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Comparison of the amide proton exchange behavior of the rapidly formed folding intermediate and the native state of an antibody scFv fragment

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Abstract We have investigated the stability of backbone amide protons of the intermediate and the native state of the scFv fragment of an antibody. Stopped flow experiments analyzed by MS and NMR detected the formation of an exchange protected intermediate within the deadtime of the stopped flow apparatus (17 ms). H/D exchange rates of the native protein identified a number of very stable backbone amide protons in the V_L and the V_H domains. In the V_L domain, this slowly exchanging core of the scFv fragment is similar to the folding core of the intermediate, while the V_H domain possesses a great number of very stable amide protons which are not stabilized to a significant degree in the folding intermediate of the scFv fragment.

Key words: scFv fragment; H/D exchange experiment; Mass spectrometry; Nuclear magnetic resonance

1. Introduction

Proton-deuterium exchange experiments and their analysis by NMR and MS have proved to be an indispensable tool for the study of protein folding [1,2]. In combination with stopped flow techniques, exchange experiments have aided in the characterization of rapidly formed protein folding intermediates in a number of cases [3–15]. In addition, the measurement of native state hydrogen exchange rates has been established as a useful tool for delineating local and global unfolding events in proteins [16]. It has been proposed that the denaturant dependence of the native state hydrogen exchange rate constants can be used to identify partially unfolded forms of a protein, which might play an important role during the folding process [17]. Furthermore, it has been suggested that the slowly exchanging regions of native proteins define the basic fold of protein domains and that the slow exchanging core defines the folding core [18]; Clarke and Fersht [19], however, have pointed out that no clear correlation between the rates of native state hydrogen exchange and the folding pathway needs to be expected. In barnase, the only correlation to be found is between the burial of a certain hydrogen bond and its exchange behavior.

We recently reported the formation of a folding intermediate with stabilized secondary structure for the scFv fragment of the phosphorylcholine binding antibody McPC603 [20]. In a subsequent step, the two variable domains, V_H and V_L, which are linked by a flexible peptide linker of the sequence $(Gly_4Ser)_3$, were shown to fold to completion in a slow and cooperative reaction. In the current study we analyzed the protection of amide protons in the scFv fragment on the millisecond to second time scale by pulsed hydrogen exchange experiments to determine when the intermediate is formed. An estimate of the protection factors for a number of amide protons in the folding intermediate was calculated and compared to the measured rate constants and protection factors of individual amide protons in the native protein. Topological differences in the protection pattern of the two variable domains in the intermediate as well as in the native state have been observed and are discussed in the light of differences in the stability and the local unfolding between the two domains.

2. Materials and methods

2.1. Protein preparation

scFv fragments were expressed in *E. coli*, using a defined medium with NH_4Cl (^{15}N labeled for the protein used in the NMR experiments) and purified as described previously [21].

2.2. Pulse labeling exchange experiments

Pulse labeling experiments were performed with a Biologic SFM-Q4 module at 10°C. The experiments were run in either the continuous (for the samples with refolding times of 17 and 78 ms) or the interrupted mode (for samples with refolding times ≥200 ms). In the continuous mode, the refolding time is determined by the adjustable volume of the delay line used divided by the flow rate. In the interrupted mode, the protein solution, diluted 25-fold to initiate folding, was allowed to stand for defined time intervals, before the labeling pulse was applied. Syringe 1 contained the denatured scFv fragment in 4 M deuterated guanidinium chloride, 10 mM borate, $pD_{read} = 8.0$, and syringe 2 was filled with 0.4 M L-arginine, 10 mM borate, 1 mM phosphorylcholine in either D_2O or H_2O. Syringe 3 contained 0.1 M borate, 1 mM phosphorylcholine, pH 9.7 in H_2O, and the quenching solution, 0.5 M H_3PO_4, was filled into syringe 4. Solution 1 and 2 were mixed in a 1:25 ratio at pH 8.0, the mixture further diluted 5-fold into solution 3 (resulting in a final pH of 9.5) and quenched by adding solution 4 (a tenth volume with respect to the end volume), resulting in a final pH of 4.9. About 1.8 ml was collected for each experiment. For the MS analysis, around 25 shots were accumulated in each experiment, while 125 shots per experiment had to be accumulated for the NMR analysis. The quenched solutions were allowed to stand for 3 h to let folding go to completion and were then concentrated by ultracentrifugation with an Amicon A8200 cell. The solution was then either dialyzed against 10 mM ammonium acetate, pH 5.0 for the MS analysis or against 2 mM KH_2PO_4, 0.02 mM phosphorylcholine for the NMR analysis. Finally, the samples were either concentrated with Centricon 10 concentrators (for the MS analysis) to the appropriate volume or lyophilized (for the NMR analysis).

For the measurement of the H/D exchange rates of the native scFv fragment, the protein was dialyzed against 6 mM phosphate buffer, containing 0.06 mM phosphorylcholine, pH 6.5, and lyophilized. Immediately before starting the NMR experiments, the protein was dissolved in 100% D_2O, resulting in 60 mM phosphate and 0.6 mM phosphorylcholine. The final protein concentration was 0.4 mM.

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2.3. MS analysis

ESI-MS was used to determine the molecular mass of the respective scFv samples, which were injected into the ion source of a Sciex API III$^+$ instrument (Sciex, Ontario, Canada). The ion spray voltage was approximately 5000 V and the nebulizer gas pressure was 50 psi. The lyophilized samples were dissolved in 10 mM ammonium acetate buffer, pH 5.0 and were mixed in a 1:1 ratio with 49.5% methanol, 49.5% H_2O, 1% formic acid to result in a final pH of approximately 3.0. 5 μl of the mixture was flow-injected into a cooled carrier solution, consisting of 49.5% methanol, 49.5% H_2O, 1% formic acid. A *m/z* range of 1200–2400 was scanned with a step size of 0.15. For calculating the number of protected deuterons, y, residual D_2O (18%) was taken into account by using the equation $y+(81-y)\cdot 0.18 = x$. Here, 81 is the number of all slowly exchanging protons and x is the measured mass difference in the intermediate, obtained by subtracting the mass of the protonated scFv fragment from the respective masses of the samples refolded in D_2O for variable times (Table 1).

2.4. NMR spectra

The spectra of the samples obtained from the stopped flow experiments were recorded on a Bruker DRX 600 spectrometer equipped with a Z-gradient unit. The temperature was 27°C and the lyophilized samples were dissolved in D_2O. Relative protein concentrations were determined by comparing the methyl resonances at −1.0 ppm in 1D spectra recorded with 64 scans. ^{15}N-^1H correlation spectra were recorded using a gradient enhanced version of a HSQC experiment [22]. The carrier was positioned on the water resonance. Residual water suppression was achieved by the application of a WATERGATE 3-9-19 refocusing pulse [23] with a pulse interval of 200 ms in the final BACK INEPT step, allowing optimal inversion of the amide resonances. The gradient strengths were 25%, 10% and 80% of a maximum gradient power of 30 G/cm for the gradients G_1, G_2 and G_3, respectively. The delay for transferring proton magnetization to nitrogen in the INEPT step was set at 2.25 ms. Decoupling during acquisition was achieved using the GARP pulse sequence [24]. To obtain phase sensitive spectra, the TPPI method was used [25]. A data matrix of 4000×128 points was acquired. Data were processed to a final size of 2000×256 points using the in-house written software CC-NMR [26].

The protection factors of a sample, which had been allowed to refold for 200 ms in D_2O before the application of the labeling pulse were obtained in the following way. The rate constants of exchange of the individual amide groups (k_{ex}) were calculated using the equation $I_S/I_{ref} = 1-e^{-k_{ex}\cdot t_s}$, where I_{ref} is the intensity of the signal of the fully protonated reference sample, I_s refers to the normalized signal intensity of the sample to which the labeling pulse was applied after 200 ms, and t_s is the length of the labeling pulse. The intrinsic rate constants for the unfolded state, k_{int}, were calculated by the equation $k_{int} = f_1\cdot k_{H_2O} + f_2\cdot k_{D_2O}$, where k_{H_2O} refers to the intrinsic rate constant for the reaction ND to NH and k_{D_2O} to the reaction NH to ND [27], taking into account the temperature (10°C) and pH (9.5) of the solution in the refolding experiments. The factors f_1 and f_2 refer to the relative fraction of H_2O (=0.8) and of D_2O (=0.2) in the buffer after the application of the labeling pulse. The protection factors were obtained by dividing the measured rate constants (k_{ex}) of the individual NH groups by the calculated rate constants of the random coil reference state (k_{int}) [27].

The H/D exchange rates of the native scFv fragment were measured on a Bruker AMX 500 spectrometer, equipped with a Z-gradient unit. A series of 20 spectra was taken in 70 h. Acquisition and processing parameters were the same as described above and the resulting values for the signal intensities were fitted by single exponentials with the Kaleidagraph Software on a Macintosh Computer.

3. Results and discussion

For measuring the rate of secondary structure stabilization by H/D exchange experiments, the denatured protein was diluted 25-fold into refolding buffer in D_2O in a first step and allowed to refold for a variable time (17 ms to 16 s). Then, in a second step, a labeling pulse of 8.3 or 11.2 ms was applied by diluting the protein solution five-fold into an H_2O buffer of pH 9.7, resulting in a final pH of 9.5. The solution was then quenched with 0.5 M H_3PO_4 to a final pH of 4.9. Folding goes to completion at this pH, where unstructured deuterons can still exchange, while native-like amide deuterons are exchange protected. As can be seen from Table 1, a total of 81 protons become protected if refolding takes place in D_2O, and no labeling pulse is applied. Due to the lower final pH of the solution and optimized conditions for preventing back exchange in the mass spectrometer (see Section 2) this number is higher than in our previous report with manual mixing. In order to see whether we could resolve the kinetics of intermediate formation, we incremented the time of refolding from 17 ms to 4 s. The results are summarized in Table 1. The average mass of all samples is near 27 122 Da, a mass which is already obtained after the shortest possible time of folding (17 ms). This shows that an intermediate has already formed after 17 ms of folding, with a number of sites already sufficiently protected to be mapped by hydrogen exchange. This mass is 32 mass units higher than the mass of the protonated reference, which translates to 21 protected protons, when the residual D_2O content (18%) of the final buffer is taken into account.

To map the protected sites in the structure, similar pulsed hydrogen exchange experiments were carried out with ^{15}N-labeled scFv fragment and analyzed by NMR. Since mass spectrometry had already indicated a constant amount of amide proton protection in the time interval between 17 ms and 16 s, we performed only experiments with refolding times of 200 ms and 2 s. The spectra of the two samples at these

Table 1
Mass spectrometric analysis of stopped flow H/D exchange experiments

Refolding time[a]	Solvent[b]	Labeling pulse[c]	Mass	Protected protons[d]	Protected protons (corrected)[e]
–	H_2O	–	27 089 ± 1	0	–
–	D_2O	–	27 170 ± 1	81 ± 2	–
17 ms	D_2O	11.2 ms	27 121 ± 2	32 ± 3	21 ± 3
78 ms	D_2O	11.2 ms	27 121 ± 2	32 ± 3	21 ± 3
200 ms	D_2O	8.2 ms	27 124 ± 3	35 ± 4	25 ± 4
500 ms	D_2O	8.2 ms	27 123 ± 2	34 ± 3	24 ± 3
1 s	D_2O	8.2 ms	27 126 ± 2	37 ± 3	27 ± 3
16 s	D_2O	8.2 ms	27 123 ± 2	34 ± 3	24 ± 3

[a]Delay time before labeling pulse.
[b]Solvent before labeling pulse or, in the absence of pulse, for the whole folding reaction.
[c]H_2O buffer, $pH_{final} = 9.5$.
[d]Uncorrected values of protected protons (x).
[e]Number of protected protons y corrected for 18% residual D_2O, using the equation $y+(81-y)\cdot 0.18 = x$ (see Section 2).

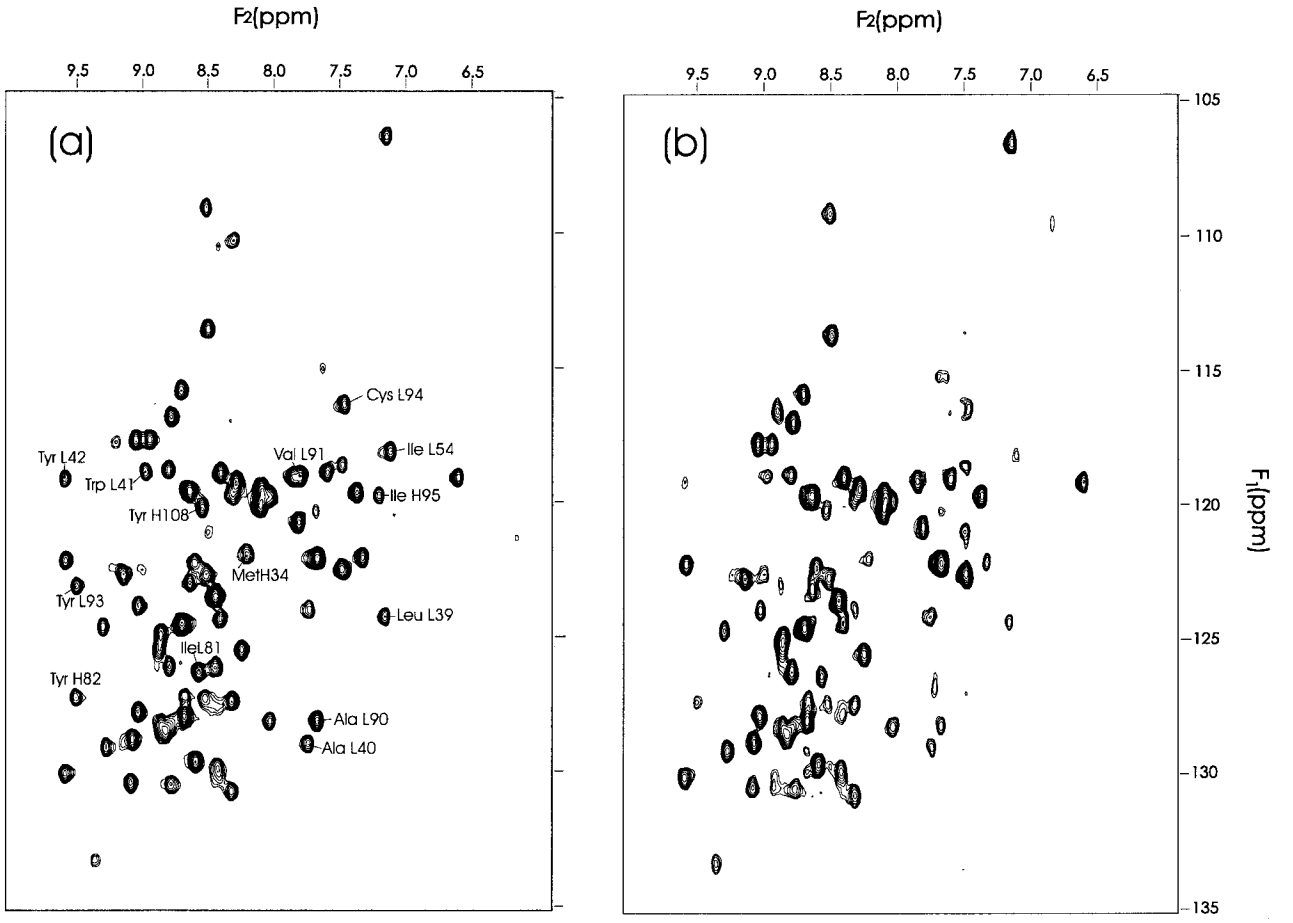

Fig. 1. a: NMR spectrum of the protonated reference sample dissolved in D_2O. b: Spectrum of the sample allowed to refold for 200 ms before application of the H_2O labeling pulse. Peaks of stable amide protons with decreased signal intensity in b are labeled in spectrum a. For these residues an estimate of the protection factors was calculated (see Table 2).

respective time points were found to be the same within experimental error. The spectrum of the sample with a refolding time of 200 ms is shown in Fig. 1b. For comparison, a reference spectrum of the protonated protein dissolved in D_2O is shown in Fig. 1a. A number of peaks with a significant loss of signal intensity, i.e. a significant degree of amide proton protection, are indicated by amino acid labels according to their occurrence in the consecutive numbering of the individual domains (Fig. 1a). They correspond well to the residues of the intermediate identified by the manual mixing experiments [20]. Two additional residues, Met H34 and Cys L94, which escaped the analysis of the manual mixing experiments because of too low signal intensity, were found to be significantly protected after 200 ms. Interestingly, both residues reside within the inner β-sheet of the individual domains, supporting our hypothesis that early stabilization of secondary structure preferentially takes place in the inner β-sheets of antibody variable domains, the side-chains of which form the V_H/V_L interface in the native scFv fragment. It remains to be investigated whether the sites of protection of the rapidly formed intermediate reflect conformational propensities of the denatured state, since NMR analysis of the urea-denatured state of a number of proteins has provided evidence that certain conformations are persistent even at a high concentration of denaturant [28–33].

In order to obtain an estimate of the stability of the protected structure of the folding intermediate, we compared the signal intensities of the two spectra shown in Fig. 1 and calculated exchange rates of the slowly exchanging amide protons in the intermediate from the duration of the labeling pulse and the final intensities. Protection factors were ob-

Table 2
Amide proton signal intensities and protection factors after 200 ms of refolding

Residue number	Signal intensity (%)	Protection factor
Met H34	57.0	5.7
Tyr H82	57.1	2.4
Ile H95	47.7	6.3
Tyr H108	43.0	5.4
Leu L39	50.1	3.6
Ala L40	64.0	1.8
Trp L41	53.9	7.0
Tyr L42	30.8	8.2
Ile L54	29.7	3.1
Ile L81	61.2	3.7
Ala L90	33.6	4.5
Val L91	28.6	15.0
Tyr L93	17.5	21.8
Cys L94	51.5	18.3

Comparison of the signal intensity of individual NH peaks of the sample which was allowed to refold for 200 ms in D_2O before dilution into H_2O buffer with the corresponding peak intensity of the reference sample. The calculated protection factors of these individual NH groups are also listed (see Section 2).

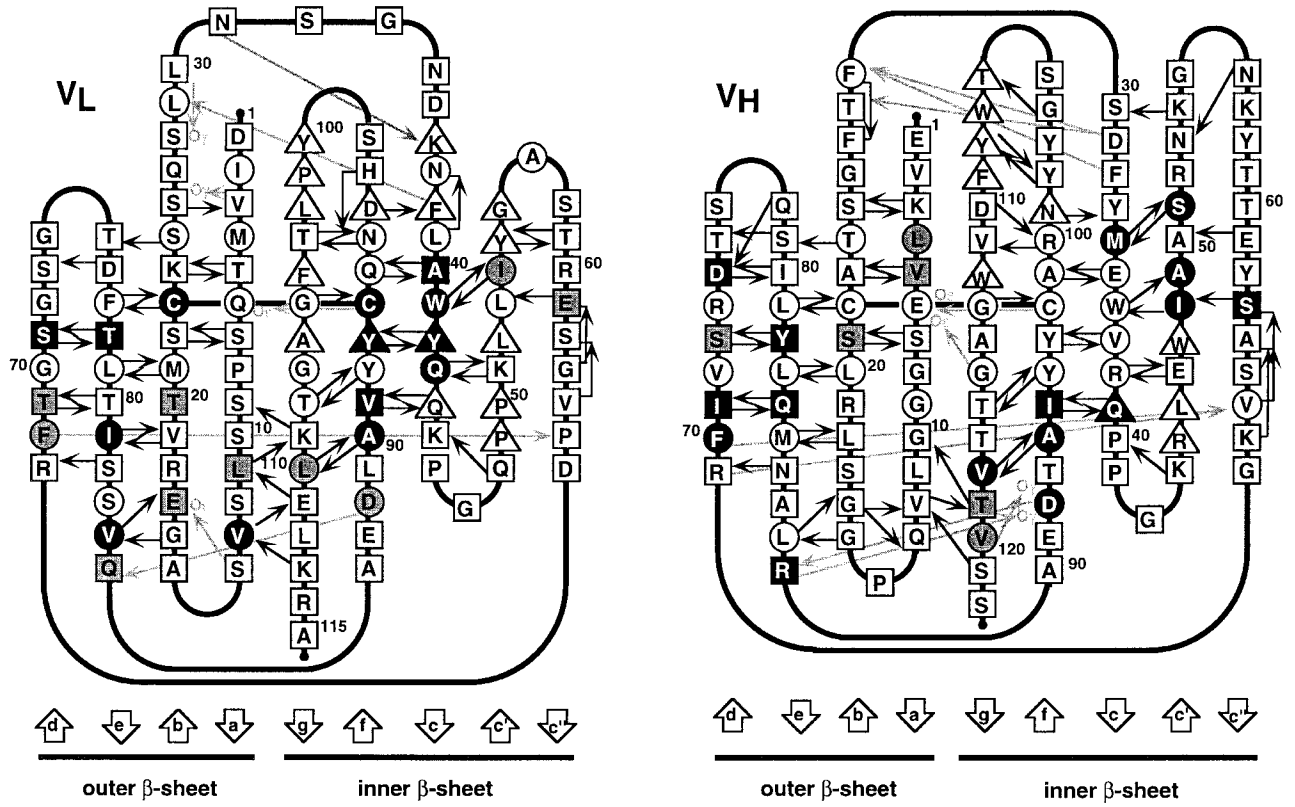

Fig. 2. 2D topology plot of the Fv fragment of the antibody McPC603 as deduced from the X-ray structure of the Fab fragment [35]. Rectangles define residues with surface exposed side-chains, triangles represent residues with side-chains becoming buried upon V_L-V_H association and circles define residues with side-chains which are buried in the domain core of the isolated V_L or the isolated V_H domain. Hydrogen bonds are displayed as arrows (NH→OC), with side-chain oxygen acceptors labeled. β-Strands are labeled consecutively from the N- to the C-terminus, and residues are numbered according to the consecutive numbering of the PDB file 2MCP. Side-chains of residues with backbone amide protection factors greater than 10^5 are shown in black, residues with amide protection factors between 10^4 and 10^5 are depicted in light gray and are listed in Table 3.

tained by using the exchange rates of Bai et al. [27] as reference values for the denatured state. As can be seen in Table 2, the largest protection factors were obtained for residues of the f strand of the V_L domain, e.g. Tyr L93 with a value of 21.8. Estimates of protection factors were deduced for residues with signal reduction of more than 20%, since the errors for residues with a small degree of protection become too large. This means that a few residues with extremely high intrinsic rate constants but still moderate protection factors might not have been taken into account. For example, if residues cysteine-23 or serine-71 of the V_L domain had a protection factor of 5, they would have escaped our analysis, since the exchange of the attached deuteron would still be too fast to be mapped under the conditions used. Exchange was assumed to proceed to a signal intensity of 0 at infinite time, not taking into account noise and signals arising from residual protonation due to small water content in the D_2O buffers. The protection factors are therefore lower estimates. Nevertheless, what becomes clear from our data is that the protection for some residues is significant, but still low compared to the native protein (see below). The major gain of stabilization of the native-like structure is obtained during the slow phase of folding.

In Fig. 2 and Table 3, the results of the H/D exchange rates of the native protein at pD = 6.9 measured by a series of HSQC spectra over a time period of three days are summarized. Residues with protection factors between 10^4 and 10^5 are

Table 3
Exchange rates and protection factors of slowly exchanging amide groups of the native scFv fragment

Residue number	k_{ex} ($min^{-1} \times 10^{-3}$)	$P(=k_{int}/k_{ex}) \times 10^4$
Leu H4	1.7 ± 0.9	5.2
Val H5	1.6 ± 0.27	1.9
Ser H21	1.7 ± 0.5	4.9
Ser H63	0.9 ± 0.6	18.7
Phe H70	1.5 ± 0.24	14.5
Ser H73	1.3 ± 0.6	10.6
Thr H119	1.9 ± 0.26	2.6
Val H120	5.0 ± 1.0	1.9
Leu L11	4.7 ± 0.75	4.7
Val L13	2.4 ± 0.35	11.1
Glu L17	12.0 ± 10	1.7
Thr L20	1.3 ± 0.2	3.8
Ile L54	2.0 ± 0.9	1.2
Glu L61	5.0 ± 1.3	2.7
Phe L68	2.3 ± 0.42	9.4
Thr L69	2.0 ± 0.4	7.2
Ser L71	2.5 ± 0.71	23.4
Thr L78	0.5 ± 0.36	27.6
Val L84	1.3 ± 0.19	20.6
Gln L85	1.2 ± 0.36	4.1
Asp L88	1.3 ± 0.12	2.5
Cys L94	1.0 ± 0.5	30.0
Leu L110	2.3 ± 0.82	3.8

The lyophilized sample was dissolved in D_2O, pD 6.9 at 300 K. Residues with no significant loss of intensity even after 3 days of measurement are not listed, but are colored black in Fig. 2.

colored light gray and residues with protection factors greater than 10^5 are colored black in Fig. 2. The latter class is referred to as the very stable amide protons of the two variable domains. These very stable protons probably exchange by a global unfolding mechanism, since the free energies of stabilization as calculated from the equation $\Delta G = RT\ln k_{int}/k_{ex}$ (assuming a protection factor of 10^5 as a lower estimate) are in the range of the unfolding transition measured by urea denaturation [34]. Interestingly, more than half of the very stable amide protons are located within the V_H domain, whereas in the folding intermediate most of the significantly protected amide groups belong to the V_L domain. Furthermore, many of the very stable amide protons of the V_L domain are already significantly protected within the folding intermediate, which shows that the slowly exchanging core of the V_L domain is formed rapidly.

On the other hand, for the V_H domain most of the very stable amide protons are not protected to any significant degree in the intermediate and become exclusively stabilized in the subsequent slow folding step. We suggest that the gain of stability of V_H depends on correct heterodimerization with V_L. The intrinsic stability of the V_H domain appears to be low, but when productive interface formation has taken place, it behaves like a stable domain with restricted motional flexibility with regard to local unfolding events. As can be seen from Fig. 2, the very stable amide groups of the native scFv fragment are spread over the whole protein and are mainly located within the regular β-sheet structure. However, the V_H domain lacks some stable amide protons in the inner β-sheet, indicating enhanced flexibility within this conserved region of the framework. On the other hand, strand c″ of the V_H domain displays a number of highly protected residues, which are absent in the V_L domain. Obviously, topologically identical parts of the two variable domains display different H/D exchange behavior. Another example is the well defined loop connecting the two β-sheets (strands e and f in Fig. 2) of the individual domains, the residues of which show measurable exchange rates for the V_L domain (residues L85 and L88, see Table 3), while they belong to the very stable residues of the V_H domain (residues H89 and H92). These residues are topologically at identical positions, have almost the same intrinsic rate constants in a random coil [27], and the N and H atoms are fully buried as defined by the X-ray coordinates [35]. Hence, the difference in the observed rate constants have to be interpreted in terms of higher local flexibility of this loop within the V_L domain as compared to the V_H domain.

We therefore conclude that in the folding intermediate of the scFv fragment the V_L domain is more stabilized than the V_H domain, whereas in the native scFv fragment the V_H domain has become highly structured and has gained a dramatic amount of free energy, as indicated by the many very stable hydrogen bonds formed. Local unfolding events leading to amide proton exchange are different for some topologically identical parts of V_H and V_L, even within conserved regions of the antibody variable domains.

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