Article No. jmbi.1999.2854 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (1999) 290, 535–546

JMB

Removal of the Conserved Disulfide Bridges from the scFv Fragment of an Antibody: Effects on Folding Kinetics and Aggregation

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Fluorescence measurements and H/^{2}H exchange experiments monitored by mass spectrometry have been applied to investigate the influence of the conserved disulfide bridges on the folding behavior and *in vitro* aggregation properties of the scFv fragment of the antibody hu4D5-8. A set of four proteins, carrying none, one, or both of the disulfide bridges have been compared regarding their stabilities, folding kinetics and tendency to aggregate. The results show that refolding of all four scFvs is ultimately limited by a slow proline isomerization in the V_L domain, since the native *cis*-conformation of proline L95 seems to be a prerequisite for formation of the native interface. Starting from short-term denatured protein, with the proline residues in their native conformation, a kinetically trapped intermediate is populated depending on the conditions, whose rate of conversion is slower than that of the fast-folding molecules. According to deuteron protection patterns determined by mass spectrometry, those domains retaining the disulfide bridge are able to form stable native-like structure, independent of native interface formation. The disulfide-free domains, in contrast, require the native interface for sufficient stabilization. The resistance of the scFvs towards aggregation seems to be critically dependent on the presence of the disulfide bridge in the V_H domain, and thus on the ability of the V_H domain to form stable structure prior to interaction with the V_L domain. The presence of a stable V_L domain in combination with a disulfide-free V_H domain appears to further promote aggregation, indicating the involvement of structured domains in the aggregates.

Keywords: protein folding; aggregation; kinetic trap; disulfide-free scFv fragments; hydrogen exchange

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Introduction

Antibody domains carry a highly conserved internal disulfide bond, connecting both β-sheets of the β-sandwich structure (Alzari *et al*., 1988; Davies *et al*., 1990; Padlan, 1996; Proba *et al*., 1997). The importance of this disulfide bond for protein stability has been demonstrated first through refolding of reduced C_L domains (Goto & Hamaguchi, 1979), and later through genetic removal of the disulfide bonds in variable domains (Glockshuber *et al*., 1992; Frisch *et al*., 1994, 1996; Wörn & Plückthun, 1998) and through the study of natural antibodies missing one of the disulfide bonds (Proba *et al*., 1997; Langedijk *et al*., 1998). There is great interest in obtaining functional disulfide-free antibody fragments for use as intracellular antibodies (intrabodies), i.e. antibodies that can be expressed functionally in the reducing environment of the cytoplasm, where disulfide bonds cannot be formed (Chen *et al*., 1994; Richardson & Marasco, 1995; Biocca & Cattaneo, 1995). Such intrabodies might become important tools in functional genomics and perhaps even in therapeutic applications

Abbreviations used: ELISA, enzyme-linked immunosorbent assay; GdnHCl, guanidine hydrochloride; Gdn^{2}HCl, guanidine deuteriumchloride; MS, mass spectrometry; PPI, peptidyl prolyl *cis-trans* isomerase; scFv, single-chain antibody fragment consisting of the variable domains of the heavy and the light chain connected by a peptide linker, V_H, variable domain of the heavy chain of an antibody; V_L, variable domain of the light chain of an antibody; wt, wild-type.

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0022-2836/99/270535–12 $30.00/0

(Biocca & Cattaneo, 1995; Marasco, 1995). It has been shown that production of an entirely cysteine-free scFv fragment is possible, either by increasing the stability by directed evolution (Proba *et al*., 1998), or by starting from an already very stable scFv (Wörn & Plückthun, 1998).

The oxidative folding of antibody fragments *in vitro* is highly complex, with chemical processes of disulfide formation and proline isomerization limiting the folding rates. However, even when starting from the disulfide-containing molecule, the folding reaction consists of a large number of phases which have been difficult to untangle. Additional factors related to docking and annealing of domains that have been shown to be rate limiting for larger proteins (Jaenicke, 1987; Seckler & Jaenicke, 1992) are almost certainly involved in antibody folding. Thus, the interaction of the domains during refolding of an antibody has been proposed to lead to a non-additive folding behavior (Goto *et al*., 1979).

The process of structure formation during *in vitro* refolding of a scFv fragment has been described in considerable detail for the scFv fragment McPC603 and some stabilized mutants thereof (Freund *et al*., 1996, 1997a,b; Jäger & Plückthun, 1997, 1999). It was found that the kinetic mechanism is quite complex. Briefly, the folding of both V_H and V_L domains is multiphasic, but a state with considerable structure is reached within seconds if the domain is stable enough. However, a complication is introduced by the slow isomerization of the peptide bond preceding ProL95, which must be *cis* for the native interface to form. As a consequence, the final correct docking of V_H and V_L, and thus the subsequent stabilization of the structure, is limited in rate by this proline *cis-trans* isomerization (Freund *et al*., 1997a; Jäger & Plückthun, 1997, 1999). Starting from short-time denatured molecules (where all prolyl-peptide bonds remain in their native configuration), a *cis-trans* re-isomerization appears to occur at the ProL95 in an early intermediate, leading to the rapid population of a kinetically trapped intermediate. An early intermediate was shown to be formed on the millisecond time-scale, regardless of the conformation at the Pro residues, and it comprises exchange-resistant amide protons, located mostly in the inner β-sheet of the V_L domain (Freund *et al*., 1996, 1997a).

One big obstacle for the use of disulfide-free antibody fragments, besides their reduced thermodynamic stability, is their increased tendency to aggregate at higher concentrations and especially upon cellular folding *in vitro*. Using a series of point mutations of widely differing stabilities measured at equilibrium, an overall correlation between the stability of a scFv and the aggregation behavior of the native protein at elevated temperatures was found (Wörn & Plückthun, 1999). On the other hand, the correlation between thermodynamic stability and *in vivo* expression is clearly more complex. One of the most important questions in this context is to define the sequence of events leading to the population of an aggregating species upon refolding in one case and to the formation of soluble protein in high yields in the other.

We wished to define the folding process in the absence of disulfide bonds to understand whether the disulfide bonds force particular intermediates to be populated. Additionally, the comparison of the folding of scFv fragments with domains containing disulfide bonds or not provides a convenient means to investigate the influence of domain stability on the folding pathway. We have chosen the hu4D5-8 scFv, an intrinsically very stable antibody scFv fragment and its disulfide-free variants (Wörn & Plückthun, 1998), as a model system to gain better insight into these processes. We investigated the folding process *in vitro* to define the factors which govern the folding and potentially limit the refolding yields and solubility, and thus the usefulness of certain antibody fragments for *in vivo* applications. Unexpectedly, we found that the most aggregation-prone fragment carries a disulfide-free V_H domain in conjunction with a disulfide-containing V_L domain. We have analyzed these findings with a detailed investigation of the folding kinetics and structure formation by mass spectroscopy.

Results

Protein expression, purification and functionality

Both 4D5 scFv fragments retaining the conserved disulfide bridge in V_H, the wild-type (wt) and the 4D5 $V_L^-V_H^+$ variant (where the superscripts indicate the presence or absence of the disulfide bridge), could be expressed in the periplasm and purified from the soluble fraction of cell extracts in high yields of 8 and 5 mg/l of liquid culture, respectively. Both mutants lacking the disulfide bridge in V_H formed inclusion bodies in the periplasm and could not be purified from the soluble fraction, but were refolded *in vitro*. All four proteins were shown to be functional by ELISA (Wörn & Plückthun, 1998). The signal intensities produced by equal amounts of protein were identical within the range of error and completely inhibitable by soluble antigen (data not shown).

The wt, the 4D5 $V_L^-V_H^-$ and the 4D5 $V_L^-V_H^+$ variants were shown to be monomeric at the concentrations used, giving a sharp and symmetric peak at the expected elution volume in analytical gel filtration (data not shown). In contrast, the 4D5 $V_L^+V_H^-$ mutant showed a high tendence to dimerize and proved to be most sensitive to aggregation, both upon concentration and refolding.

Equilibrium transition measurements

The V_H domain with its five tryptophan residues contributes proportionally more to the total fluorescence intensity of the scFv fragment, thus dominating the spectrum. The emission maxima of the

variants lacking the disulfide bridge in V_L are blue-shifted compared to the wt, illustrating the quenching effect of the disulfide bridge on the only tryptophan residue in V_L, TrpL35 (Cowgill, 1967; Figure 1). Surprisingly, no such effect on the structurally homologous TrpH36 is observed upon removal of the disulfide bridge in V_H.

While the equilibrium transition of the 4D5 $V_L^-V_H^+$ seems to be occurring in one single cooperative step, the transition of the wt and the 4D5 $V_L^+V_H^-$ mutant is clearly not two-state, indicated by the gradual increase after the main transition (Figure 1). As shown by kinetic and mass spectrometry measurements (see below), an equilibrium intermediate is populated in the case of the wt at concentrations of around 2 M GdnHCl. Since it occurs only in variants carrying the disulfide bridge in V_L, this intermediate most likely retains native structure in V_L in the presence of an unfolded V_H. This is further confirmed by partial proteolysis in the presence of 2 M GdnHCl, where a fragment is accumulated which contains an intact V_L domain (Wörn & Plückthun, 1999). Because of the small contribution of V_L to the fluorescence intensity, its denaturation leads only to a small shift in emission maximum, and thus this step is difficult to observe. Whereas full reversibility is retained in the wt and the 4D5 $V_L^-V_H^+$ mutant (as judged from fluorescence intensity measurements, data not shown), the transition becomes only partially reversible upon removal of the conserved disulfide bridge from V_H, due to aggregation upon refolding at low concentrations of GdnHCl.

Due to his obvious lack of two-state behavior in the wt, and the incomplete reversibility resulting from aggregation in the case of the 4D5 $V_L^-V_H^-$, curves have not been normalized nor fitted to obtain ΔG values. It is clear from the data, however, that the wt has a higher midpoint of denaturation in its first transition than all other variants. Its second transition at about 2.5 M GdnHCL is only observed in those variants which contain the disulfide bridge in V_L, and thus this transition has been identified as the denaturation of V_L, supported by kinetic data described below.

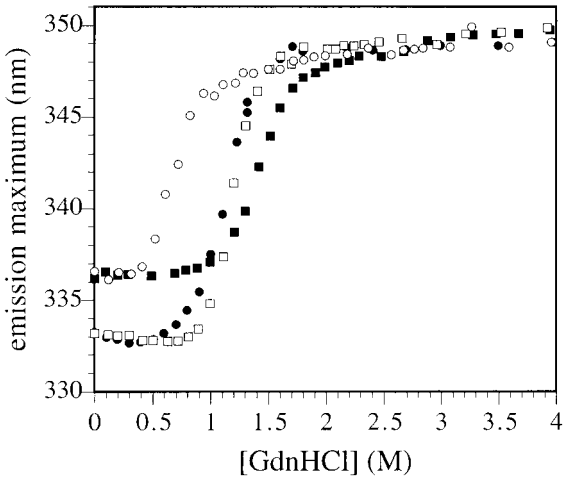

Figure 1. GdnHCl-induced equilibrium transitions at pH 6, 10 °C, monitored by the emission maximum. 4D5 wt (■), 4D5 $V_L^-V_H^+$ (□), 4D5 $V_L^+V_H^-$ (○) and 4D5 $V_L^-V_H^-$ (●).

The 4D5 $V_L^-V_H^-$ and the 4D5 $V_L^-V_H^+$ fragments, on the other hand, do not seem to differ markedly from each other in stability, as their main transition occurs at the same denaturant concentration. The transition of the 4D5 $V_L^-V_H^+$ fragment is, however, much more cooperative than that of 4D5 $V_L^-V_H^-$ with the shift in the fluorescence maximum beginning only at higher denaturant concentrations. If the transition is followed by fluorescence intensity, which is difficult due to much more pronounced scattering of the data compared to the shift in emission maximum, a marked decrease of fluorescence intensity is seen for 4D5 $V_L^-V_H^-$ before the main transition, pointing towards the loss of protein due to aggregation at these denaturant concentrations.

Surprisingly, the variant retaining the disulfide bridge in V_L, but not in V_H appears to be the least stable of all by far (Figure 1, open circles). Remarkably, this fragment is less stable than the one having no disulfide bridge at all, suggesting the formation of a stabilized, non-native form at intermediate GdnHCl concentrations, which depopulates the native state and depends on the presence of native V_L. One possible explanation for the occurrence of this non-native form might be the formation of V_L homodimers at the expense of heterodimerization.

Unfolding kinetics

Unfolding is monophasic for 4D5 $V_L^-V_H^-$ and 4D5 $V_L^-V_H^+$, i.e. both proteins lacking the disulfide bridge in V_L. Moreover, unfolding rates and their dependence upon GdnHCl concentration are identical in the range of error for these two proteins (Table 1), reflecting their similar stabilities.

Unfolding of the wt fragment and the 4D5 $V_L^+V_H^-$ mutant, in contrast, occurs in two kinetic phases: a fast phase, characterized by a large decrease in fluorescence intensity, typical for the destruction of the interface, and a slow phase with a small increase in fluorescence intensity, as would be expected for the unfolding of an isolated domain (Table 1). We interpret this as a fast denaturation of the less stable V_H, with concomitant loss of the interface, followed by a much slower denaturation of the more stable V_L. If unfolding of the wt is carried out in intermediate GdnHCl concentrations of around 2 M, the fast unfolding phase still goes to completion, while the slow unfolding phase is not observed any more. Thus, an equilibrium intermediate, which most likely retains native structure in V_L in the presence of a largely unfolded V_H (see above), can be populated at these intermediate denaturant concentrations. This interpretation is supported by mass spectrometry data (see below).

Table 1. Rate constants (s^{-1}) for unfolding at 10 °C

	Final GdnHCl concentrations (M)					
	5	4	3.5	3	2.5	2
4D5 wt[a]	0.08	0.05	0.0015	0.0005		
			4.6	3		0.1
4D5 $V_L^-V_H^+$		0.2		0.022	0.007	0.0023
4D5 $V_L^-V_H^-$		0.6	0.095	0.022	0.007	0.0023

Errors were estimated from at least duplicate measurements and were generally within 3 % of the mean.
[a] The rate constants for unfolding are shown. Wherever two numbers are given, a biphasic reaction was observed. In all other cases, monoexponential fits gave random residuals.

Refolding kinetics observed by fluorescence spectroscopy

Refolding of equilibrium denatured protein

Refolding kinetics of equilibrium denatured scFv fragments, i.e. with an equilibrium distribution of proline *cis-trans* isomers, seems to be limited by proline *cis-trans* isomerization of at least one of the two *cis*-proline residues, ProL95 and ProL8, for all mutants investigated (cf. M. Jäger, unpublished results), consistent with experiments for the antibody McPC603 (Freund *et al*., 1996, 1997a; Jäger & Plückthun, 1997). In this case, the isomerization could be assigned to ProL95, since replacement of ProL8 by an alanine residue did not abolish the slow phase (Jäger & Plückthun, 1997). The rates of the isomerization phase at low GdnHCl concentrations are similar for all mutants and in the range of 6×10^{-4} to 9×10^{-4} s^{-1}, although their denaturant dependencies differ slightly (Table 2). Figure 2 shows the appearance of the slow folding species (relative amplitude of the slowest phase) as a function of denaturation time, due to proline *cis-trans* isomerization occurring in the denatured state, for the 4D5 $V_L^-V_H^-$ variant. All other fragments behave similarly (data not shown).

Starting from equilibrium denatured protein, those variants with a disulfide bridge in V_H, i.e. the wt and $V_L^-V_H^+$ fragment, display an additional faster phase, which is not observed in the 4D5 $V_L^-V_H^-$ and the 4D5 $V_L^+V_H^-$ mutant fragments (Figure 3(a) and (b)). The rate of this phase is about ten times faster than that of the slow isomerization reaction (Table 2). The relative amplitude of this faster phase differs, however, between the two proteins, amounting to only about 30 % of the total observed amplitude in the case of 4D5 $V_L^-V_H^+$, compared to 60 % for the wt.

An indication about the nature of the process responsible for this additional refolding phase is given by the observation of a kinetic folding intermediate for the wt (Figure 4(a)). The intermediate was detected by monitoring the amplitude of an unfolding reaction, initiated by immediate denaturation after different times of refolding. The conditions of this unfolding reaction (2 M GdnHCl) were chosen such that only the intermediate, but not the native wt V_L domain would unfold. The formation of the intermediate occurs with approximately the same rate as the additional faster phase in wt refolding ($k = 0.004$ s^{-1}). If, on the other hand, the appearance of the native protein is probed by unfolding at higher GdnHCl concentrations (4 M), its formation is detected to occur at a slower rate than that of the intermediate, corresponding to the slow proline *cis-trans* isomerization phase (data not shown).

Table 2. Rates constants k (s^{-1}) and relative amplitudes A (% total amplitude) for refolding starting from different conditions

	Equilibrium denatured protein						Short-term denatured protein					
	Final GdnHCl concentrations (M)											
	0.2		0.4		0.8		0.2		0.4		0.8	
	k	A	k	A	k	A	k	A	k	A	k	A
4D5 wt	0.0045	45	0.0045	50	0.003	50	0.03	18	0.04	25	0.05	65
	0.0009	25	0.0008	30	0.0008	30	0.007	12	0.007	15	0.009	10
4D5 $V_L^-V_H^+$	0.007	20	0.006	25	0.005	20	0.02	35	0.03	40	0.022	60
	0.0009	40	0.0007	45	0.0004	50	0.004	25	0.004	20	0.003	10
4D5 $V_L^-V_H^-$	0.0005	65	0.0003	80	0.0002	90	0.03	35	0.05	45	0.08	85
							0.004	25	0.005	25	0.01	15

The fraction of the total amplitude that is missing to 100 % in some cases is reached in the dead-time of manual mixing. The total amplitude has been determined from a fluorescence intensity *versus* GdnHCl plot (data not shown) by extrapolation of the baselines to the relevant GdnHCl concentration.
Errors for the rate constants were estimated from at least duplicate measurements and were generally within 8 % of the mean.

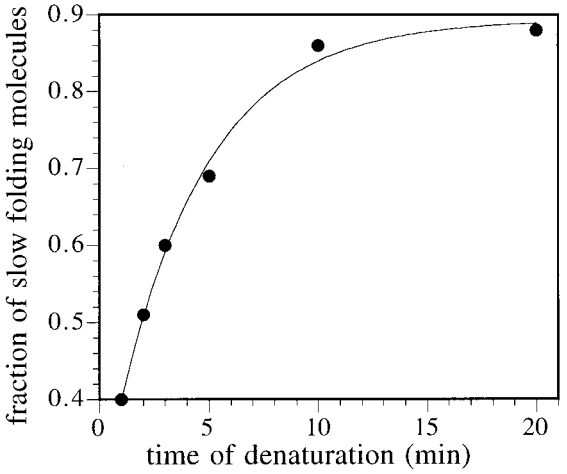

Figure 2. Appearance of the slow folding species of 4D5 $V_L^-V_H^-$ through proline *cis-trans* isomerization upon denaturation in 4 M GdnHCl at 10 °C. Amplitudes of the slow isomerization phase upon refolding (10 °C, final GdnHCl concentration 0.4 M, 0.4 μM protein) are plotted *versus* the time of denaturation. The continuous line represents a fit with one single exponential, yielding a half time of 170 seconds for the isomerization reaction at 10 °C.

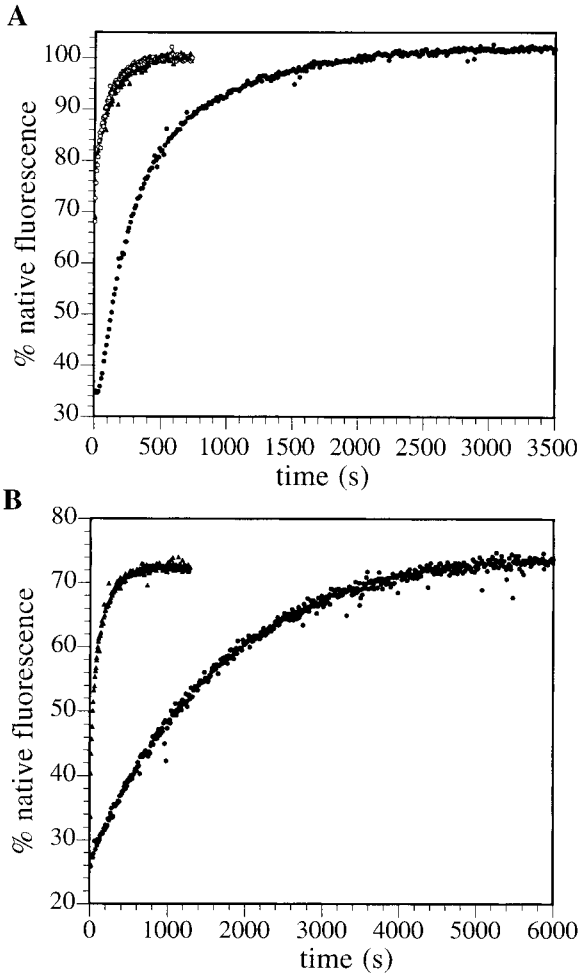

Figure 3. Fluorescence traces of refolding of the (a) 4D5 wt and the (b) 4D5 $V_L^-V_H^-$ mutant at 10 °C. Refolding was started from equilibrium denatured protein (●), short-term denatured protein (▲) and an equilibrium intermediate populated at 2 M GdnHCl (○). The intensity at 326 nm was followed. Final protein concentrations were 0.4 μM in all cases at final GdnHCl concentrations of 0.4 M.

In the case of 4D5 $V_L^-V_H^-$, on the other hand, fluorescence refolding kinetics as well as the appearance of the native species, probed by unfolding at different GdnHCL concentrations, occur in one slow phase (Figures 3(b) and 4(b)), corresponding in rate to the limiting proline isomerization reaction ($k = 0.0004\ s^{-1}$). Thus, there is no indication for the existence of a significantly populated kinetic folding intermediate for the 4D5 $V_L^-V_H^-$ protein, when starting from equilibrium unfolded protein. In contrast, in 4D5 $V_L^-V_H^+$, the additional phase ($k = 0.007\ s^{-1}$) is also seen (Table 2). This suggests that the kinetic intermediate mentioned above must be dependent on the presence of a V_H domain having sufficient stability on its own, and thus the ability to form structure independently and before the rate-limiting isomerization reactions in V_L have taken place. If, however, V_H is not stable enough by itself, it only folds into a stable structure once V_L provides a suitable interface, whose formation most likely depends primarily on the conformation of ProL95 due to its location in the interface.

Refolding of short-term denatured protein

When refolding is started from short-term denatured protein at high GdnHCl concentrations, i.e. from protein with its proline residues in their native conformation, all 4D5 scFv variants behave very similarly. The slow proline *cis-trans* isomerization phase disappears completely, but refolding nevertheless occurs in two phases: the slower phase has a rate similar to that of the faster phase of refolding started from equilibrium. The other phase is about ten times faster (Table 2 and Figure 3(a) and (b)). With increasing final GdnHCl concentrations, the fast phase increases in amplitude, while the amplitude gained in the dead-time of manual mixing as well as that of the slower phase decrease (Table 2). For the wt, the slow phase disappears only above 0.8 M GdnHCl (data not shown). At GdnHCl concentrations close to the transition region, refolding thus occurs in one fast step. This means that folding of short-term denatured protein is fastest at the highest possible denaturant concentrations, which still allow formation of the native structure. However, neither the rates nor the amplitudes of the two phases are concentration-dependent over the fourfold concentration range tested (0.2-0.8 μM), such that

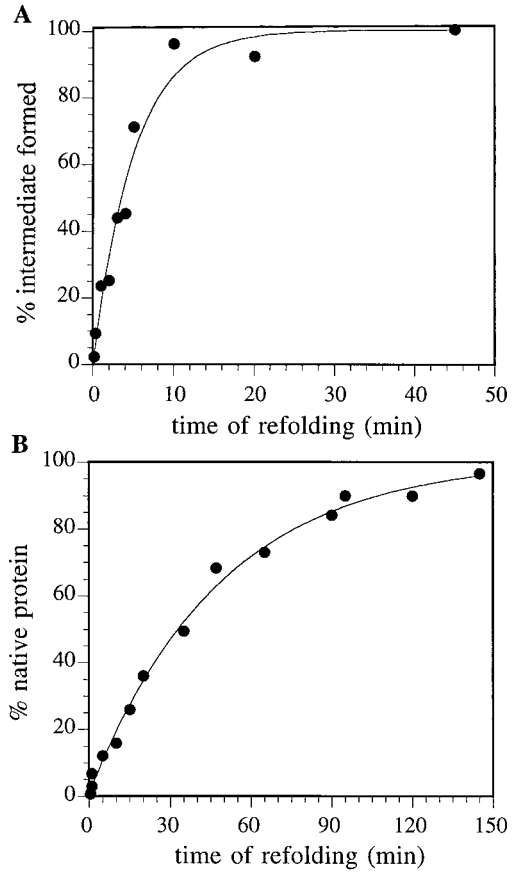

Figure 4. (a) Appearance of an intermediate during the time-course of refolding of the 4D5 wt starting from equilibrium denatured protein at 10 °C, expressed in percentage of its maximal population. (b) Appearance of the native species of the 4D5 $V_L^-V_H^-$ mutant upon refolding from equilibrium denatured protein at 10 °C. Plotted are the amplitudes of the respective unfolding kinetics at 10 °C of either the intermediate (at 2 M GdnHCl) or the native species (at 3 M GdnHCl) after increasing times of renaturation at 10 °C and 0.2 M GdnHCl.

aggregation phenomena can be ruled out as a cause for the additional fast phase.

This disappearance of the slow refolding phase at increasing GdnHCl concentrations can be interpreted as GdnHCl destabilizing an off-pathway intermediate, whose conversion to an on-pathway intermediate is causing this slow reaction. In summary, these data can be explained by the existence of a kinetic trap on the refolding pathway, the population of which is dependent on the refolding conditions. The rates of 0.003-0.007 s^{-1} (the faster reaction in equilibrium denatured protein and the slow reaction in short-term denatured protein) thus most likely constitute the rate at which this off-pathway intermediate returns to a productive on-pathway intermediate. The faster rate (0.02-0.05 s^{-1}, occurring only in short-term denatured proteins) constitutes the fraction of molecules which avoid the trap. The slowest rate (0.0004-0.0008 s^{-1}, occurring only in equilibrium denatured protein) corresponds to the folding reactions which take place only once the isomerization at ProL95 has occurred.

Refolding from intermediate GdnHCl concentrations

If refolding of the wt is started from the equilibrium intermediate populated at 2 M GdnHCl (Figure 3(a)), kinetics identical with those starting from short-term denatured protein are observed. Traces of refolding show the same biphasic behavior, lacking the slow proline isomerization phase. This argues further for the existence of a native-like V_L domain in the wt equilibrium intermediate, with its proline residues still in the native *cis*-conformation. Thus, in both cases (starting from 2 M GdnHCl or from short-term denatured protein), the reaction starts from a V_L domain with *cis*-proline-peptide bonds.

The fact that the additional slow folding phase, described above for the short-term denatured protein, is also observed if refolding is started from the wt equilibrium intermediate retaining a native V_L, provides strong evidence that this slower phase is not due to an isomerization reaction of proline residues and thus supports the earlier suggestion that kinetic complexity may rather arise through the population of a kinetic trap on the folding pathway.

Interestingly, when refolding of the 4D5 wt fragment is started from the equilibrium intermediate (in 2 M GdnHCl), both phases gain in amplitude upon increasing GdnHCl concentrations at the expense of the amplitude gained in the dead-time, while their ratio remains the same (data not shown). Since the biphasic behavior persists with increasing GdnHCl concentrations in the refolding mixture, which should contain native-like V_L at all times, it appears as if the formation of the trap occurs under all of these conditions, and may be inevitable in the presence of native V_L. The constant relative amplitude suggests that the observable phases are sequential processes, when starting from the equilibrium intermediate. In contrast, with short-term unfolded protein only the amplitude of the faster phase increases at the expense of both the slower phase and the dead-time amplitude with increasing GdnHCl. In short-term denatured protein, the postulated kinetically trapped intermediate apparently becomes populated in parallel with a pathway of fast refolding, and the relative flux along the two pathways depends on the final GdnHCl concentration. We currently do not know the nature of the species giving rise to the dead-time amplitude in manual mixing. Mass spectrometry (see below) shows that in the dead-time of manual mixing no molecule with fully native protection is obtained, even though a large number of protons is already protected. This excludes native molecules formed in the dead-time of manual mixing.

H/^{2}H exchange measurements

The number of maximally exchange-protected deuterons in fully deuterated protein differs in the various mutants, reflecting their different stabilities. In the wt, 72 deuterons are protected if mass spectrometry measurements are performed under the acidic conditions used routinely (see Material and Methods). This number decreases to 65 for the 4D5 $V_L^-V_H^+$ variant and 54 for the 4D5 $V_L^-V_H^-$ mutant. The 4D5 $V_L^-V_H^-$ mutant is the only variant that is prone to complete back-exchange of all protected deuterons upon prolonged incubation in H_2O, probably due to cooperative unfolding events.

H/^{2}H exchange measurements during the time-course of refolding, starting from equilibrium denatured protein, were carried out to further test the interpretation of the kinetic results obtained with fluorescence measurements. The formation of a very early intermediate with about 12 protected deuterons is common to all three variants (Table 3). While its formation seems to occur in the dead-time of the manual mixing experiment for the wt, it is slowed down in the two variants lacking cysteine residues in V_L. The first intermediate is most likely of the same nature as the one described for the McPC603 ScFv fragment (Freund *et al.*, 1996). Early formation of inner β-sheet secondary structure had already been suggested from the experiments to be of general relevance in the refolding of antibody variable domains.

In the case of equilibrium denatured wt (Table 3 and Figure 5(a)), the native number of protected deuterons is reached with a rate corresponding to the faster phase observed in fluorescence measurements. After only five minutes, the native species is already populated to more than 50 %, with no intermediate being observed. Consequently, no phase is detectable by mass spectrometry that would correspond in rate to the slow phase of proline isomerization seen in fluorescence, which has an amplitude of 40 %. It thus seems as if stable secondary structure formation within the domains occurred already in the faster phase of refolding. Both domains retaining the conserved disulfide bridge thus refold independently of the conformation at the proline residues, as judged by the gain in protected deuterons. The slow phase detected in fluorescence must thus be due to native interface formation detectable by a change in fluorescence intensity, following the formation of native structure within the domains. The *cis*-conformation of the limiting proline residues might be a prerequisite for this interface formation. Apparently, this

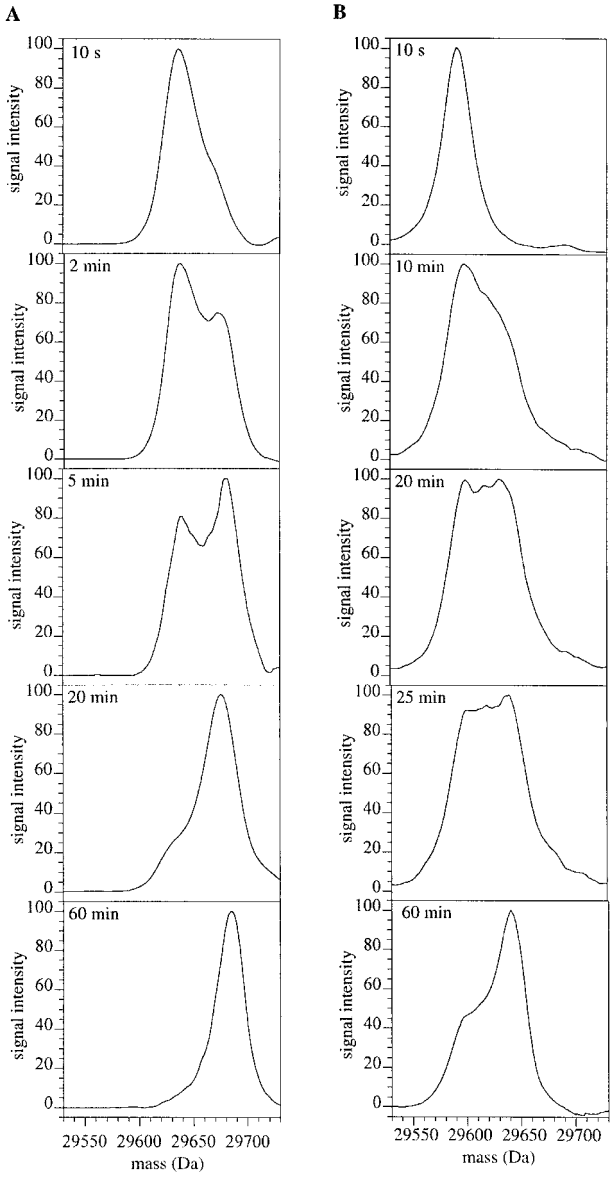

Figure 5. Mass spectrometric analysis of the (a) equilibrium denatured 4D5 wt and the (b) 4D5 $V_L^-V_H^+$ mutant after different times of refolding in ^2H_2O buffer as indicated.

Table 3. Number of deuterons protected upon refolding at 10 °C

	Deuterated	Length of refolding in ^2H_2O							
	Reference	0 s	10 s	2 min	5 min	10 min	20 min	25 min	60 min
4D5 wt	72 ± 2	12	11	13	14	15			
				62	62	69	70	71	72
4D5 $V_L^-V_H^+$	65 ± 2	5	8	10	10	12	12	13	13
							36	39	
							58	61	64

Errors were estimated from at least duplicate measurements and were generally within 8 % of the mean.

rearrangement involves native-like domains, which do not lose their protected deuterons in the reaction.

The 4D5 $V_L^-V_H^+$ mutant (Table 3 and Figure 5(b)), on the other hand, shows a strikingly different behavior in protection experiments. The native number of protected deuterons is reached with a much slower rate than in the case of the wt, corresponding to that of the slow isomerization phase. Upon closer inspection of the protection patterns between ten and 25 minutes, it becomes obvious that protection of the exchangeable sites does not occur in one single cooperative step. Rather, two folding reactions appear to take place sequentially, since the population of an intermediate can be observed with a mass of about 29,616, corresponding to about 36 protected deuterons. It seems reasonable to postulate that folding first occurs in V_H, where the disulfide bridge is retained, and that folding of V_L, which is destabilized by the lack of the disulfide bridge, requires the stabilizing presence of the native interface within the scFv. Formation of the native V_L domain, therefore, only occurs with a rate corresponding to that of the proline isomerization reaction, because that in turn is required for docking of V_H.

The low solubility and aggregation properties of the 4D5 $V_L^-V_H^-$ mutant, lacking both disulfide bridges, have unfortunately rendered impossible its detailed investigation by mass spectrometry up to now. Since refolding monitored by fluorescence is monophasic and slow, formation of native structure most likely takes place only in this slow cooperative reaction, corresponding in rate to the proline isomerization. No intermediates would be expected to be populated, as refolding of both domains will be dependent on their mutual stabilization in the scFv fragment, i.e. the formation of the native interface and hence the *cis*-conformation at the proline residues.

If refolding experiments with the wt are initiated from the equilibrium intermediate populated in 2 M Gdn^2HCl (postulated from fluorescence measurements to retain native V_L), the protection pattern is completely different (Table 4). Refolding occurs in one fast phase, with the native number of protected deuterons being gained in seconds. There is a very high number of deuterons (54) protected already in the dead-time of mixing, but a species with the native number of protected deuterons (72) is not populated. Thus, there are no native molecules formed in the dead-time of manual mixing. Upon increasing Gdn^2HCl concentrations in the refolding mixture, the number of deuterons protected in the dead-time is reduced drastically, from 54 to 34. The decrease of the fluorescence intensity amplitude gained in the dead-time of mixing upon increasing denaturant concentrations (see above) is thus paralleled by a decrease of the number of deuterons protected in this dead-time. This suggests that less structure is formed in the V_H domain in the dead-time, and that consequently a species with a native V_L domain and a largely unstructured V_H domain may be present transiently at higher GdnHCl concentrations. The deuterons protected in the dead-time upon refolding at 1 M GdnHCl have most likely already been present at the start, and thus most likely constitute deuterons present in V_L.

Table 4. Number of protected deuterons upon refolding of the 4D5 wt starting from the equilibrium intermediate

Gdn^2HCl	Length of refolding in ^2H_2O			
concentration (M)	0 s	10 s	2 min	∞
0.2	54	67	70	72
0.5	41	65	70	72
1	34	63	69	71

Aggregation behavior

Since the 4D5 $V_L^-V_H^-$ and the 4D5 $V_L^-V_H^+$ variants have a similarly reduced stability compared to the wt, together with identical unfolding rates and similar rates of refolding, and since in both cases, the proline isomerization reaction is limiting for the formation of the native structure, the question arises what may be causing their widely different aggregation behavior. Both the wt and the 4D5 $V_L^-V_H^+$ mutant are highly soluble proteins that can be concentrated to several milligrams per milliliter, and they refold with yields of around 100 % in all GdnHCl concentration before the onset of the equilibrium transition (below 1.1 M). However, 4D5 $V_L^-V_H^-$, and even more so 4D5 $V_L^+V_H^-$, i.e. both variants lacking the disulfide bridge in V_H, are extremely prone to aggregation, both upon concentration and refolding, with yields reaching 75-80 % at the most at 0.4 μM protein and in the absence of arginine. This *in vitro* behavior thus seems to reflect very well the situation *in vivo*, where the wt and 4D5 $V_L^-V_H^+$ can be expressed as soluble periplasmic proteins, whereas 4D5 $V_L^-V_H^-$ and 4D5 $V_L^+V_H^-$ form periplasmic inclusion bodies.

The only obvious kinetic difference between those variants that do refold in high yields and those that tend to aggregate is the additional fast phase ($k = 0.003$-$0.007\ s^{-1}$) observed in the ''well-folding'' proteins in fluorescence upon refolding from equilibrium denatured protein. $H/^2H$ exchange measurements have shown that in the case of the wt both domains, and in the case of the 4D5 $V_L^-V_H^+$ variant at least V_H, reach the native number of protected deuterons during this fast phase, independently of native interface formation and of the conformation of the prolines. Those variants with a high tendency to aggregate, on the other hand, have apparently lost the ability of independent structure formation in V_H due to the lack of the stabilizing disulfide bridge. It is possible that this ability of the stable V_H domain, namely to fold to its native state without interaction of the V_L domain, might help to prevent premature interaction of the two domains and thus avoid the

formation of intermediates that are prone to aggregation.

An unstable V_H domain requiring native interactions for folding, however, does not seem to be the only factor influencing the tendency of the different variants to aggregate. It is very instructive to take a closer look at the refolding behavior of the different variants under various conditions, especially concerning the yields of native protein obtained. If the wt is refolded from equilibrium denatured protein, yields are around 100 % at all final denaturant concentrations below the equilibrium transition (up to 1.1 M), i.e. conditions still allowing formation of native protein. When wt refolding is started from short-term unfolded protein, i.e. with both V_H and V_L refolding fast, independent of proline *cis-trans* isomerization, yields are almost as high. However, if refolding is started from the equilibrium intermediate populated at 2 M GdnHCl, retaining native structure in V_L, yields decrease substantially (from 100 to 75 %) when folding is completed at final GdnHCl concentrations from 0.1 to 1.0 M. Increasing GdnHCl concentrations appear to increase the fraction of molecules which fold with phases resolvable in manual mixing and not in the dead-time, when started from 2 M GdnHCl or short-term denatured protein. The loss of dead-time amplitude thus seems to correlate with increasing aggregation, in their dependence on GdnHCl concentration. Since the total amplitude must at least be indirectly caused by V_H folding, V_L being native, it follows that aggregation correlates with late V_H folding, as evidenced by small dead-time amplitude. $H/^2H$ exchange experiments are also consistent with less structure formation in the dead-time of manual mixing with increasing GdnHCl concentrations (Table 4). In conclusion, we postulate that an increased population of a state with mostly unfolded V_H in the presence of a native V_L leads to aggregation. This situation probably never arises upon refolding of the fully unfolded wt, even from short-term unfolded protein, since V_H will be able to form some early structured intermediate, before V_L reaches its native state.

It is, however, plausible that such an intermediate would become populated during refolding of 4D5 $V_L^-V_H^-$, starting from short-term, and to a lesser extent from equilibrium denatured protein. As discussed above, in this case neither domain is able to fold independently, and especially the less stable V_H requires the stabilizing presence of the native interface.

These findings might also help to explain the excessively low refolding yields and solubility of the 4D5 $V_L^+V_H^-$ mutant. Not only does the disulfide bridge in V_L in the absence of the one in V_H not show any stabilizing effect compared to the 4D5 $V_L^-V_H^-$ variant, but, on the contrary, it further destabilizes the mutant, making it even more prone to aggregation. In this case, an intermediate state with a native V_L and a largely unfolded V_H could possibly be populated to even higher extents.

Discussion

The results clearly show that the sequence of events taking place upon refolding of the four hu4D5-8 scFvs depends on the intrinsic stabilities of the two domains. In those cases, where the conserved disulfide bridge is retained, the respective domain is able to fold to a native-like state with protected protons before the proline *cis-trans* isomerization has occurred, with the proline-limited formation of the native interface occurring subsequently. If, however, one or both domains are destabilized by the removal of the disulfide bond, the formation of native structure becomes dependent on the presence of the stabilizing native interface and thus the folding of the other domain. By destabilizing V_H through removal of its conserved disulfide bridge, biphasic kinetics are shifted to a monophasic reaction. Folding of V_H becomes dependent upon the presence of a folded V_L to stabilize it. A cooperative transition without population of any detectable kinetic intermediates is thus observed. This is consistent with similar observations that have been made in other proteins (Khorasanizadeh *et al*., 1996; Jackson, 1998).

As is the case for many proteins containing *cis*-proline residues in their native fold, structure formation of each of the four 4D5 scFvs can proceed along two parallel pathways, the slower being limited by proline *cis-trans* isomerization in the V_L domain. This is in accordance with observations made with the McPC603 scFv (Freund *et al*., 1996, 1997a; Jäger & Plückthun, 1997). If refolding is started from protein having its proline residuess in V_L in their native *cis* conformation, the isomerization in V_L is not longer rate limiting. Similar kinetics are then observed for all variants. The additional slow phase, observed for short-term denatured protein at low denaturant concentrations, seems to be due to the transient population of an off-pathway species whose conversion to the native state is slower than the parallel fast track. Unlike in the antibody McPC603 (Freund *et al*., 1997a; Jäger & Plückthun, 1997), however, the population of the kinetic trap does not seem to be dependent on the conformation of the proline residues in the molecules investigated here. Rather than arising through re-isomerization of proline residues in an early folding intermediate, the population of the trapped intermediate in the 4D5 scFv thus seems to depend only on the refolding conditions. Possibly, premature interface formation (that cannot be prevented even at high GdnHCl concentrations in the presence of a native V_L) and subsequent slow refolding or rearrangement could be the reason for the additional slow folding phase.

Interestingly, our results allow us to give an explanation for the widely differing aggregation behavior observed in the four variants. The observations suggest that two structural features correlate with the tendency of these scFvs to aggregate. Most important is the presence of a largely unfolded V_H domain. The simultaneous presence

of a native or native-like V_L domain seems to further increase the tendency to aggregate. Independent structure formation in the V_H domain might prevent wrong interactions with an already largely native V_L domain at too early a stage from taking place and thus avoid the population of an aggregation-prone species with an unfolded V_H domain. It would be interesting to see whether this behavior is of a more general nature.

Thus, there seems to be a kinetic partitioning between the development of native secondary structure in the individual domains and subsequent formation of the correct interface on the one hand, and the population of aggregation-prone off-pathway intermediates through interaction at too early a stage on the other hand. The decision of which pathway is predominant is apparently critically dependent on the ability of V_H to fold (at least partially) independently, but also on the relative rate of structure formation of the two domains.

In conclusion, the folding of disulfide-free antibody domains, at least at the level of analysis possible from fluorescence spectroscopy and H/^{2}H exchange followed by mass spectrometry, is not substantially different from that of disulfide-containing ones. Rather, they behave as unstable antibody domains, and thus not different from disulfide containing, unstable antibody domains.

Materials and Methods

Protein expression and purification

The gene for the hu4D5-8 scFv fragment (Kelley *et al*., 1992) was cloned between the *Eco*RV and *Eco*RI sites of the secretion vector pIG6 (Ge *et al*., 1995) in a V_L-linker-V_H orientation with a non-repetitive 20-mer linker (Tang *et al*., 1996; Proba *et al*., 1997). For disulfide-free variants of this scFv, the following pairwise mutations were made in either or both domains: CysH22 was replaced by Val, CysH92 by Ala, CysL23 by Val and CysL88 by Ala (numbering according to Kabat *et al*. (1991), as described by Wörn & Plückthun, 1998). The resulting four variants are labeled with the superscripts +, denoting the presence, and −, denoting the absence of the disulfide bond: 4D5 wt (containing both conserved disulfide bridges), 4D5 $V_L^-V_H^-$ (having all four cysteine residues replaced), 4D5 $V_L^-V_H^+$ (V_H with disulfide bridge only) and 4D5 $V_L^+V_H^-$ (V_L with disulfide bridge only).

All variants were expressed in the periplasm of *Escherichia coli* JM83 and purified essentially as described (Wörn & Plückthun, 1998), with the following modifications. Proteins purified from soluble cell extract (both variants with the cysteines in V_H) were purified in addition by chromatography on Protein A Sepharose (Pharmacia Biotech) (Akerström *et al*., 1994) followed by a buffer exchange *via* gel filtration chromatography on PD10 Sephadex G-25 M column (Pharmacia-Biotech).

For proteins purified from periplasmic inclusion bodies (both variants lacking the cysteines in V_H), the initial immobilized metal ion affinity chromatography was omitted. Instead, solubilized inclusion bodies were refolded directly in a buffer containing 0.4 M arginine (pH 7) and purified by chromatography on Protein A Sepharose (Pharmacia Biotech) (Akerström *et al*., 1994) followed by a buffer exchange *via* gel filtration chromatography on PD10 Sephadex G-25 M column (Pharmacia-Biotech). In all four cases the pH of the elution buffer used for Protein A chromatography was at 3.5 to minimize aggregation upon elution. The addition of arginine to the refolding buffer and the Protein A chromatography result in monomeric scFv fragments.

ELISA measurements

ELISA measurements were carried out as described (Wörn & Plückthun, 1998).

Analytical gel filtration

Analytical gel filtration was carried out as described (Wörn & Plückthun, 1998) on a SMART system in 20 mM Mes (pH 6.0), 200 mM NaCl with 0.005% Tween-20. Fragments were injected at an initial concentration of 2.5 μM in a volume of 50 μl.

Fluorescence measurements

All fluorescence measurements were performed with a PTI Alpha Scan spectrofluorimeter (Photon Technologies Inc.) at 10 °C, using excitation and emission wavelengths of 280 nm and 326 nm, respectively. The buffer used in all cases was 20 mM Mes (pH 6.0) (filtered, degassed), containing varying amounts of GdnHCl. Protein concentrations were 0.2 μM for equilibrium measurements and 0.4 μM for kinetic measurements.

For equilibrium denaturation measurements, either completely denatured or native protein was incubated overnight at 10 °C in Mes buffer containing different amounts of GdnHCl. Equilibrium transitions were followed by measuring intensities at 326 nm upon excitation at 280 nm, as well as the shift in emission maximum. Curves were not normalized, nor could ΔG values be derived, because of the obvious lack of two-state behavior for several of the proteins and because of incomplete reversibility due to aggregation in some of the mutants.

For kinetic measurements, the proteins, either native or unfolded as described in the text, were diluted rapidly (between 1:100 and 1:25) into buffer containing GdnHCl at various final concentrations. Kinetics were followed by the emission intensity at 326 nm upon excitation at 280 nm. All kinetic traces were evaluated using the Kaleidagraph software (Synergy software, Reading, UK) with either single exponential functions or a sum of two exponential functions for biphasic kinetics.

In order to follow the time-course of formation of either the native species or of an intermediate, the amplitude of the unfolding reaction was recorded that resulted from a further dilution with concentrated GdnHCl after different times of refolding. The final concentrations of GdnHCl are described in the text.

H/^{2}H exchange experiments

Purified proteins were denatured in ^2H_2O containing Gdn^{2}HCl at a final concentration of 3-4 M. Denatured samples were lyophilized and redissolved in ^2H_2O. This process was repeated three times to ensure complete deuteration of all exchangeable sites.

For initiating folding reactions, the deuterated proteins were diluted 1:25 or 1:50 into 20 mM Mes (pH 6.0) in ^2H_2O at 10 °C. The pH of the solutions was not isotope-

corrected. After different times of refolding, a further 1:5 dilution was performed into an H_2O-buffer containing 20 mM Mes (pH 6.0) at 10 °C. The reaction was then allowed to proceed to completion overnight at 10 °C. Subsequently, samples were desalted by gel filtration chromatography on PD10 Sephadex G-25 M (Pharmacia-Biotech) in 5 mM ammonium acetate in H_2O (pH 6.0) and concentrated with either Microcon and Centricon (Amicon Inc.) or Ultrafree-CL and ML concentrators (Millipore Corp., low binding regenerated cellulose, for the less soluble variants) to approximately 10 μM. All samples were measured within three hours of the desalting procedure, as freezing or prolonged storage was observed to lead to a loss of protected protons.

MS analysis

Molecular masses were determined using ESI-MS by flow injection (8 μl/minute) of the scFv into the ion source of a Sciex API III instrument. The ion spray voltage was 5000 V and the nebulizer gas pressure 50 psi.

Just before injection, samples were mixed 1:1 with a cooled solution (−20 °C), to minimize back exchange (Freund *et al*., 1997b) of 95 % methanol and 0.5 % formic acid in H_2O to result in final pH of approximately 3. A 10 μl sample of the solution was injected within less than ten seconds into a cooled (−20 °C) carrier solution, consisting of 50 % methanol, 0.25 % formic acid in H_2O. An m/z range of 840-2000 was scanned with a step size of 0.2 Da and a dwell time of five seconds.

For calculating the number of protected deuterons y, residual H_2O (20 %) was taken into account by using the equation $y + (z - y)\ 0.2 = x$, where z is the number of all slowly exchanging protons and x is the actually measured mass difference (Freund *et al*., 1997a,b).

Acknowledgments

The authors thank Arne Wörn for helpful discussions and Alain Tissot for critically reading the manuscript. This work was supported by a predoctoral grant from the Roche Research Foundation to K.R. and by the Schweizerische Nationalfonds grant 31-47302.96.

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Edited by I. A. Wilson

(Received 29 January 1999; received in revised form 3 May 1999; accepted 5 May 1999)