Protein Science (2000), *9*:552–563. Cambridge University Press. Printed in the USA.

Direct evidence by H/D exchange and ESI-MS for transient unproductive domain interaction in the refolding of an antibody scFv fragment

MARCUS JÄGER AND ANDREAS PLÜCKTHUN

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

(RECEIVED August 2, 1999; FINAL REVISION January 7, 2000; ACCEPTED January 13, 2000)

Abstract

The refolding kinetics of a single-chain Fv (scFv) fragment, derived from a stabilized mutant of the phosphorylcholine binding antibody McPC603, was investigated by H/D exchange and ESI-MS and compared with the folding kinetics of its constituting domains V_H and V_L. Both V_H and V_L adopt essentially native-like exchange protection within the dead time of the manual-mixing H/D exchange experiment (10 s) and in the case of V_L, which contains two *cis*-prolines in the native conformation, this fast protection is independent of proline *cis*/*trans* isomerization. At the earliest time point resolvable by manual mixing, fewer deuterons are protected in the scFv fragment than in the two isolated domains together, despite the fact that the scFv fragment is significantly more stable than V_L and V_H. Full H/D exchange protection in the scFv fragment is gained on a time scale of minutes. This means that the domains in the scFv fragment do not refold independently. Rather, they associate prematurely and in nonnative form, a kinetic trap. Unproductive domain association is observed both after equilibrium- and short-term denaturation. For the equilibrium-denatured scFv fragment, whose native structure formation is dependent on a *cis* conformation of an interface proline in V_L, this *cis*/*trans* isomerization reaction proceeds about one order in magnitude more slowly than the escape from the trap to a conformation where full H/D exchange protection is already achieved. We interpret these data in terms of a general kinetic scheme involving intermediates with and without domain association.

Keywords: antibody fragments; folding intermediates; H/D exchange; mass spectrometry; protein folding

Due to their modular structure (Padlan, 1994), antibodies are useful model systems to study the folding and assembly of large proteins, which is generally far more complex than the folding of small single-domain proteins (Jaenicke, 1987). The refolding of the Fv fragment, a noncovalently associated heterodimer consisting of the domains V_H and V_L, from a chemically denatured state involves several discrete folding steps with rate constants differing by almost five orders of magnitude (Jäger & Plückthun, 1999a). These experiments have been carried out with domains and fragments of the phosphorylcholine binding antibody McPC603 (Satow et al., 1986), where the domain association can conveniently be stabilized by the hapten, which binds in a cleft between V_H and V_L. The denatured and dissociated domains fold rapidly and independently into compact intermediates. As the V_L domain contains two *cis*-prolines, the folding rate of the V_L domain is significantly slower than that of the V_H domain. The rate-limiting step for the formation of the Fv heterodimer is a slow proline *cis*/*trans* isomerization at ProL95 in the V_L domain, which must be in *cis* for productive domain assembly (Freund et al., 1997a; Jäger & Plückthun, 1997). This residue is directly located in the heterodimer interface and residues involved in crucial domain interactions are adjacent on either side (Satow et al., 1986).

Covalently tethering the V_H and V_L domain to a single-chain (scFv) fragment does not change the rate-limiting step for folding of the equilibrium-denatured protein (*cis*/*trans* isomerization at ProL95), but fluorescence refolding studies provided evidence for transient, unproductive domain interaction after short-term unfolding (Freund et al., 1997a; Jäger & Plückthun, 1997, 1999a). The assembly of the Fv heterodimer from a native V_H domain and a short-term denatured V_L domain, which maintains all proline-peptide bonds in the native conformation, is fast and monophasic. In contrast, refolding of the short-term denatured scFv fragment is characterized by the persistence of the slowest phase, which results from a spontaneous *cis*/*trans* reisomerization at ProL95. Addition

Reprint requests to: Andreas Plückthun, Biochemisches Institut, Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland; e-mail: plueckthun@biocfebs.unizh.ch.

Abbreviations: ANS, 8-anilino-1-naphthalene sulfonic acid; C_L, constant domain of an antibody light chain; ESI-MS, electrospray-ionization mass spectrometry; Fv, Fv fragment of an antibody, consisting of V_H and V_L; GdmCl, guanidinium hydrochloride; H/D exchange, hydrogen-deuterium exchange; PC, phosphorylcholine; scFv, single-chain Fv fragment; V_H, variable domain of an antibody heavy chain; V_L, variable domain of an antibody light chain.

of PPIase to the short-term unfolded scFv fragment completely abolishes the fast folding phase, indicating that ProL95 is accessible to the enzyme early during folding and the structure around ProL95 flexible enough. We also observed that the V_L domain shows no and the V_H domain only moderate transient binding of the fluorescent dye 8-naphthalene-1-sulfonic acid (ANS) (Jäger & Plückthun, 1999a) during their respective folding reactions. ANS binds to solvent-exposed hydrophobic patches typically found in partially structured folding intermediates (Ptitsyn et al., 1990). In contrast, much higher transient dye binding is seen in the refolding of the scFv fragment with significantly slower desorption kinetics, suggesting that exposed hydrophobic segments persist longer and their burial is impaired, when the domains are covalently linked (Jäger & Plückthun, 1999a).

Using hydrogen-deuterium exchange (H/D exchange) (Kim, 1986; Englander et al., 1996) in combination with electrospray ionization mass spectrometry (ESI-MS) (Miranker et al., 1993; Hooke et al., 1995), we now provide direct evidence for premature domain interaction (trap) in the refolding of the scFv fragment, but not during refolding of the isolated domains V_H and V_L. Consistent with optical spectroscopy, the escape from this trap is not rate limiting for the refolding of the equilibrium-denatured scFv fragment as maximal H/D exchange protection is gained at a rate much faster than the slow proline *cis/trans* isomerization at ProL95. However, this escape is slow enough to allow the re-isomerization of native prolines into nonnative conformers in a significant fraction of the short-term denatured scFv fragment.

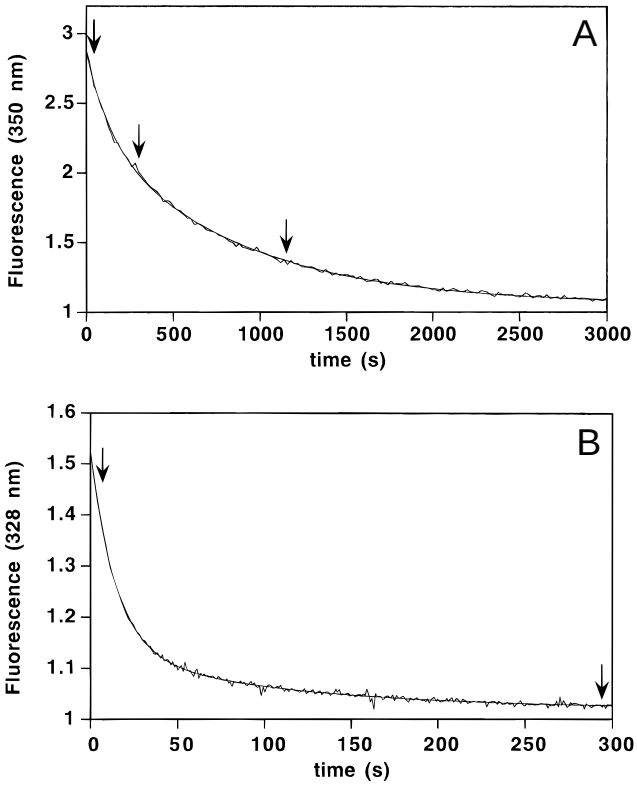


Fig. 1. A: Fluorescence refolding trace of the equilibrium-denatured V_L domain. Excitation was at 295 nm; emission was at 350 nm. The arrows indicate the time points of the manual H/D quenching steps. **B:** Fluorescence refolding trace of the equilibrium-denatured V_H domain. The arrows indicate the time points of the first two manual H/D quenching steps. Excitation was at 295 nm; emission was at 328 nm. In both panels, the fluorescence intensity was normalized to the fluorescence of the native protein, which was set to 1.0. The experiments were carried out at pH 6.0 and 10 °C.

Results

Refolding of the V_L domain monitored by fluorescence

The refolding kinetics of the isolated V_L domain, resolvable by manual mixing, monitored by the change in fluorescence at 350 nm, are biphasic (k_{fast}: 0.0072 s^{-1}; relative amplitude 0.32, k_{slow}: 0.0012 s^{-1}, relative amplitude 0.68) (Fig. 1A). Yet, only about 25% of the total folding amplitude is recovered in the manual mixing experiment, and most of the fluorescence decrease occurs in a major fast phase with a rate constant of 10 s^{-1}, which is detectable by stopped-flow mixing (Jäger & Plückthun, 1999a). Both slow phases are PPIase-catalyzed and disappear almost quantitatively after short-term unfolding, indicating that they are both proline-limited (Freund et al., 1996; Jäger & Plückthun, 1999a). The slower of the two fluorescence phases observed in manual mixing could be assigned to *cis/trans* isomerization at ProL95 (Jäger & Plückthun, 1997, 1999a). The time points of the manual H/D quenching step are indicated by arrows in Figure 1A.

Refolding of the V_H domain monitored by fluorescence

The fluorescence refolding trace of the isolated V_H domain, monitored at 328 nm, is also biphasic (k_{fast}: 0.068 s^{-1}; relative amplitude 0.78, k_{slow}: 0.0095 s^{-1}, relative amplitude 0.22) (Fig. 1B). Folding of the V_H domain is thus considerably faster than the V_L domain, when monitored by fluorescence spectroscopy, which is due to the fact that there are only two prolines in the native V_H domain, both in *trans* (Satow et al., 1986). The two phases of the manual mixing experiment can also be observed, with the same amplitude ratio but with opposite sign, in the presence of a molar excess of native V_L domain (Jäger & Plückthun, 1999a). The phases are believed to arise from two parallel, proline-independent folding reactions in the V_H domain, since both apparently lead to interface formation. The arrows in Figure 1B indicate the time points of the manual H/D quenching steps (only the first two time points are covered by the fluorescence trace shown).

Refolding of the V_L domain monitored by H/D exchange

The mass spectrum of the V_L domain (Fig. 2A) shows a single series of related peaks and a narrow distribution of low m/z values, consistent with the proteins being native after refolding (Loo et al., 1991). This spectrum was obtained from protein that was refolded in D_2O for 3 h, before refolding was completed in H_2O to exchange labile protons, and it represents the data from which the last spectrum in Fig. 2B has been obtained. The insert shows the +8 charge state and demonstrates that the symmetrical distribution obtained in the deconvoluted spectra (Fig. 2B) is also observed in the m/z peak.

Figure 2B shows the refolding of the equilibrium-denatured V_L domain monitored by H/D exchange and ESI-MS. The mass of the protonated V_L is 12,926 Da (for a summary of the mass spectrometric data see Table 1). The detection of species with this mass in the H/D exchange experiment would be an indicator for the pres-

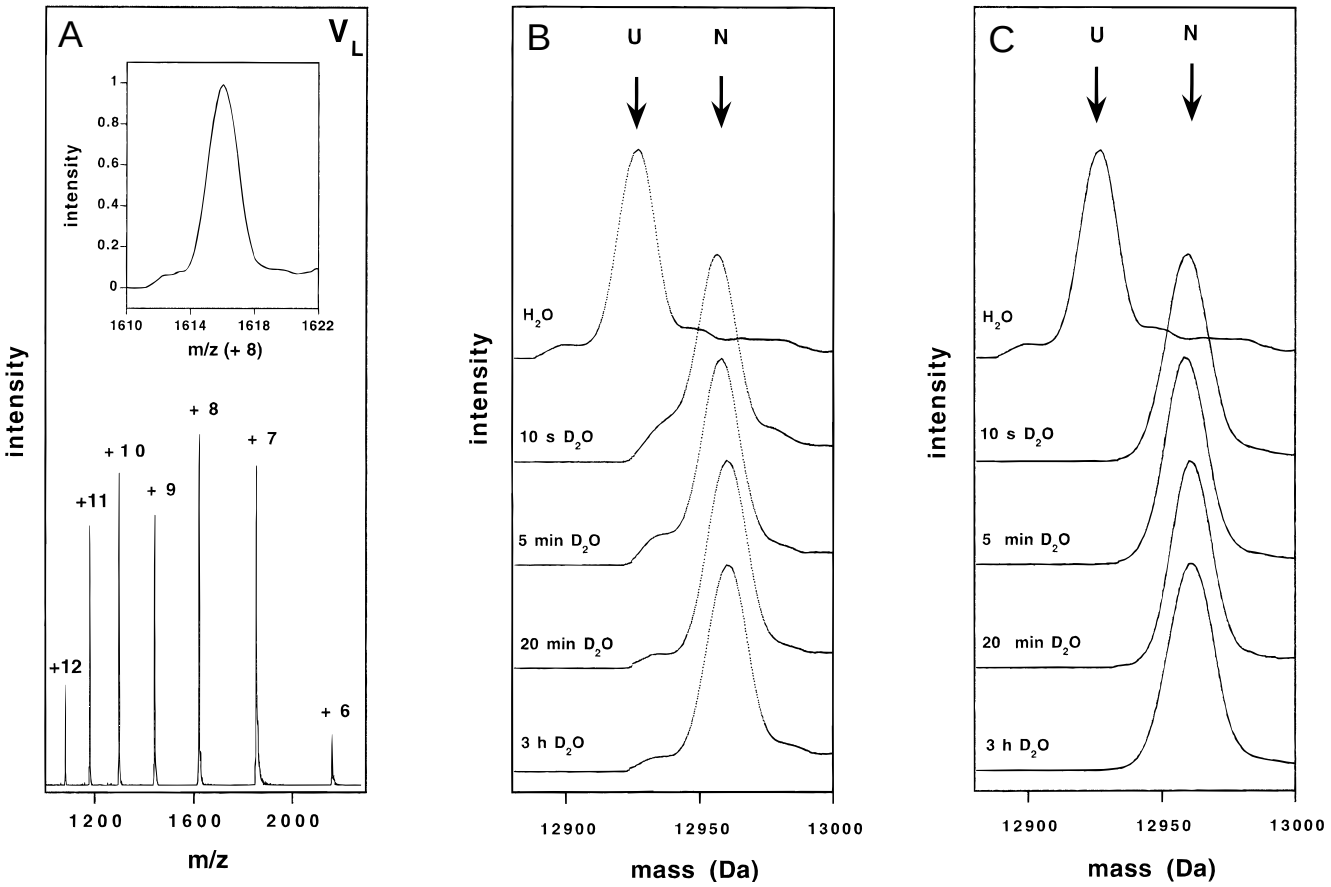


Fig. 2. A: Electrospray mass spectrum of the V_L domain. The different *m/z* peaks represent different charge states of the same molecule. This is the same sample for which the deconvoluted spectrum is shown in **B**, lowest trace (3 h folding in D_2O buffer). The insert is a blow-up of the +8 charge peak. **B:** Mass spectrometric analysis of the H/D exchange experiment of the equilibrium-denatured V_L domain. Indicated is the refolding time of the V_L domain in D_2O, before H/D exchange was quenched by manual dilution into H_2O buffer. **C:** Same as **B**, but with short-term denatured protein. The final refolding was carried out at pH 5.2 and 10 °C.

ence of unstructured conformations still present at the time point of the H/D quenching step, since all labile deuterons rapidly exchange with solvent H_2O and the original molecular weight would be regenerated (Kim, 1986; Englander et al., 1996). If the V_L domain is fully refolded in D_2O, a molecular mass of 12,960 Da is obtained. This corresponds to an incorporation of 34 deuterons, bound tightly enough to escape back-exchange with solvent protons during sample concentration, desalting and ionization in the mass spectrometer. Already after a pulse of 10 s in D_2O (the shortest pulse achievable by manual mixing), the unfolded protein disappeared almost completely. Instead, we observe a peak with a mass of 12,956 Da, corresponding to 30 stable deuterons in the V_L domain, which indicates that the reaction leading to protection from H/D exchange is essentially completed within the dead time of the manual mixing experiment and must thus be considerably faster than the two slow, proline-dependent phases monitored by fluorescence spectroscopy (Fig. 1A). Also visible is a small shoulder (see below) at a molecular weight between that of the unfolded (protonated) and native (maximally deuterated) protein, which disappears slowly within the first 20 min of refolding.

As the V_L domain contains seven proline residues (ProL8 and ProL95 are in *cis* in the native protein) (Satow et al., 1986; Steipe et al., 1991), a considerable fraction of nonnative proline conformers must be present after equilibrium denaturation. Nevertheless, the H/D exchange data show that a native-like V_L core is formed much faster than the slow proline *cis/trans* isomerization steps, the implication being that these slow isomerizations must occur in an already highly structured environment. This view is directly supported by repeating the H/D exchange experiment with a short-term unfolded V_L domain (Fig. 2C). Similar to the experiment with equilibrium-denatured protein, H/D protection is complete within the dead time of manual mixing (Table 1). In this case, there is no remaining shoulder and the peak is symmetrical (Fig. 2C). While this is speculative at present, the shoulder might be due to the formation of a small amount of microaggregates or dimeric V_L, whose formation is favored in the presence of one or more nonnative prolyl-peptide bonds, present during folding under equilibrium-denaturation conditions.

The number of deuterons trapped in short-term unfolded V_L is (within error) identical to that seen in equilibrium-denatured V_L, despite the fact that deuteration was performed in the presence of residual H_2O (25%). This suggests that there must be additional H/D exchange taking place within the first 10 s of the refolding reaction, during which time the residual protons, which should be present when the V_L domain equilibrates with solvent after short-term unfolding, are exchanged for solvent deuterons. Otherwise,

Table 1. *Mass spectrometric analysis of the kinetic H/D exchange experiments of the domains V_H and V_L*

Protein	Unfolding mode[a]	Refolding time in D_2O[b]	Mass (Da)	Protected deuterons
V_L	—	—	12,926	—
V_L	Equilibrium denaturation	10 s	12,956 ± 1	30 ± 1
V_L	Equilibrium denaturation	5 min	12,958 ± 1	32 ± 1
V_L	Equilibrium denaturation	20 min	12,959 ± 1	33 ± 1
V_L	Equilibrium denaturation	3 h	12,960 ± 1	34 ± 1
V_L	Short-term denaturation	10 s	12,958 ± 1	32 ± 1
V_L	Short-term denaturation	5 min	12,959 ± 1	33 ± 1
V_L	Short-term denaturation	20 min	12,958 ± 1	32 ± 1
V_L	Short-term denaturation	3 h	12,959 ±1	33 ± 1
V_H	—	—	13,644	—
V_H	Equilibrium denaturation	10 s	13,680 ± 2	36 ± 2
V_H	Equilibrium denaturation	5 min	13,682 ± 1	38 ± 1
V_H	Equilibrium denaturation	20 min	13,681 ± 2	37 ± 2
V_H	Equilibrium denaturation	3 h	13,683 ± 2	39 ± 2

[a] Protein unfolding was achieved either by equilibrium-denaturation (>12 h in 6 M GdmCl) or by short-term denaturation (40 s in 6 M GdmCl).
[b] Folding time in D_2O buffer before the H/D exchange was quenched by a 1:10 dilution into H_2O buffer.

only about 26 deuterons (75% of 34) should have been retained in the V_L domain after complete refolding in D_2O buffer. Such a back-exchange is, however, only possible if the rate of folding into an exchange-resistant conformation is slower than the rate of H/D exchange at the pD used (6.9, not isotope corrected). As the exchange rates for deuterons in unfolded proteins are known (Englander et al., 1996), we conclude that the rate of folding into an exchange-resistant conformation must be slower than or on the order of 10 s^{-1}. Interestingly, this estimate is similar in magnitude to the stopped-flow phase of the V_L domain (Jäger & Plückthun, 1999a), which might thus directly monitor the formation of the exchange-protected species. However, a direct support of this hypothesis will require further H/D exchange experiments performed on a millisecond time scale with rapid mixing (Freund et al., 1997b).

Refolding of the V_H domain monitored by H/D exchange

The mass spectrum of the V_H domain (Fig. 3A) also shows a single series of related peaks and a narrow distribution of low *m/z* values, indicating again a native or native-like state (Loo et al., 1991). The only very minor contaminant detectable is one of V_L (asterisks in Fig. 3A), resulting from fact that the V_H domain was obtained by chain separation of the Fv fragment. The spectrum was obtained from protein that was refolded in D_2O for 3 h, before refolding was completed in H_2O to exchange labile protons. The insert shows the +8 charge state and demonstrates that the symmetrical distribution obtained in the deconvoluted spectra (Fig. 3B) is also observed in the *m/z* peak.

Similar to the V_L domain, the H/D protection kinetics of the V_H domain is fast relative to the rate of H/D exchange and nearly completed within the dead time of the manual mixing experiment (Fig. 3B; Table 1). The protonated V_H domain has a mass of 13,644 (theoretical mass 13,644.5). During the time course of refolding, a total number of 39 deuterons are trapped in the V_H domain. When comparing these results with earlier ones using a less stable V_H domain in the context of an scFv fragment (Freund et al., 1996), we note that the number of protected deuterons found here exceeds by far the number of deuterons found in the V_H moiety of that scFv fragment (24 deuterons). The difference in protection is even more pronounced early in the refolding reaction. After a refolding pulse of 10 s in D_2O, only two highly protected deuterons are detectable by NMR spectroscopy in the V_H moiety of the scFv fragment (Freund et al., 1996), compared to the 37 very stable deuterons found by ESI-MS in the isolated V_H domain used in this study. Thus, the V_H domain used here with five framework mutations seems to be more stable, even in isolated form, than the one used earlier in the context of the scFv fragment, which carried only three mutations in V_H and none in V_L.

Refolding of the scFv fragment monitored by fluorescence

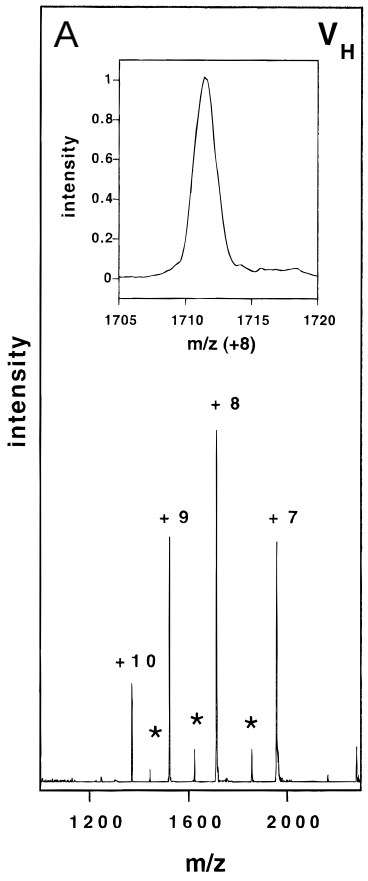
Fluorescence traces of equilibrium and short-term unfolded scFv fragment are shown in Figures 4A and 4B, respectively. Both traces are biphasic. In the equilibrium denatured scFv fragment, there is an initial fluorescence decrease (k_{fast}: 0.008 s^{-1}), followed by a slow fluorescence increase (k_{slow}: 0.00080 s^{-1}). The slow fluorescence increase monitors the formation of the native heterodimer interface, which is limited by the *cis/trans* isomerization at ProL95 (Freund et al., 1997a; Jäger & Plückthun, 1997, 1999a). In the double-jump experiment, both phases show an increase in fluorescence (k_{fast}: 0.009 s^{-1}; k_{slow} 0.00077 s^{-1}). The persistence of the slow phase in the double-jump experiment is believed to arise from a spontaneous *cis/trans* reisomerization at ProL95 in a kinetically trapped intermediate (Freund et al., 1997a; Jäger & Plückthun, 1997, 1999a). The arrows indicate the time points of the manual H/D quenching step.

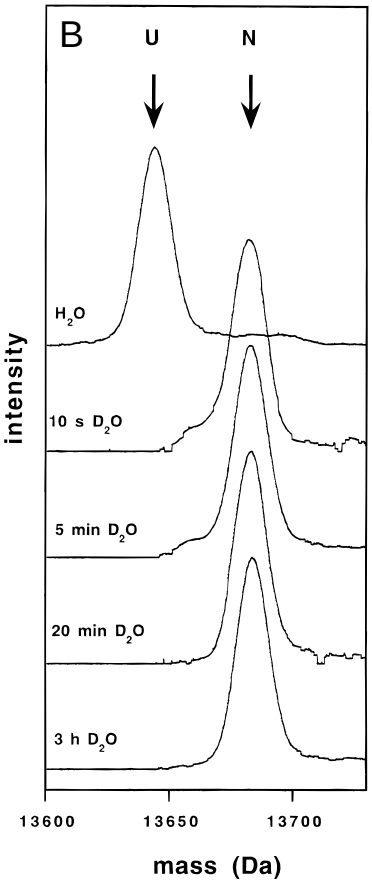
Refolding of the scFv fragment monitored by H/D exchange

The mass spectrum of the scFv fragment (Fig. 5A) shows a single series of related peaks and distribution of low *m/z* values, again being consistent with the protein having reached a native conformation after refolding. This spectrum was obtained from protein that was refolded in D_2O for 2 min, before refolding was completed in H_2O to exchange labile protons and represents the data from which the spectrum in Figure 5B have been obtained. The insert shows the +20 charge state, and demonstrates that the shoulder observed in the deconvoluted spectra (Fig. 5B, 4th trace from the top) is also observed in the *m/z* peak.

The H/D exchange kinetics of the equilibrium-denatured scFv are shown in Figure 5B. The fully deuterated scFv fragment has a mass of 28,216 Da, the protonated scFv a mass of 28,124 Da. This means that 92 deuterons are incorporated into the scFv fragment during refolding. This number is slightly higher than the number of deuterons trapped by the isolated domains together (75 deuterons). This difference in the extent of deuteration is possibly due to the higher stability of the scFv fragment because of mutual domain stabilization in the heterodimer (Jäger & Plückthun, 1999b; Wörn & Plückthun, 1999).

After a refolding time of 10 s in D_2O, the peak corresponding to the unfolded scFv fragment has disappeared completely. Instead, we observe a rather broad peak. The asymmetric peak shape suggests that this peak might represent an ensemble of conformations with slightly differing H/D exchange protection properties. At this





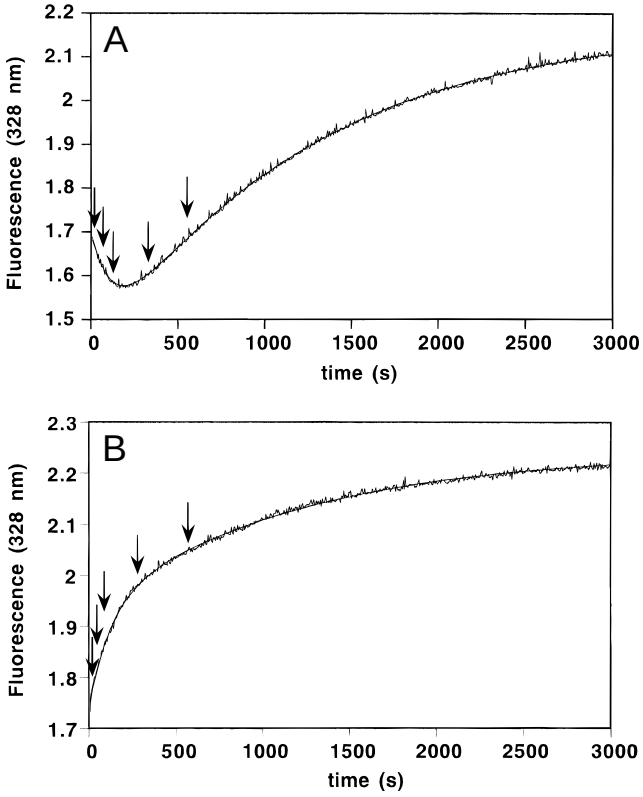
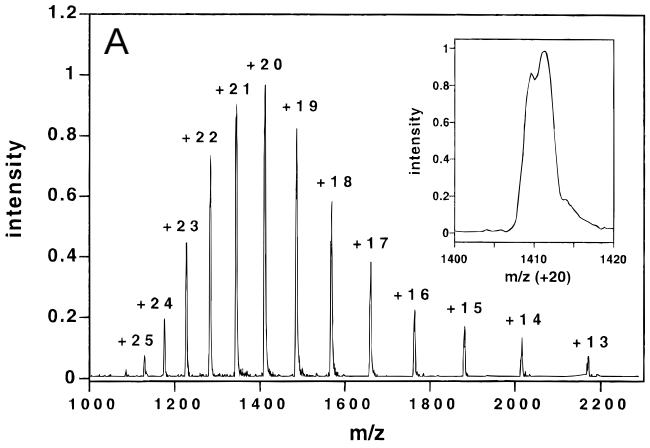


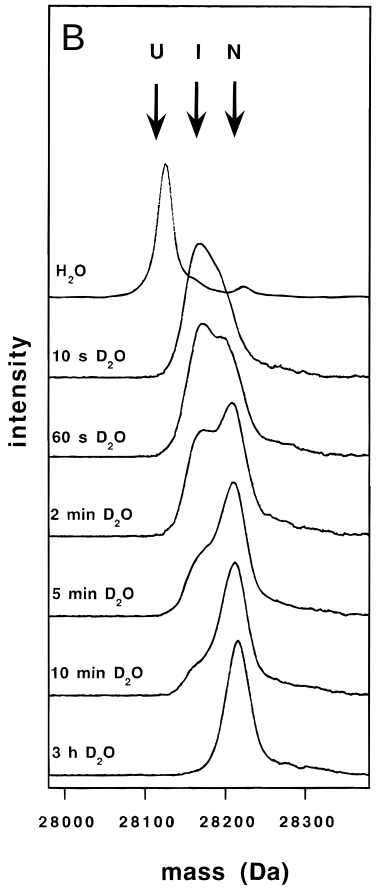
Fig. 4. Fluorescence refolding traces of (**A**) equilibrium-denatured and (**B**) short-term denatured scFv fragment. Excitation was at 295 nm; emission was at 328 nm. The fluorescence intensity was normalized to the fluorescence of the unfolded protein, which was set to 1.0. The arrows indicate the time points of the manual H/D quenching step where mass spectrometric analysis was carried out.

early time point, the peak maximum is at 28,169 Da, which corresponds to 45 deuterons trapped in the heterodimer, compared to the 66 deuterons found in the two isolated domains together after an identical folding time under identical conditions. This implies that the V_H and V_L domain, once covalently tethered by a flexible peptide linker, do not fold independently, as in such a case the level of deuteration in the scFv fragment should at least match that in the less stable isolated domains. We propose that this scFv species, with only 45 deuterons trapped, less than the sum of deuterons in the isolated V_H and V_L domain after 10 s, is the direct demonstration for early premature domain interaction in the scFv fragment, which we name "trap" in the following.

Upon longer folding times in D_2O, the population of the partially structured folding trap seems to decrease at the cost of con-

Fig. 3. A: Electrospray mass spectrum of the V_H domain. The different m/z peaks represent different charge states of the same molecule. This is the same sample for which the deconvoluted spectrum is shown in **B**, lowest trace (3 h folding in D_2O buffer). The asterisks indicate peaks from a V_L contamination. The insert is a blow-up of the +8 charge peak. **B:** Mass spectrometric analysis of the H/D exchange experiment of the equilibrium-denatured V_H domain. Indicated is the refolding time of the V_H domain in D_2O, before H/D exchange was quenched by manual dilution into H_2O buffer. The final refolding was carried out at pH 5.2 and 10 °C.





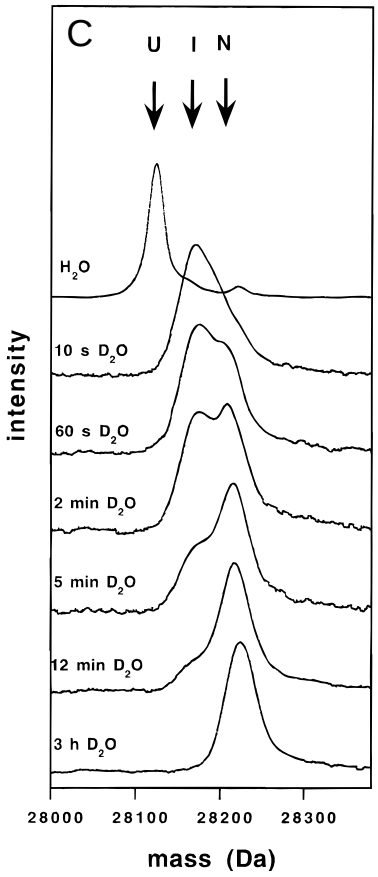


Fig. 5. A: Electrospray mass spectrum of the scFv fragment. The different *m/z* peaks represent different charge states of the same molecule. This is the same sample for which the deconvoluted spectrum is shown in **B**, 4th trace from top (2 min folding in D_2O buffer). The insert is a blow-up of the +20 charge peak. **B:** Mass spectrometric analysis of the H/D exchange experiment with equilibrium-denatured and (**C**) short-term denatured scFv fragment. The refolding time of the scFv fragment in D_2O is indicated, before H/D exchange was quenched by manual dilution into H_2O buffer. The final refolding was carried out at pH 5.2 and 10 °C.

formers with native-like H/D exchange protection properties. There is a small shift in the mass peak maximum of the native-like peak to higher molecular masses, while no such a shift is detectable in the intermediate peak (Table 2). This observation suggests that the escape from the trap to the native state might not be a simple two-state process, but instead might involve several discrete folding intermediates with similar H/D exchange characteristics (see also below).

After a refolding time of 10 min, the folding trap is only populated at hardly detectable levels, as indicated by the presence of essentially a single symmetric peak with a mass already very close to that of the native scFv fragment (Table 2). Therefore, the folding reaction of the scFv fragment appears considerably faster when monitored by H/D exchange than by fluorescence, the implication being that H/D protection cannot be limited by proline *cis/trans* isomerization. The scFv fragment is similar to the isolated domains in this respect. This uncoupling of protection and interface formation is only observed, if the domains of the scFv fragment have been stabilized by the mutations described above (Freund et al., 1996, 1997b).

As shown in Figures 4A (vertical arrows) and 4B, the time scale of the H/D exchange experiment is in the same range as the first phase in the refolding experiment monitored by the change in intrinsic Trp fluorescence at 328 nm. On the other hand, the sub-

Table 2. *Mass spectrometric analysis of the kinetic H/D exchange experiments of the scFv fragment*

Protein	Unfolding mode[a]	Refolding time in D_2O[b]	Mass (Da)		Protected deuterons	
			Peak 1[c]	Peak 2[d]	Peak 1	Peak 2
scFv	—	—	28,124 ± 2	—	—	—
scFv	Equilibrium denaturation	10 s	28,169 ± 3	n.d.[e]	45 ± 3	n. d.
scFv	Equilibrium denaturation	60 s	28,170 ± 3	28,206 ± 4	46 ± 3	82 ± 4
scFv	Equilibrium denaturation	2 min	28,170 ± 4	28,208 ± 4	46 ± 4	84 ± 4
scFv	Equilibrium denaturation	5 min	n.d.	28,210 ± 3	n.d.	86 ± 3
scFv	Equilibrium denaturation	10 min	n.d.	28,212 ± 4	n.d.	88 ± 4
scFv	Equilibrium denaturation	3 h	—	28,215 ± 3	—	91 ± 3
scFv	Short-term denaturation	10 s	28,168 ± 2	28,205 ± 3	44 ± 2	81 ± 2
scFv	Short-term denaturation	60 s	28,171 ± 3	28,207 ± 2	47 ± 3	84 ± 3
scFv	Short-term denaturation	2 min	28,172 ± 2	28,209 ± 4	48 ± 2	85 ± 2
scFv	Short-term denaturation	5 min	n.d.	28,212 ± 3	n.d.	88 ± 3
scFv	Short-term denaturation	10 min	n.d.	28,214 ± 4	n.d.	90 ± 4
scFv	Short-term denaturation	3 h	—	28,216 ± 2	—	92 ± 2

[a] Protein unfolding was achieved either by equilibrium denaturation (>12 h in 6 M GdmCl) or by short-term denaturation (40 s in 6 M GdmCl).
[b] Folding time in D_2O buffer before H/D exchange was quenched by a 1:10 dilution into H_2O buffer.
[c] Mass of the kinetic intermediate (except line 1, which shows the mass of the protonated scFv in H_2O buffer).
[d] Mass of the native-like intermediate.
[e] Exact masses could not be determined.

sequent slow fluorescence increase, which monitors proline *cis/trans* isomerization at ProL95 and the formation of the correct heterodimer interface, does not correspond to a detectable further change in the H/D exchange experiment, indicating that this slow phase monitors the correct docking of essentially native-like domains.

We interpret the initial fluorescence decrease in the scFv fragment (Fig. 4A), which occurs about one order of magnitude more slowly than the corresponding fluorescence decrease in the Fv fragment (Jäger & Plückthun, 1999a), as the return from the trap (see Discussion), and this provides a simple explanation, why a phase with such a rate is only detectable in the refolding of the scFv fragment, but not in the refolding of the corresponding Fv fragment, which lacks the interdomain linker peptide. In the Fv fragment, the formation of the trap is disfavored at low protein concentrations (lower μM range) and after equilibrium denaturation, as the equilibrium is on the side of the individual domains at low protein concentration, which are furthermore disfavored to associate before *cis*-ProL95 has formed, a requirement for productive chain association. These factors favor the fast and independent domain folding into structured conformations in the Fv fragment and the individual domains, as indicated by the H/D exchange experiments reported here. The linker peptide in the scFv fragment results in a high effective domain concentration, which favors the premature collision of domains, leading to the trap. While it might seem formally possible that the linker itself interacts with the domains, previous NMR experiments have clearly shown the absence of any stable interaction between linker and antibody domains (Freund et al., 1993).

The trap is formed independently of proline cis/trans isomerization

To prove directly that the H/D exchange protection is not affected by proline *cis/trans* isomerization in the scFv fragment, the H/D exchange experiment was repeated with short-term unfolded scFv fragment (Fig. 5C). Both the shape of the mass spectra as well as the peak mass centers are comparable within experimental error to those obtained in the equilibrium-denaturation experiment (Table 2), and within the limits of the experiment, there is also no evidence for an increased population of native protein present at 10 s, nor is there a significant difference in the number of protected deuterons detectable after short-term and equilibrium unfolding. This indicates again that the residual protons present in the unfolded polypeptide chain are back-exchanged for solvent deuterons early in the refolding reaction, similar to the situation in the V_L domain described above.

As in the experiment with equilibrium-denatured protein (Figs. 4A, 4C, 5B), there is general agreement between the time scale of the faster of the two manual mixing phases in the fluorescence refolding experiment (Fig. 4B) and the time scale of folding monitored by H/D exchange (time points of the H/D quenching steps are indicated by vertical arrows in Fig. 5C). The only difference is that the fast fluorescence phase now shows a fluorescence increase (Fig. 4B).

Apparent rate constants for folding into and out of the trap

Figure 6A (trace Fv) shows the refolding reaction of the Fv fragment monitored by the change in fluorescence intensity at >305 nm. A large decrease in fluorescence is seen, and the fluorescence trace can be fitted to a double exponential with kinetic rate constants of 1.87 s^{-1} (relative amplitude: 0.43) and 0.091 s^{-1} (relative amplitude: 0.57) for the fast and slow phase, respectively. Assigning the two phases to a discrete folding step in one of the two domains is difficult, as each of the two isolated domains gives biphasic fluorescence kinetics, but with different sign of amplitude (Jäger & Plückthun, 1999a). Nevertheless, the slower of the two phases reported here is comparable to the slow stopped-flow phase of the isolated V_H domain. At these concentrations and in the presence of

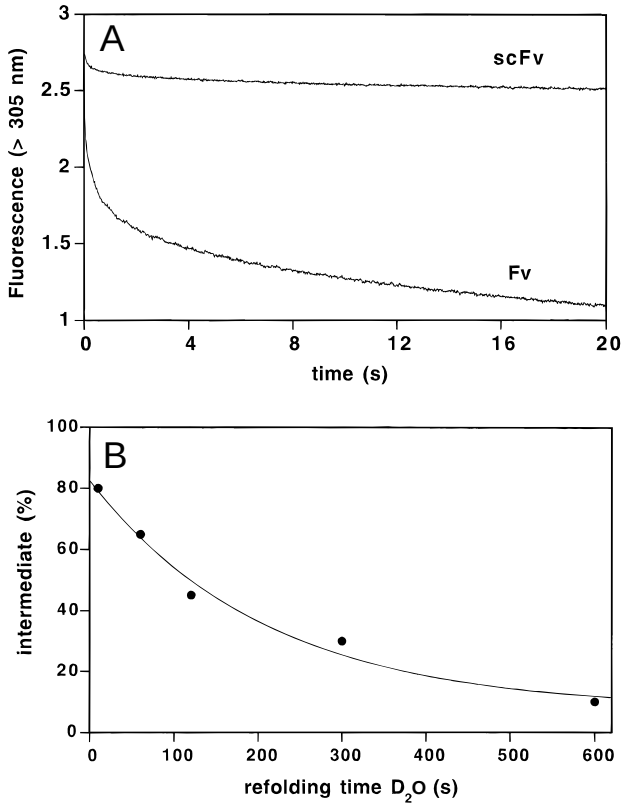


Fig. 6. A: Evidence for very early domain interactions in the scFv fragment. Unfolded Fv or scFv fragment in 4 M GdmCl was diluted 1:10 (v:v) into refolding buffer in a stopped-flow apparatus and the change in fluorescence at >305 nm was monitored using a cut-off filter. Protein excitation was at 295 nm, and the final protein concentration after initiation of refolding was 2 μM. The arrows indicate the fluorescence emission intensity after extrapolation to zero time. The experiments were carried out at pH 6.0 and 10 °C. **B:** Decrease in the relative population of the scFv-specific trapped intermediate as a function of the refolding time, from the data in Figure 5. The amount of trap formed at the H/D quenching time points was estimated by visual inspection of the percentage of the intermediate peak area on the total peak area. The half-life of the trapped intermediate was approximated by fitting the points to a single exponential. The experiments were carried out at pH 6.0 and 10 °C.

antigen, the bimolecular reaction is fast and not rate limiting (Jäger & Plückthun, 1999a).

In contrast, a much smaller decrease is obtained with the scFv fragment at identical conditions (Fig. 6A, trace scFv). The decrease in fluorescence accounts for only 15% of the change seen in the Fv fragment. The fluorescence trace is also biphasic, with rate constants of 2.38 s^{-1} (relative amplitude: 0.35) and 0.059 s^{-1} (relative amplitude: 0.65) for the fast and slow phase, respectively. The rate constants (but not the amplitudes) are therefore comparable to the rate constants obtained for the Fv fragment. This observation immediately suggests that only a small fraction of the unfolded scFv fragment displays the fast refolding behavior of the Fv fragment, while the major fraction (~85%) of unfolded scFv fragment does not fold in an analogous way on a stopped-flow time scale, as indicated by the missing fluorescence amplitude. Interestingly, a similar conclusion has been obtained from the H/D exchange experiments described above, where a small fraction of native-like protein was already present after 10 s, whereas the bulk protein shows only half the maximally protected number of deuterons. We therefore suggest that the missing folding amplitude in the scFv fragment is directly related to the formation of the trap, which immediately suggests that the trap is formed very rapidly. At least the data show that both domains in the scFv fragment interact within the dead time of the stopped-flow experiment.

The rate constant for the escape from the folding trap to productive folding into the native state can only be approximated, because of the limited number of H/D quenching points available. Figure 6B shows that the decrease in the population of the trap, monitored in the H/D exchange experiment, can be satisfactorily fitted to a single exponential. Interestingly, the obtained rate constant of 0.004 s^{-1} is identical to the major ANS desorption phase seen in the scFv fragment (0.004 s^{-1}), which is much faster and smaller in amplitude in the corresponding Fv fragment (Jäger & Plückthun, 1999a) and is still comparable to the fast phase seen in the fluorescence experiment (~0.007 s^{-1}). Again, an analogous fluorescence phase is missing in the refolding of the Fv fragment (Jäger & Plückthun, 1999a). The fluorescence decrease seen in the Fv fragment occurs at a ten times faster rate and is consistent with being due to the folding of the V_H domain before heterodimerization with the V_L domain into the Fv heterodimer takes place. The minor differences in the rate constants evaluated by the H/D exchange (~0.004 s^{-1}) and ANS desorption experiments (~0.004 s^{-1}) (Jäger & Plückthun, 1999a) on the one hand and the intrinsic Trp fluorescence (0.007–0.009 s^{-1}) on the other hand most likely result from the heterogeneity of the trap, as already inferred from the manual H/D exchange experiment (see above).

Discussion

The aim of this study was to complement our previous fluorescence spectroscopic studies on the Fv and scFv fragment of the antibody McPC603 (Freund et al., 1996, 1997a, 1997b; Jäger & Plückthun, 1997, 1999a) and to provide *direct* evidence for the existence of an scFv-specific folding trap (Ramm et al., 1999). For this purpose, the folding of a stabilized variant of this scFv fragment was compared with the folding of the isolated domains using H/D exchange and ESI-MS. In contrast to NMR, it can be used to directly monitor distinct folding intermediates, in addition to unfolded and native states (Miranker et al., 1993; Hooke et al., 1995).

Folding of the single domains and the Fv fragment

Models derived from a combination of earlier fluorescence data with the mass spectrometric data described here are summarized in Figures 7A and 7B. The isolated V_L domain gains native-like protection within the dead time of the manual H/D exchange experiment (10 s) and independent of the fact of whether the folding reaction was initiated from equilibrium-denatured or short-term unfolded protein. While still to be proven by H/D exchange experiments in the ms time scale, it is reasonable to assume that the major fluorescence decrease detectable in the stopped-flow refolding experiment (rate constant: 10 s^{-1}) (Jäger & Plückthun, 1999a) is also the reaction where the H/D exchange protection is gained, as all subsequent phases are much slower in rate. As all protection is complete after 10 s, the slow proline *cis*/*trans* isomerization in the refolding of the V_L domain must occur in a highly structured intermediate (I_L) with native-like H/D exchange properties.

A biphasic reaction is observable on the measured time scale, when the folding reaction is monitored by the change in fluores-

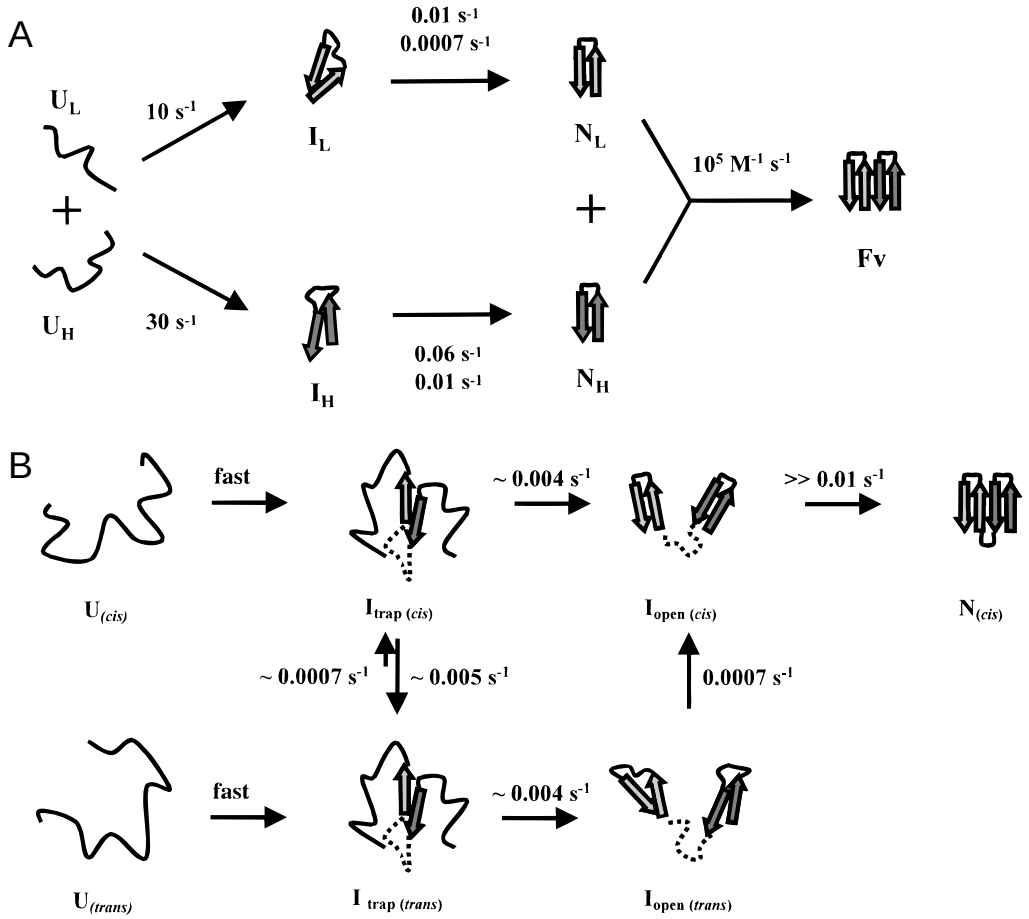


Fig. 7. Proposed refolding schemes for (**A**) the V_L and V_H domains and the Fv fragment and (**B**) the scFv fragment. See text for explanations.

cence emission intensity of the single core TrpL36. However, the nature of these slow steps (I_L to N_L in Fig. 7A, for reasons of simplicity not detailed further) subsequent to the completion of protection are the subject of further investigations. It is possible that one of them involves dimeric species or microaggregates, and both steps are catalyzed by proline *cis/trans* isomerase (Jäger & Plückthun, 1999a).

Association of the V_L domain with the V_H domain into the Fv heterodimer occurs only after the rate-limiting *cis/trans* isomerization at ProL95, as a *cis*-ProL95 is required for stable heterodimerization (Jäger & Plückthun, 1999a). Only a monoexponential reaction is visible in which the native interface is formed (except for the ~10% of V_L molecules, which contain a *cis*-ProL95 under equilibrium denaturation conditions, and which heterodimerize with the V_H domain in the dead time of manual mixing at micromolar protein concentrations) (Jäger & Plückthun, 1999a). This requires that all molecules go through a native state with ProL95 in *cis* before association (Fig. 7A, N_L to Fv).

The V_H domain also shows full H/D protection after 10 s of folding in D_2O buffer (I_H), and the number of trapped deuterons is even slightly higher than that in the V_L domain. Despite the formation of a native-like intermediate in the dead time of manual mixing, the V_H domain is not yet association competent with the native V_L domain at this stage of folding, as evidenced by the fact that the fluorescence increase, which is indicative for correct interface formation, takes longer to develop (Jäger & Plückthun, 1999a). Association competence is gained in two slower, apparently parallel pathways (about 70% of the V_H molecules refold via the fast folding track), which are only detectable by fluorescence spectroscopy and which might involve only subtle changes in solvent-exposed regions of the V_H domain. Whether this heterogeneity, leading to two parallel pathways, already exists on the level of the first intermediate I_H or is formed later in two diverging steps from I_H to N_H remains to be shown.

Folding of the scFv fragment

The folding of the scFv fragment is summarized in Figure 7B, which is an extension and modification of an earlier folding scheme, which had been solely based on fluorescence kinetic data (Jäger & Plückthun, 1997). Upon refolding of the equilibrium denatured scFv fragment $U_{(trans)}$, about 90% of the unfolded molecules fold rapidly into an intermediate $I_{trap(trans)}$ within the deadtime of the stopped-flow experiment (a few ms).

The intermediate $I_{trap(trans)}$ is partially structured with 45 trapped deuterons observable in the mass spectrum at the shortest time point resolvable by manual mixing (10 s). Since this is *less* than the sum of V_H and V_L at identical time (66 deuterons), we conclude that

the two domains must mutually retard their folding at a nonnative, yet significantly structured stage. This difference in protection is even more significant, as the native scFv fragment shows *higher* protection than the sum of the two native domains.

We therefore define this intermediate as I_{trap}, as it retards the formation of the two native domains. While it is, strictly speaking, on-pathway for the equilibrium denatured molecules, it constitutes still a "detour," compared to the isolated domains. How does the folding proceed from $I_{trap(trans)}$? There are two conceivable routes, one of proline *cis/trans* isomerization at this stage (leading to $I_{trap(cis)}$), and the other a continuation to $I_{open(trans)}$, the equivalent of two native domains (except for the prolines), tethered by the linker.

To distinguish these possibilities, we have to first consider the behavior of $I_{trap(cis)}$. The mass spectrometric data clearly indicate that the trap $I_{trap(cis)}$ is also formed after short-term unfolding, that is, from $U_{(cis)}$. The main argument is that the mass spectra are virtually identical at different time points for short- and long-term denatured protein (Fig. 5B,C). While the molecules do reisomerize from $I_{trap(cis)}$ to $I_{trap(trans)}$, this is only about half the population, as seen by fluorescence spectroscopy (see below). If only molecules at $I_{trap(trans)}$ were retarded in their folding, a much faster native-like protection should be seen in the double-jump experiment, which is not found. It thus follows that $I_{trap(cis)}$ also retards folding. The kinetics of escape from both I_{trap} species, *cis* and *trans*, to their respective open species I_{open} appear to be about the same.

Therefore, the isomerization within I_{trap} might well occur from *trans* to *cis*, but there is no reason to assume that it is faster than the *trans* to *cis* isomerization within I_{open}, that is, about 0.0007 s^{-1}. Therefore, the main flux will be from $I_{trap(trans)}$ to $I_{open(trans)}$, as this reaction is faster. However, there is no doubt that the reverse *cis* to *trans* isomerization takes place at the I_{trap} stage, which would argue for a nonnative structure around the proline(s) in question stabilizing the *trans* state and favoring the reaction going in this direction. The main argument is the reappearance of the very slow fluorescence phase despite double-jump experiments. This slow phase (about 0.0007 s^{-1}) is due to proline *cis/trans* isomerization. Once in $I_{trap(cis)}$, the molecules escape only slowly with kinetics on the minutes time scale (approximate rate constant: 0.004 s^{-1}), according to the H/D exchange experiment. This leads to a kinetic competition between the escape from $I_{trap(cis)}$ and subsequent fast folding into $N_{(cis)}$ on the one hand (faster of the fluorescence phases in Fig. 4B) and proline *cis/trans* reisomerization, which leads to the accumulation of $I_{trap(trans)}$ species and which give rise to the slower of the fluorescence phases (Fig. 4B).

The slow reaction that is monitored in the manual mixing H/D exchange experiment must then be the escape from the trap, a reaction by which the constituting domains gain their full H/D exchange protection. Native scFv protein $N_{(cis)}$ is only formed after the *trans* to *cis* isomerization at ProL95, which is rate limiting for the overall folding reaction and which can be observed by fluorescence spectroscopy (slow fluorescence increase in Fig. 4A), but not by H/D exchange. By the time this isomerization occurs, the domains are essentially native.

This current outline model also provides an explanation of why PPIase catalyzes the formation of the *trans* state starting from $U_{(cis)}$ (Freund et al., 1997b; Jäger & Plückthun, 1999a). If the nonnative structure of I_{trap} favors the *trans* conformation of ProL95 and PPIase acts directly on $I_{trap(cis)}$, it would accelerate the formation of $I_{trap(trans)}$. At the same time, the rate acceleration effect of PPIase on the refolding of $U_{(trans)}$, by catalyzing the conversion $I_{open(trans)}$ to $I_{open(cis)}$, levels off at high stoichiometric enzyme concentrations, possibly since the escape from $I_{trap(trans)}$ becomes rate limiting (Jäger & Plückthun, 1997).

Can the escape from the trap be followed by fluorescence spectroscopy? We note that folding both after equilibrium denaturation and double-jump experiments is biphasic, with the slower phase always giving rise to a fluorescence increase at 328 nm with the same slow kinetics that monitors the interface formation, subsequent to proline *cis/trans* isomerization (about 0.0007 s^{-1}) (Jäger & Plückthun, 1997, 1999a). The faster of the two rates in manual mixing has opposite signs (Fig. 4A,B) in both experiments. After short-term denaturation, the observed *increase* would reflect molecules that reach the native monomer while still carrying *cis* prolines. They must be held up by the escape from the trap, $I_{trap(cis)}$ to $I_{open(cis)}$, but the open form $I_{open(cis)}$ then immediately converts to the native scFv fragment, such that the rate of interface formation is in fact limited by the escape reaction. In contrast, if the molecules that escape from the trap have a *trans*-ProL95, the native heterodimer (monitored by a fluorescence increase) cannot be formed before ProL95 slowly isomerizes to *cis*, with the consequence that the two domains first dissociate after escaping from the trap, which is manifest as a fluorescence *decrease*. We caution that the interpretation of the magnitude of the rate constants of this fluorescence phase is difficult (about 0.008 or 0.009 s^{-1}; see Fig. 4A,B), as both domains themselves give rise to slow fluorescence phases (always as an intensity decrease), and it is clear that they would mix into the observed values.

Comparison to other scFv fragments

The H/D exchange results obtained in this study are different from those obtained with another, less stable derivative of the same scFv fragment, which carries only three out of the eight mutations (Freund et al., 1996). In that case, protection was much slower in the equilibrium-denatured than in the short-term denatured protein and comparable in rate to the rate-limiting *cis/trans* isomerization reaction at ProL95. This means that in the less stable protein, additional protection depends on the correct interface formation as a prerequisite for mutual domain stabilization (Wörn & Plückthun, 1999). Interface formation, in turn, is dependent on the ProL95 configuration, and thus the denaturation method is reflected in the protected deuterons observed (Freund et al., 1997b), as they monitor native interface formation. Quite in contrast, in the present case, the domains of the engineered scFv fragment are stable enough to fold by themselves and keep the trapped deuterons in an exchange-resistant state. Therefore, they are not influenced much by interface formation, and thus no difference is seen between equilibrium- and short-term unfolded scFv.

We believe that this investigation further helps in understanding the folding pathway and side reactions of immunoglobulin domains and will be useful for the engineering of improved antibody variants, in particular for minimizing nonproductive intermediates. Nevertheless, detailed structural knowledge of the on- and off-pathway intermediates at residue resolution will now be required to understand this process in greater detail.

Materials and methods

Proteins

All residue numbering is according to Kabat (Kabat et al., 1991). The V_H domain carries the mutations P(H40)A, S(H63)A,

A(H64)D, S(H79)N, I(H80)T, which improve in vivo and in vitro folding efficiency (Knappik & Plückthun, 1995; Jäger & Plückthun, 1997, 1999a; H. Bothmann & A. Plückthun, unpubl. data). The V_L domain used in this study carries the three stabilizing framework mutations A(L15)L, S(L56)P, and N(L90)Q (Steipe et al., 1994; Ohage et al., 1997). The two C-terminal amino acids of the V_L domain were removed and replaced by a His_5 tag (Lindner et al., 1992). The scFv fragment was obtained by fusing the C-terminus of the V_H domain with the N-terminus of the V_L domain by a flexible $(G_4S)_5$ linker. The scFv fragment was expressed in the *Escherichia coli* strain JM83 and purified as described (Jäger & Plückthun, 1999a). The isolated domains V_H and V_L were obtained from pure Fv fragment using a previously published chain separation procedure under native conditions (Jäger & Plückthun, 1999a). Freshly purified proteins were dialyzed extensively against 20 mM Mes, pH 6.0 and stored at 4 °C until use.

Refolding experiments detected by fluorescence

Manual refolding experiments were performed in 20 mM Mes, pH 6.0, 200 mM arginine and were followed on a PTI Alpha Scan spectrofluorimeter (Photon Technologies Inc., Ontario, Canada). In the case of the scFv fragment, 10 mM PC was added to the refolding buffer to irreversibly trap the heterodimer in the native conformation. Folding reactions were initiated by a manual 1:100 dilution of unfolded protein into refolding buffer, while monitoring the change in fluorescence at 328 nm (scFv and V_H domain) or 350 nm (V_L domain) upon excitation at 295 nm. The final protein concentrations were 2 μM for V_L domain, 1 μM for the V_H domain and 0.8 μM the scFv fragment. Equilibrium denatured protein, with all prolyl-peptide bonds at equilibrium, was prepared by a 1:4 dilution of native protein into 8 M GdmCl (6 M final denaturant concentration), followed by incubation for >12 h at 10 °C. Short term unfolded protein was prepared by a 1:4 dilution of native protein into 8 M GdmCl (6 M final denaturant concentration) for only 20 s, followed by an immediate 1:100 dilution step into refolding buffer to initiate folding. Stopped-flow experiments confirmed complete unfolding of the scFv fragment and the V_L domain within <20 s unfolding in 6 M GdmCl (Jäger & Plückthun, 1999b).

Stopped flow refolding experiments were performed with an Applied Photophysics model SX-17MV spectrofluorimeter (Applied Photophysics, Leatherhead, England) at 10 °C. Unfolded protein (20 μM Fv or scFv fragment) in 20 mM Mes, pH 6.0, 4 M GdmCl was diluted 1:10 (v:v) into refolding buffer (20 mM Mes, pH 6.0, 200 mM arginine), and the change in fluorescence at >305 nm was recorded using a cut-off filter. A bandpass of 1.5 and 2.0 mm was used for excitation and emission, respectively. Protein excitation was at 295 nm.

H/D exchange experiments

Two types of H/D exchange experiments are described. In the first type of experiments, refolding was started from an equilibrium-denatured and fully deuterated protein. To achieve complete deuteration, affinity-purified protein was extensively dialyzed against water, lyophilized, dissolved in D_2O buffer (20 mM MOPS, pD 6.7, containing 6 M GdmCl) and incubated for >12 h at room temperature. The pD of the solution was not isotope-corrected. For the initiation of refolding, the protein was diluted 1:100 into 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pD 6.7, 200 mM arginine, dissolved in D_2O. After various times, the protein was diluted again 10-fold into H_2O buffer containing 50 mM ammonium acetate, pH 5.2 to allow all labile deuterons to exchange for protons. The final volume was typically 20 mL. The reaction was then allowed to go to completion (3 h at 10 °C). The protein solution was concentrated at 4 °C (Centricon 10), loaded onto a PD-10 column (Pharmacia, Uppsala, Sweden), equilibrated in 10 mM ammonium acetate, pH 5.0 in H_2O to remove buffer salts and arginine, concentrated to a final volume of ~30–40 μL and stored on ice until used for mass determination.

A slightly different protocol was used for labeling short-term denatured protein. Native and fully protonated scFv or V_L was first concentrated to ~500 μM. Deuteration was then achieved by a 1:4 dilution of the protein into 20 mM MOPS, pD 6.7, 8 M GdmCl in D_2O (6 M final denaturant concentration). Unfolding of both proteins is complete in <20 s under these conditions (Jäger & Plückthun, 1999b). The exchange rate for labile, unprotected protons in an unfolded protein is ~10 s^{-1} at pD 6.7 (Englander et al., 1996). After 40 s incubation in 6 M GdmCl, which is long enough to unfold the protein completely and to achieve maximal possible deuteration (75%), the protein was further diluted 1:100 into 20 mM MOPS, pD 6.7, 200 mM arginine in D_2O to initiate the refolding reaction. All subsequent steps were performed as described above.

Mass spectrometry

Electrospray ionization mass spectra were collected using an API III triple-quadrupole instrument (PE-Sciex, Ontario, Canada). Protein solutions were infused into the ion source at a flow rate of 8 μL/min by a syringe pump. The samples were mixed in a 1:1 ratio with precooled methanol containing 0.5% formic acid, resulting in a pH of ~2.5, and 5 μL of this mixture were immediately flow-injected into ice-cold carrier solution (50% methanol, 0.25% formic acid). The use of cooled solutions and the adjustment of the pH value of the protein samples to pH 2.5 for the mass spectrometric measurements were found to minimize back-exchange considerably. An ion spray voltage of 5,000 V and an orifice voltage of 75 V were applied for all measurements. The resolution of the mass spectrometer was tuned to give a constant peak width of ~1 Da (full-width at half-maximum) across the mass range of interest. Mass spectra were recorded in the m/z range from 1,000 to 2,300 Da with a scan step size of 0.2 Da and a scan duration of 5 s.

Acknowledgments

Financial support was from the Schweizerischer Nationalfonds, Grant 3100-46624.96. We would like to thank Dr. P. Gehrig for help with the mass spectrometry experiments, Prof. J. Robinson (Organisch-chemisches Institut, Universität Zürich) for the use of his stopped-flow fluorimeter and Prof. H.R. Bosshard for his interest in this work and for helpful comments on the manuscript.

References

Englander SW, Sosnick TR, Englander JJ, Mayne L. 1996. Mechanisms and uses of hydrogen exchange. *Curr Opin Struct Biol 6*:18–23.

Freund C, Gehrig P, Baici A, Holak TA, Plückthun A. 1997a. Parallel pathways in the folding of a short-term denatured scFv fragment of an antibody. *Fold Des 3*:39–49.

Freund C, Gehrig P, Holak TA, Plückthun A. 1997b. Comparison of the H/D-exchange behavior of the rapidly formed folding intermediate and the native state of the scFv fragment of an antibody. *FEBS Lett 407*:42–46.

Freund C, Honegger A, Hunziker P, Holak TA, Plückthun A. 1996. Folding nuclei of the scFv fragment of an antibody. *Biochemistry 35*:8457–8464.

Freund C, Ross A, Guth B, Plückthun A, Holak T. 1993. Characterization of the linker peptide of the single-chain Fv fragment of an antibody by NMR spectroscopy. *FEBS Lett 320*:97–100.

Hooke SD, Eyles SE, Miranker A, Radford SE, Robinson CV, Dobson CM. 1995. Cooperative elements in protein folding monitored by electrospray ionization mass spectrometry. *J Am Chem Soc 117*:7548–7549.

Jaenicke R. 1987. Folding and association of proteins. *Prog Biophys Mol Biol 49*:117–237.

Jäger M, Plückthun A. 1997. The rate-limiting steps for the folding of an antibody scFv fragment. *FEBS Lett 418*:106–110.

Jäger M, Plückthun A. 1999a. Folding and assembly of an antibody Fv fragment, a heterodimer stabilized by antigen. *J Mol Biol 285*:2005–2019.

Jäger M, Plückthun A. 1999b. Domain interactions in antibody Fv and scFv fragments: Effects on unfolding kinetics and equilibria. *FEBS Lett 462*:307–312.

Kabat EA, Wu TT, Reid-Miller M, Perry HM, Gottesmann KS, Foeller C. 1991. Sequences of proteins of immunological interest. 5th ed. NIH Publication no. 91-3242. Washington, DC: U.S. Department of Health and Human Services.

Kim PS. 1986. Amide proton exchange as a probe of protein folding pathways. *Methods Enzymol 131*:136–160.

Knappik A, Plückthun A. 1995. Engineered turns of a recombinant antibody improve its *in vivo* folding. *Protein Eng 8*:81–89.

Lindner P, Guth B, Wülfing C, Steipe B, Müller F, Plückthun A. 1992. Purification of native proteins from the cytoplasm and periplasm of *Escherichia coli* using IMAC and histidine tails: A comparison of proteins and protocols. *Methods 4*:41–56.

Loo JA, Loo RR, Udseth HR, Edmonds CG, Smith RD. 1991. Solvent-induced conformational changes of polypeptides probed by electrospray-ionization mass spectrometry. *Rapid Comm Mass Spectrom 5*:101–105.

Miranker A, Robinson CV, Radford SE, Aplin RT, Dobson CM. 1993. Detection of transient protein folding populations by mass spectrometry. *Science 262*:896–900.

Ohage EA, Graml W, Walter MM, Steinbacher S, Steipe B. 1997. Turn propensities as paradigms for the analysis of structural motifs to engineer protein stability. *Protein Sci 6*:233–241.

Padlan EA. 1994. X-ray crystallography of antibodies. *Adv Prot Chem 49*:57–133.

Ptitsyn OB, Pain RH, Semisotnow GV, Zerovnik E, Razgulajev OI. 1990. Evidence for a molten globule state as a general intermediate in protein folding. *FEBS Lett 262*:20–26.

Ramm K, Gehrig P, Plückthun A. 1999. Removal of the conserved disulfide bridges from the scFv fragment of an antibody: Effects on folding kinetics and aggregation. *J Mol Biol 290*:535–546.

Satow Y, Cohen GH, Padlan EA, Davies D. 1986. Phosphorylcholine binding immunoglobulin Fab McPC603. *J Mol Biol 190*:593–604.

Steipe B, Plückthun A, Huber R. 1991. Refined crystal structure of a recombinant immunoglobulin domain and a complementarity-determining region I grafted mutant. *J Mol Biol 225*:739–753.

Steipe B, Schiller B, Plückthun A, Steinbacher S. 1994. Sequence statistics reliably predict stabilizing mutations in a protein domain. *J Mol Biol 240*:188–192.

Wörn A, Plückthun A. 1999. Different equilibrium stability behavior of scFv fragments: Identification, classification, and improvement by protein engineering. *Biochemistry 38*:8739–8750.