization at ProL8, while the higher plateau value of the P(L8)A mutant indicates that this mutant is limited by a different rate-limiting step. Alternatively, PPIase may already efficiently catalyze the isomerization of ProL8 in the 'wt' at the highest concentrations, and thus both 'wt' and P(L8)A mutant may be rate-limited by the same type of non-proline step, which occurs 2-fold faster in the mutant for steric reasons.

3.5. *Complex refolding kinetics of short-term unfolded scFv fragment*

To test whether the plateau-values in Fig. 2c represent maximal intrinsic folding rates of the scFv fragment, we performed a double-jump experiment. Native protein is only briefly denatured and rapidly transferred to refolding buffer to initiate refolding. The brief unfolding time keeps the proline-peptide bonds in the native conformation, as the isomerization reaction is a slow process [14,15]. Unfolding of native scFv fragment in 4 M GdmCl was a mono-exponential reaction with a kinetic rate constant of 1.2 s^{-1} at 10°C (data not shown), indicating that the unfolding reaction is indeed fast under these conditions.

The refolding kinetics of short-term denatured scFv P(L8)A is shown (Fig. 3a). The fast fluorescence decrease, seen after refolding of long-term denatured protein, was no longer detectable. Instead, two phases, each giving rise to a fluorescence increase, were observed. The fast fluorescence increase (40% relative amplitude) showed a kinetic rate constant of 0.016 s^{-1}, which is faster than the mono-exponential fluorescence increase after refolding of long-term unfolded scFv fragment, even at the highest PPIase concentrations. We therefore conclude that this phase is not limited by proline *cis/trans* isomerization, and refolding of short-term denatured scFv is faster than PPIase-catalyzed refolding of long-term denatured protein. This suggests that the plateau-values reached at high PPIase-concentration (Fig. 2c) must be attributed to folding processes other than proline-isomerization, which become rate-determining under these conditions, and which are not on the 'fast-track' folding pathway of short-term denatured protein.

The presence of a second, slow phase in refolding of short-term denatured scFv, which is also characterized by a fluorescence increase (60% relative amplitude) is at first unexpected, since the fast unfolding should result in molecules with native prolyl-peptide bonds exclusively, and thus a fast mono-exponential refolding reaction should be observed. Notably, the kinetic rate constant of 0.00078 s^{-1} of this additional phase was almost identical to the rate constant of the rate-determining step of long-term denatured protein (0.00072 s^{-1}), which was shown to be *cis/trans* isomerization at ProL95. Thus, we propose that kinetic heterogeneity is introduced by an isomerization at ProL95 during the experiment. This might happen either as a consequence of an unusually rapid isomerization during the unfolding reaction or in an early folding intermediate, formed after initiation of refolding.

In order to distinguish between these two possibilities, we repeated again the experiment in the presence of PPIase in the refolding buffer. Even stoichiometric amounts of PPIase (0.45 μM) had a drastic effect on the refolding kinetics of short-term denatured scFv P(L8)A (Fig. 3a). The fast fluorescence increase (kinetic rate constant: 0.016 s^{-1}), seen only after refolding of short-term unfolded protein and in the absence of PPIase, disappeared completely at the expense of the appearance of a fluorescence decrease (kinetic rate constant: 0.013 s^{-1}), while the slow fluorescence increase was only marginally accelerated (0.00072 s^{-1} in the absence of PPIase; 0.00137 s^{-1} in the presence of 0.45 μM PPIase). Higher concentrations of PPIase still abolished the fast fluorescence increase completely, but at the same time accelerated the slow fluorescence increase in a similar concentration dependent manner as shown in Fig. 3b for long-term denatured protein. A plot of the ratio of the kinetic rate constants for the slow phase of long- and short-term denatured scFv P(L8)A at different PPIase concentrations is shown in Fig. 3b (closed circles). This ratio is about 1.0 under all conditions, suggesting that the same rate-limiting step governs the folding of long- and short-term denatured scFv, provided small amounts of PPIase remove the 'fast-track' molecules.

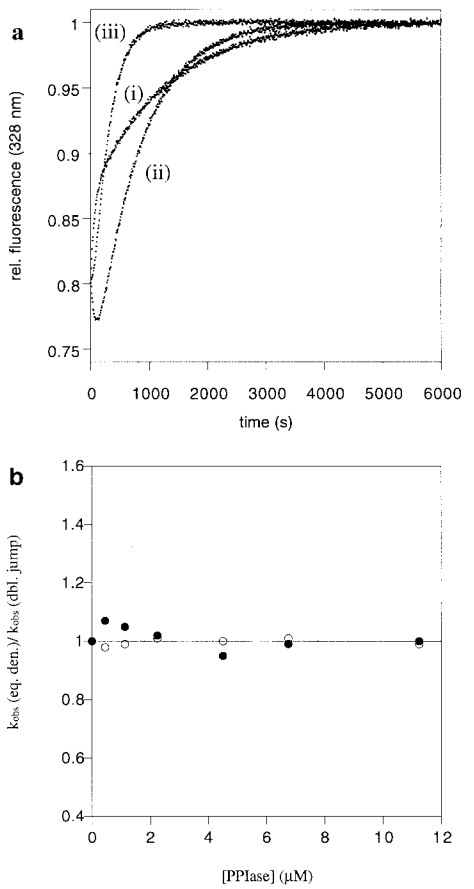

Fig. 3. a: Fluorescence emission trace obtained after refolding of short-term denatured scFv P(L8)A mutant (0.40 μM) in (i) the absence of PPIase, (ii) the presence of 0.45 μM PPIase, (iii) the presence of 4.5 μM PPIase. b: Plot of the relative ratio of the kinetic rate constants of the slow phase of long-term denatured scFv fragment divided by that of the short-term denatured scFv fragment vs. [PPIase] in the refolding buffer. Closed circles: scFv P(L8)A. Open circles: scFv 'wt'.

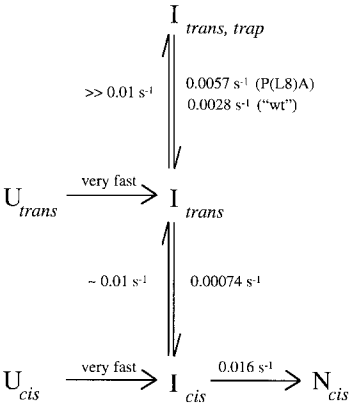

Fig. 4. Proposed kinetic scheme for refolding of long- and short-term denatured scFv fragment. For explanations, see text.

3.6. *PPIase competes with 'fast-track' folding by introducing non-native prolyl-peptide bonds at position L8 and L95*

To check whether the second *cis*-proline (ProL8) is also recognized by PPIase, we repeated the double-jump experiments with the scFv 'wt'. Unfolding and refolding of short-term denatured scFv 'wt' was comparable to the P(L8)A mutant, and similarly to the P(L8)A mutant, inclusion of PPIase to the refolding buffer resulted in a complete disappearance of the fast fluorescence decrease (data not shown). Identical kinetic rate constants were also obtained for long- and short-term denatured scFv 'wt' (Fig. 3b, open circles). Thus, the same transition state governs folding in the presence of PPIase, no matter whether folding is started from short- or long-term denatured scFv fragment for the 'wt' as well as for the P(L8)A mutant. Differences between 'wt' and P(L8)A exist, however, in the absolute folding rates at high PPIase concentrations, with the 'wt' being the slower folding molecule under these conditions.

Together, these data rule out an unusually rapid re-isomerization of proline peptide bonds in the unfolded state as a molecular explanation for the biphasic refolding kinetics of short-term denatured scFv fragment. If this were the case, the concentration of the fast folding species (and thus the relative amplitude of the fast fluorescence increase) should not depend on the refolding conditions, but only on the time the protein resides in the denatured state. However, we observe that PPIase, present during refolding, completely abolishes the fast phase in the refolding of short-term denatured scFv. Therefore, the additional slow phase in the double-jump refolding experiment must have arisen from a spontaneous *cis*→*trans* re-isomerization at the ProL95-peptide bond in an early folding intermediate, with kinetics similar to the 'fast-track' folding molecules (~0.01 s^{-1}).

3.7. *Proposed kinetic model for refolding of the scFv fragment*

We summarize these results in a kinetic model (Fig. 4). For reasons of simplicity, only the isomerization state of ProL95 is indicated as a subscript. Refolding of long-term unfolded protein (both 'wt' and P(L8)A mutant) starts from U_{trans} and immediately goes to $I_{trans,trap}$. From there, it can only slowly return to the native state N_{cis} at a rate of 0.00074 s^{-1}, as the rate-limiting step is the *cis*→*trans* re-isomerization at ProL95. This isomerization is catalyzed by PPIase, but conversion to N_{cis} is not faster than a maximal rate of 0.0028 s^{-1} (scFv 'wt') or 0.0057 s^{-1} (scFv P(L8)A mutant), indicating that the iso-

merization from $I_{trans,trap}$ to I_{trans} becomes rate limiting under these conditions. Short-term denatured protein starts from U_{cis}, goes immediately to I_{cis} and partitions to the native state N_{cis} and the intermediate I_{trans}, from where it falls rapidly into the trap $I_{trans,trap}$. At this point, long- and short-term denatured protein behave the same. PPIase efficiently catalyzes the interconversion between I_{trans} and I_{cis}. This speeds up the build-up of $I_{trans,trap}$ at the expense of the fast folding molecules that directly reach the native state N_{cis} at a rate of 0.016 s^{-1}. In the reverse direction, PPIase catalyzes the isomerization at ProL8 and ProL95, until the conversion of $I_{trans,trap}$ to I_{trans} becomes rate-limiting.

Despite the lack of extensive structural information on the nature of I_{trans}, I_{cis} and $I_{trans,trap}$, we can draw a few interesting conclusions. I_{trans} and I_{cis} cannot be native-like, since I_{trans} is formed spontaneously from I_{cis} during refolding of U_{cis}, indicating that non-native prolines are favored in this intermediate. PPIase accelerates this re-isomerization to the non-native conformer, thereby acting both on ProL95 and ProL8 (ProL8 only in 'wt'). This suggests that the V_L domain must be flexible enough in order to allow these processes. On the other hand, $I_{trans,trap}$ is likely to be a state in which V_H and V_L have interacted, as this intermediate does not occur in the folding of the corresponding Fv fragment (M. Jäger and A. Plückthun, manuscript in preparation). It is therefore probable that $I_{trans,trap}$ is stabilized by the presence of an interdomain linker, which keeps the domains at high local concentrations during folding and may lead to the formation of this trap. NMR data indicate stable secondary structure formation only with the slowest phase [6]. This suggests that stable structure formation actually depends on the reaction I_{cis} to N_{cis}, reinforcing our view that $I_{trans,trap}$, I_{trans} and I_{cis} are non-native species.

4. Discussion

In principle, a short-term denatured scFv fragment can refold relatively fast, with a kinetic rate constant of 0.016 s^{-1}. However, under the experimental conditions, ProL95 re-isomerizes back to *trans* during the refolding reaction with comparable rate. This unproductive reaction is catalyzed by PPIase, even at low concentration (0.45 μM). This directly shows that the native scFv fragment cannot form, unless ProL95 is in the *cis*-conformation, presumably because this prolyl-peptide bond is critical for correct interface formation (Fig. 1) [3,4].

Yet, even at high amounts of PPIase, the rates of the 'fast-track' molecules after short-term denaturation are never reached. This indicates that either, ProL95 is quite inaccessible for the enzyme, or that another step becomes rate limiting, which is still slower than the 'fast-track' molecules, and thus different. This new rate-limiting step is identical, no matter whether the reaction starts with short-term or long-term denatured molecules. Apparently, the *trans*-conformation of ProL95 is sufficient to direct the scFv fragment into a kinetic trap, but the escape from this trap seems to be slowed down by a factor of two by the presence of a proline residue at position L8.

While the rate, achievable at maximal acceleration of ProL95 isomerization is faster for the P(L8)A mutant than for the 'wt', it is still not that of the 'fast track', for neither molecule. This suggests that the isomerization of ProL8 is not solely rate-limiting here, but that another step at least partially limits the rate in going to the native state. This step depends on the nature of the residue at position L8, and perhaps isomerization of ProL8 is partially involved and accounts for it being slower in the 'wt' than in the P(L8)A mutant.

Similar observations have previously been made for two other proteins, human carbonic anhydrase II [16], and the Fab fragment derived from the antibody MAK33 [17]. The characteristics of these multidomain proteins, including the scFv fragment of this study, is a complex fold, a high content of prolines and significant β-sheet structure. The kinetic studies reported here will allow us now to structurally investigate the nature of these trapped intermediates and search for potential similarities among proteins giving rise to such intermediates.

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