Domain interactions in antibody Fv and scFv fragments: effects on unfolding kinetics and equilibria

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Abstract The equilibrium denaturation and unfolding kinetics of the domains V_L and V_H have been compared with those of the Fv and single-chain Fv (scFv) fragment of an engineered variant of the antibody McPC603 in the presence and absence of the antigen phosphorylcholine. The scFv fragment is significantly more stable than the isolated constituting domains. Antigen binding stabilizes the heterodimeric assembly even further. Domain dissociation and domain unfolding are coupled processes, giving rise to a highly cooperative unfolding transition. For the Fv fragment, cooperative unfolding is only observed in the presence of antigen. At low protein concentrations and in the absence of antigen, the Fv fragment is significantly destabilized, leading to quantitative domain dissociation before significant domain unfolding takes place. The kinetic unfolding of V_H, V_L and the scFv fragment is monophasic. Unfolding of the scFv fragment is much slower, when extrapolated to zero denaturant, than either of the isolated domains, suggesting that the higher thermodynamic stability of the scFv fragment is at least partially due to a high-energy transition state for unfolding. These studies emphasize the enormous importance of mutual domain stabilization in engineering stable antibodies.

Key words: Single-chain Fv fragment; Protein stability; Two-domain protein; Kinetic stabilization; Domain folding

1. Introduction

Folding pathways of several small, monomeric proteins have been mapped to considerable detail [1–3]. However, much less information is available on the folding and assembly of larger multidomain proteins. The quaternary structure in oligomeric proteins adds another layer of complexity to the problem of understanding the mechanism by which the amino acid sequence directs the folding to a stable conformation [4]. One particularly interesting question is to what extent the folding and stability of the structural domains in multidomain proteins is influenced by the presence of the other domains of the protein. This situation arises both in oligomeric proteins and in large monomeric proteins where a chain can consist of several interacting domains. These aspects are difficult to study experimentally, as a rigorous analysis requires the availability of both the intact protein and its domains or subunits in isolated form. Yet, subunits of many oligomeric proteins either tend to aggregate or are populated only at insignificant amounts at equilibrium [4]. Often, the isolated domains can only be formed and maintained by special, non-native conditions such as low temperature, high hydrostatic pressure or the presence of substantial amounts of chaotropic substances, thereby making a rigorous biophysical study and comparison with the oligomer or multisubunit protein difficult [4].

The antibody McPC603 [5] is a useful model system to study these aspects of large, multidomain proteins in detail. The Fv fragment is a non-covalent heterodimer, composed of the variable domains of the light (V_L: 12.4 kDa) and heavy chains (V_H: 13.6 kDa), while the single-chain Fv (scFv) fragment is a covalently linked form, in our study of the form V_H-$(G_4S)_5$-V_L. The Fv and scFv fragments, but not V_H and V_L alone, bind the small hapten phosphorylcholine (PC) ($K_D = 8 \times 10^{-6}$ M^{-1}) [6,7]. The binding site for PC is located in a pocket close to the pseudo two-fold axis relating V_L and V_H in the interface. Residues of both V_L and V_H contribute to antigen binding [5]. PC does not show any intrinsic fluorescence, and its binding to the fully associated Fv fragment is spectroscopically silent at the Trp-specific excitation at 295 nm [7]. Once engineered for stability and folding efficiency [8,9], V_H and V_L can easily be obtained in isolated and fully functional form by chain separation under native conditions [10].

We now describe experiments to investigate the equilibrium and kinetic unfolding behavior of these molecules. By comparing the thermodynamic stability of V_H and V_L in isolated form with those of the Fv and scFv fragments, we can now make quantitative statements on the effect of mutual domain stabilization and the additional effect of the interdomain linker. Finally, we compare the mechanism of kinetic and equilibrium unfolding of these proteins and investigate the effect of antigen, which is bound in a pocket in the interface between domains. These studies will be important for guiding further engineering of recombinant antibodies and for a better understanding of the implications of the scFv format for antibody stability.

2. Materials and methods

2.1. Protein purification and characterization

Mutations were introduced into V_H and V_L to improve stability and folding efficiency [10]. The scFv fragment used is in the orientation V_H-$(G_4S)_5$-V_L. A His_5 tag was attached to the C-terminus of V_L to facilitate protein purification. The Fv and scFv fragments were

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Abbreviations: CDR, complementarity determining region; GdmCl, guanidinium chloride (guanidine hydrochloride); em_{max}, emission maximum; Fv, antibody fragment consisting of the variable domains of the heavy and the light chain in non-covalent assembly; PC, phosphorylcholine; 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

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expressed in *Escherichia coli* strain JM83 and purified as described [10]. V_H and V_L were obtained from affinity-purified Fv fragment by chain separation under native conditions [10]. Purified proteins were stored at 4°C in 10 mM MES, pH 6.0 (V_H, V_L) or in 10 mM MES, pH 6.0, 10 mM PC (Fv, scFv) and used within 48 h.

2.2. Determination of thermodynamic stabilities

Equilibrium unfolding curves were obtained by mixing protein in 10 mM MES, pH 6.0 in the presence or absence of 50 mM PC and increasing amounts of guanidinium chloride (GdmCl) (0–3.6 M). Denaturant concentrations were determined refractometrically [11]. After incubation for 12 h at 20°C, fluorescence emission spectra were recorded at 20°C with a PTI Alpha Scan spectrofluorimeter (Photon Technologies Inc., Ontario, Canada). The protein was excited at 280 nm (V_L) or 295 nm (V_H, scFv). Fluorescence emission maxima were determined by fitting the peak regions of the emission spectra to a third-order polynomial [8], using the program Kaleidagraph (Synergy Software, Reading, MA, USA). Four fluorescence spectra per sample were averaged, and changes in free energy upon unfolding (ΔG_{N-U}) were estimated by a six-parameter least-squares fit, assuming a two-state unfolding model [12]:

$$F_{obs} = ((F_{folded} + m_{folded}[D]) + (F_{unfolded} + m_{unfolded}[D])\cdot\exp(-(\Delta G_{N-U} - m_G[D])/RT))/(1 + \exp(-(\Delta G_{N-U} - m_G[D])/RT)) \quad (1a)$$

F_{obs} is the observed fluorescence intensity at denaturant concentration $[D]$, F_{folded}, $F_{unfolded}$, m_{folded} and $m_{unfolded}$ represent intercepts and slopes of native and unfolded baselines, respectively, m_G is a cooperativity parameter related to exposure of hydrophobic groups upon global unfolding [13], R is the gas constant and T the absolute temperature. Unfolding transitions were normalized to the fraction of unfolded protein (F_U):

$$F_U = ((F_{folded} + m_{folded}[D]) - F_{obs})/((F_{folded} + m_{folded}[D]) - (F_{unfolded} + m_{unfolded}[D])) \quad (1b)$$

where F_{obs} is the observed fluorescence signal at $[D]$.

2.3. Kinetics of protein unfolding

Unfolding rates were measured at 20°C using an Applied Photophysics SX17 spectrofluorimeter (Applied Photophysics, Leatherhead, UK). Protein stock solutions in 10 mM MES, pH 6.0; 50 mM PC were diluted 1:10 (v/v) into a solution of GdmCl, dissolved in 10 mM MES, pH 6.0; 50 mM PC. Protein excitation was at 280 nm (V_L, scFv) or 295 nm (V_H). The change in fluorescence emission intensity at >360 nm (V_L, scFv) or >335 nm (V_H) was recorded with a cut-off filter. Typically, 4–7 single fluorescence traces were averaged and fitted to a sum of exponentials using Kaleidagraph. Unfolding rates in the absence of denaturant were estimated by linear extrapolation [14]:

$$\ln(k_u(obs)) = \ln(k_u(H_2O)) + m_u[D]/RT \quad (2)$$

where $k_u(obs)$ is the apparent first-order rate constant for unfolding at denaturant concentration $[D]$, $k_u(H_2O)$ is the apparent rate constant for unfolding in the absence of denaturant, and m_u is the change in solvent accessibility of the unfolded state, R is the gas constant and T is the absolute temperature.

3. Results

3.1. Thermodynamic stabilities of V_H and V_L

Fig. 1a shows the fluorescence spectra of the native domains V_H and V_L relative to the fluorescence spectra of the respective domains in 4 M GdmCl. V_L contains a single Trp residue (TrpL36), completely buried in the hydrophobic core [5]. In native V_L, the fluorescence of TrpL36 is essentially quenched by the juxtaposed disulfide bond, but a large increase in fluorescence is observed upon unfolding. Besides the homologous TrpH35, V_H contains three additional, mainly solvent-exposed Trp residues (TrpH47, TrpH100i, TrpH103), located at the edges of the complementarity determining regions (CDRs) [5,10]. Similar to V_L, unfolding of V_H can be followed by a fluorescence increase.

Fig. 1b shows the reversible equilibrium unfolding curves of V_L and V_H measured by fluorescence at 350 nm (V_L) or 370 nm (V_H). For a direct comparison of the two domains, the unfolding transitions shown were normalized to the fraction of unfolded protein (F_U) (Eq. 1b). A least-squares fit of the unfolding transitions (Eq. 1a) gives a free energy of unfolding (ΔG_{N-U}) of 23.5 ± 0.2 kJ mol^{-1} (V_L) and 18.0 ± 1.1 kJ mol^{-1} (V_H) and unfolding cooperativities (m_G) of 13.9 ± 0.2 kJ mol^{-1} M^{-1} (V_L) and 13.6 ± 0.8 kJ mol^{-1} M^{-1} (V_H) (Table 1).

3.2. Mutual domain stabilization in the scFv fragment

The fluorescence emission spectrum of the native scFv fragment differs clearly from the computational addition of the spectra of native V_H and V_L at a 1:1 stoichiometry (Fig. 2a). The emission maximum of the scFv is blue-shifted to 331 nm, accompanied by a fluorescence increase at <360 nm and a fluorescence decrease at >360 nm. As shown previously, the spectral changes result from a burial of the CDR tryptophans in V_H in a hydrophobic interface upon domain association [10]. The unfolding of the scFv fragment will thus be followed by the shift in the emission maximum (em_{max}) and/or the increase in fluorescence emission intensity at 370 nm (F_{370nm}).

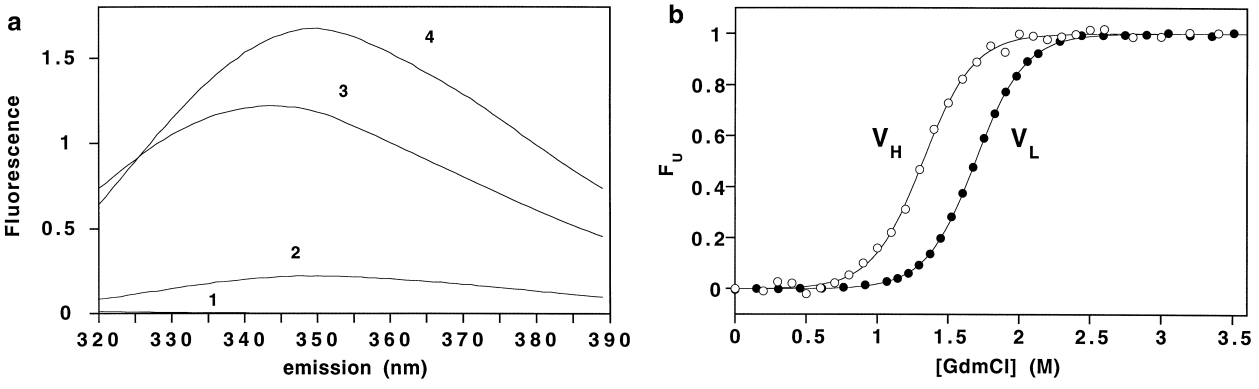

Fig. 1. a: Fluorescence emission spectra of (1) native V_L, (2) unfolded V_L, (3) native V_H, (4) unfolded V_H. Protein excitation was at 295 nm. Protein concentration was 1 μM. b: Equilibrium unfolding curves of the V_H (○) and V_L (●) domains, monitored by the change in fluorescence at 350 nm (V_L) or 370 nm (V_H). Solid lines represent fits to a two-state unfolding model (Eq. 1a) after normalization to the fraction of unfolded protein (F_U) (Eq. 1b). Protein concentration was 1 μM.

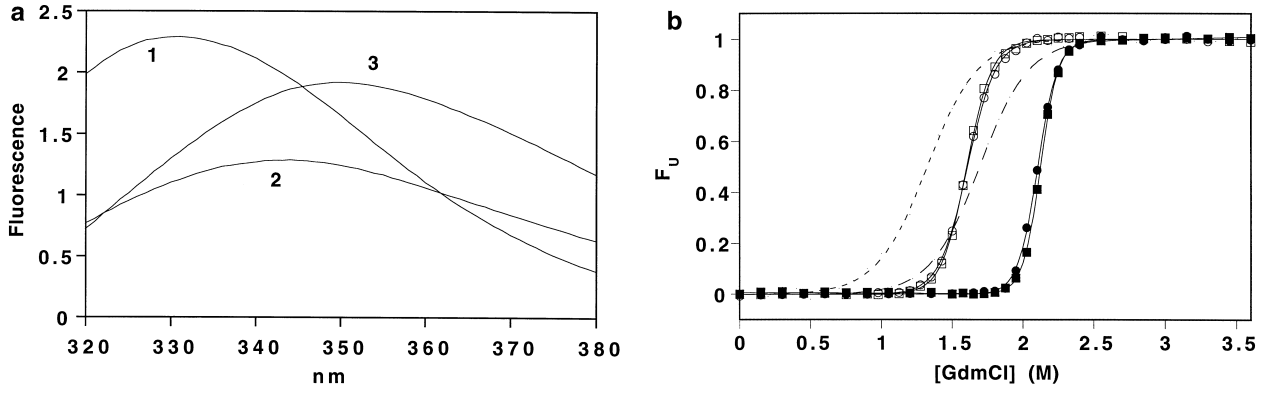

Fig. 2. a: Fluorescence emission spectra of (1) native scFv fragment, (2) a 1:1 computational addition of the emission spectra of native V_L and V_H (equivalent to a hypothetical scFv fragment with completely dissociated interface but native domains), (3) unfolded scFv fragment. b: Equilibrium unfolding transition of the scFv fragment. Data sets shown were obtained with the em_{max} (no PC: □; 50 mM PC: ■) or the F_{370nm} method (no PC: ○; 50 mM PC: ●). Solid lines represent fits to a two-state unfolding model (Eq. 1a) after normalization to the fraction of unfolded protein (F_U) (Eq. 1b). Also shown in panel b are the normalized transitions of V_H (——) and V_L(- - -) from Fig. 1b. Protein excitation was at 295 nm. Protein concentration was 1 μM.

As shown in Fig. 2b, both types of data analysis give highly cooperative and nearly superimposable unfolding transitions. Unfolding transitions were analyzed by a least-squares fit assuming the validity of a two-state model for unfolding (Eq. 1a). Free energy changes (ΔG_{N-U}) and unfolding cooperativities (m_G) of the scFv fragment are comparable for F_{370nm} and em_{max} data (Table 1). The minor differences in the thermodynamic values between em_{max} and F_{370nm} measurements might be due to well known intrinsic differences, which are due to the fact that the observed fluorescence emission maximum does not have to be linearly related to the fraction of molecules in both states [14,15]. The stability of the scFv fragment in the absence of PC is clearly higher than the stability of the isolated domains, indicating that both domains stabilize each other in the scFv fragment. Binding of PC leads to a further gain in stability of the scFv fragment of ~30 kJ mol^{-1}. This shows that hapten binding is a major determinant in the stability of the domain assembly. Control experiments verified that the stability of V_H and V_L was not influenced by PC, and gel filtration studies confirmed that the scFv fragment was in a monomeric state up to concentrations of at least 100 μM (data not shown). The stabilizing effect of PC on the heterodimer is thus likely to be due to the fact that the hapten interacts with residues in both V_H and V_L [5], thereby contributing directly to the effective interface energy. The good agreement between the normalized transitions, obtained by measuring either spectral shift or intensity changes, together with the high cooperativity of unfolding (m_G), suggests that a single transition is observed. The unfolding of the scFv fragment of McPC603, containing the stabilizing mutations detailed above, can therefore most likely be described by a two-state unfolding model with only native and unfolded species populated to significant amounts.

Table 1
Thermodynamic parameters of the equilibrium unfolding reaction of the Fv and scFv fragments and the constituting domains V_H and V_L (see Eqs. 1a and 1b)

Protein	ΔG_{N-U} (kJ mol^{-1})	m_G (kJ mol^{-1} M^{-1})
V_L	23.5 ± 0.2	13.9 ± 0.2
V_H	18.0 ± 1.1	13.6 ± 0.8
Fv (0.1 μM, +50 mM PC)	36.6 ± 1.0[a]	22.6 ± 0.6[a]
	30.9 ± 1.1[b]	18.0 ± 1.0[b]
Fv (3.8 μM, +50 mM PC)	43.6 ± 1.0[a]	23.9 ± 0.5[a]
	40.8 ± 1.0[b]	21.8 ± 0.5[b]
scFv (−PC)	43.5 ± 1.0[a]	27.1 ± 0.5[a]
	39.1 ± 0.9[b]	24.4 ± 0.5[b]
scFv (+50 mM PC)	77.2 ± 1.3[a]	37.0 ± 0.6[a]
	71.8 ± 1.7[b]	34.1 ± 0.9[b]

[a]Thermodynamic data obtained from fitting a two-state model (Eq. 1a) to the em_{max} data.
[b]Thermodynamic data obtained from fitting a two-state model (Eq. 1b) to the F_{370nm} data.

3.3. Uncoupling of domain dissociation and domain unfolding in the Fv fragment

From direct domain titrations and earlier estimates it is known that the Fv fragment dissociates with a K_D of about 2×10^{-7} M into V_H and V_L [6,7,10]. Consequently, at low protein concentrations (0.1 μM) *and* in the absence of PC, the Fv fragment is present to only ~30% in the assembled heterodimeric form, consistent with the fairly red-shifted fluorescence emission maximum of 340 nm (Fig. 3a, ●). Almost complete chain dissociation is observed at moderate denaturant concentrations (1 M GdmCl), i.e. at denaturant concentrations where the isolated domains are still essentially folded (Fig. 1b). Unfolding of the single domains contributes only little to the shift in the maximum, and is only detectable by a small further red shift in the emission maximum between 1.0 and 2.0 M GdmCl. Higher protein concentrations (3.8 μM) favor the formation of the heterodimer. The Fv fragment is now fully assembled in the absence of denaturant (emission maximum: 331 nm) and more resistant towards denaturant-induced chain dissociation (Fig. 3a, ○). The stability of the scFv fragment (Fig. 3a, ▲) is, however, not reached. This means that the effective domain concentration in the scFv fragment must be ⩾3.8 μM, the highest concentration used for the Fv fragment in the present study.

In the presence of antigen, the stability of the Fv heterodimer is significantly increased. Even at sub-micromolar concentrations (0.1 μM) of the Fv fragment, the heterodimeric form is quantitatively assembled and unfolding is cooperative (Fig. 3b, ●). A slight shift (1–2 nm) of the maximum in the pre-transition region, compared to the scFv fragment, might indicate a small amount of domain dissociation (Fig. 3b).

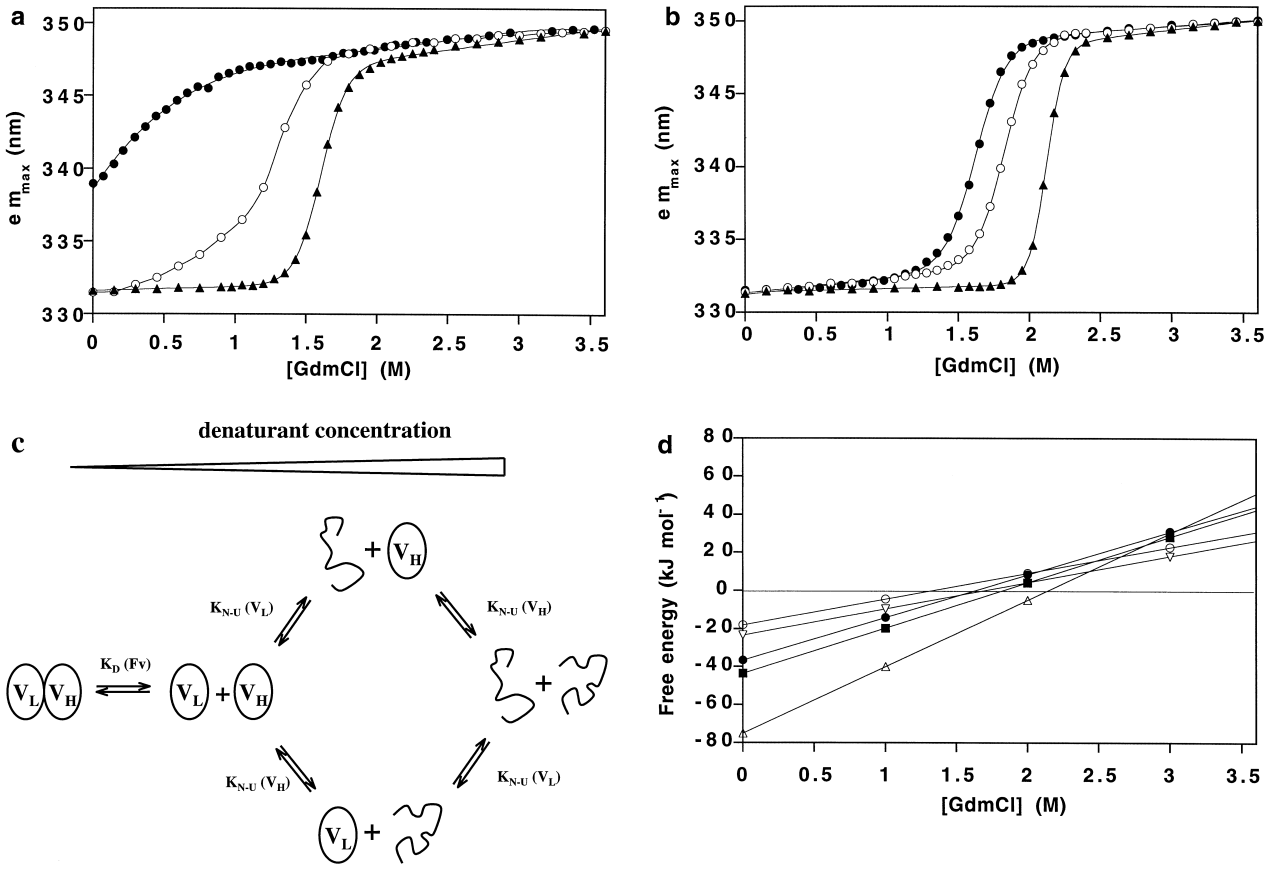

Fig. 3. Equilibrium unfolding transitions of the Fv fragment obtained (a) in the absence of PC, (b) in the presence of 50 mM PC. The emission maximum (em_{max}) is shown as a function of the GdmCl concentration. Protein concentrations were 0.1 μM (●) and 3.8 μM (○). Also shown in panels a and b are the unfolding transitions of the scFv fragment (▲) shown in Fig. 2, but with the unfolding transitions not normalized. Solid lines in panel b represent fits to a two-state unfolding model (Eq. 1a), while in panel a only the data of the scFv fragment are fitted to Eq. 1a. c: Schematic representation of the equilibrium unfolding behavior of the Fv fragment. For explanations see text. d: Plot of the data in Table 1 to illustrate the relative stability of the various species investigated at intermediate denaturant concentration. A linear dependence of free energy on denaturant is assumed. The midpoint of unfolding of each species is the denaturant concentration where the line crosses the line of zero free energy. At any given denaturant concentration, species in equilibrium with each other will be populated according to their relative free energy. The data of the Fv and scFv fragments were obtained in the presence of 50 mM PC, such that the native proteins will be exclusively in the form of the PC complex. ○: V_H; ▽: V_L; ●: Fv (0.1 μM); ■: Fv (3.8 μM); △: scFv.

ΔG_{N-U} and m_G values of the Fv fragment are higher than that of the individual domains (Table 1) at all protein concentrations investigated. As expected from the law of mass action for a dimeric protein, the apparent stability of the Fv fragment increases with higher protein concentrations (Table 1). Again, a comparison with the scFv fragment (Fig. 3b, ▲) shows that the effective domain concentration in the scFv fragment is ⩾3.8 μM, the protein concentration used in the Fv fragment. From a plot of $RT\ln[Fv]$ vs. ΔG_{N-U}, we would estimate an effective domain concentration in the lower millimolar range in the present scFv fragment (data not shown), but this determination clearly requires more data points and attention to the linker length.

Taken together, these findings show that three unfolding equilibria must exist for the Fv fragment in the *absence* of PC and at low protein concentrations (0.1 μM or lower) (Fig. 3c). The first equilibrium ($Fv \rightleftharpoons V_{H(native)} + V_{L(native)}$) describes the concentration-dependent monomer-heterodimer dissociation equilibrium. The second and third equilibria describe the concentration-independent unfolding of the V_H and V_L domain ($V_{H(native)} \rightleftharpoons V_{H(unfolded)}$; $V_{L(native)} \rightleftharpoons V_{L(native)}$) at denaturant concentrations where the heterodimer is already quantitatively dissociated into the constituting domains. Binding of PC or higher total protein concentrations or both, as well as covalent linking of the two domains in the scFv, shift the dissociation equilibrium towards the assembled Fv heterodimer, thereby lowering the concentration of free $V_{H(native)}$ and $V_{L(native)}$. Above a certain protein concentration, favored by PC or the covalent linking of the domains, the stability of the heterodimer overcomes that of the isolated domains, resulting in cooperative unfolding transitions ($Fv{\cdot}PC \rightleftharpoons V_{H(unfolded)} + V_{L(unfolded)} + PC$).

We have summarized the stabilities of the domains and the domain assemblies in Fig. 3d. This plot illustrates which species is predominant at any denaturant concentration and uses the data from Table 1. The scFv fragment in the presence of PC is more stable than any of the domains over the whole range of denaturant concentrations before it crosses the zero line, illustrating a two-state behavior. In contrast, the Fv fragment in complex with PC approaches the isolated domains at intermediate concentrations of denaturant, and in the absence of PC, the stability is even significantly lower (Fig. 3a,b), suggesting that its stability line will cross that of the single domains before crossing the zero line. This means that the

isolated domains will be more stable than the Fv fragment at intermediate concentrations, in other words, the equilibrium unfolding of the Fv fragment first involves domain dissociation. This plot also shows how the increase in effective domain concentration, or more generally, interaction energy between the domains, moves the denaturation toward a two-state transition. With increasing stability (from ● to ■ to △), the line moves away from those of the single domains, indicating that the folded V_H-V_L assembly will directly unfold both domains.

3.4. Unfolding kinetics

The unfolding kinetics of all three proteins investigated (isolated V_H and V_L domain, scFv fragment) are monoexponential at all denaturant concentrations tested. Burst-phase unfolding in the dead time of the stopped-flow apparatus (a few ms) can be ruled out, as the initial fluorescence intensities, obtained after extrapolation of the measured fluorescence back to zero time, was only insignificantly higher than the fluorescence of the native protein without denaturant (data not shown). The apparent unfolding rate (k_u) of V_L increased in a linear fashion with the denaturant concentration (Fig. 4, ○). This suggests that no changes occur in the rate-limiting step of unfolding. The slope (m_u) of the unfolding rate constant is 3.29 kJ mol^{-1} M^{-1}, and an unfolding rate constant in buffer without denaturant ($k_u(H_2O)$) of 3.6×10^{-3} s^{-1} can be estimated by linear extrapolation (Eq. 2) (Table 2).

The logarithms of the unfolding rates (k_u) obtained for the isolated V_H domain were not linearly dependent on the denaturant concentration (Fig. 4, ●), which makes an extrapolation to buffer without denaturant problematic. Nevertheless, an estimate of $k_u(H_2O)$ of V_H could be obtained by fitting the linear part of the plot (~2.5–3.5 M GdmCl) to Eq. 2. The approximate value (~2.4×10^{-3} s^{-1}) is comparable to that of the V_L domain. A curvature in the log(k_u) vs. [GdmCl] plot has also been detected in other proteins, such as the Arc P22 repressor [16] or the ribosomal protein U1A [17], and was explained by changes in the solvent exposure of the transition state with denaturant concentration.

Unfolding of the scFv fragment below 3.8 M GdmCl was too slow to be studied by stopped-flow mixing, but above 3.8 M GdmCl, monoexponential kinetics were obtained. The rates increased in a linear fashion with denaturant ($m_u = 4.52$ kJ mol^{-1} M^{-1}), and an unfolding rate in buffer ($k_u(H_2O)$) of 2×10^{-5} s^{-1} was obtained by linear extrapolation (Fig. 4, ●). The fact that unfolding of the scFv fragment is monophasic and about one order of magnitude slower than for V_L and V_H implies that the heterodimer must unfold cooperatively. Significant domain interaction must persist in the transition state for unfolding. Direct support for this view can be obtained by calculating the increase in solvent exposure of the transition state for unfolding (β_T) [18], relative to the native state, according to the relationship $\beta_T = m_u/m_G = 0.06$ (94% native-like contacts present in transition state). The kinetic data therefore imply that the higher stability of V_H and V_L in the scFv fragment result at least partly from a higher activation energy barrier towards unfolding. In other words, in the antibody McPC603, the unfolding pathway of one domain is kinetically blocked by the presence of the other domain.

Table 2
Kinetic parameters of the unfolding reaction of the scFv fragment and the isolated domains V_H and V_L (see Eq. 2)

Protein	$k_u(H_2O)$ (s^{-1})	m_u (kJ mol^{-1} M^{-1})
V_H	2.4×10^{-3}	variable
V_L	3.6×10^{-3}	3.3
scFv	2.0×10^{-5}	4.5

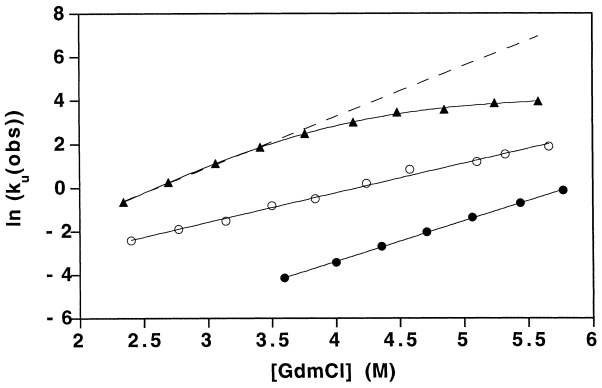

Fig. 4. Dependence of the apparent rate constants for unfolding (k_u(obs)) on the GdmCl concentration for V_L (○), V_H (▲) and the scFv fragment (●). In the case of V_L and the scFv fragment, the solid lines are theoretical fits to Eq. 2. In the case of V_H, the solid line is only supplied to visualize the curvature. The dotted line represents a fit to Eq. 2 of the linear part of the plot only.

4. Discussion

We compared the equilibrium stability and unfolding kinetics of the Fv and the covalently linked scFv heterodimer of the antibody McPC603 with its constituting domains. While the equilibrium stability of scFv fragments has been investigated before [19,20], this report provides the first study in which the stability of V_H, V_L, Fv and scFv fragments has been compared, including the influence of the small antigen PC, which binds in the interface.

Besides in antibodies, the stability of multidomain proteins has been investigated with other model systems, such as γII-crystallin [21,22], phosphoglycerate kinase [23], CD4 [24], tryptophan synthase [25] and diphtheria toxin [26]. The relative contribution of the intrinsic domain stability and the stability provided by the domain assembly has been used to interpret these findings in several examples [26,27].

The unfolding of the scFv fragment is a highly cooperative process, suggesting that domain dissociation and domain unfolding are thermodynamically coupled processes. V_H and V_L are stabilized in the scFv fragment by about 25–30 kJ mol^{-1} and this effect is even more pronounced (ca. 50 kJ mol^{-1}) in the presence of PC, which binds into an interface cleft and makes contact with residues of both chains. The Fv fragment, in which the domain subunits are associated non-covalently with a K_D of 2×10^{-7} M in the absence of PC, is only partially associated at micromolar protein concentrations. Quantitative domain association can be achieved in the presence of PC under strongly native conditions. As expected for a heterodimeric protein, the thermodynamic stability of the Fv fragment increases with higher protein concentrations, but the stability of the scFv fragment was not reached in the concentration range investigated (0.1–3.8 μM). The high thermodynamic stability of the scFv fragment thus results from a high effec-

tive domain concentration, provided by the $(G_4S)_5$ linker, which we estimate to be in the lower millimolar range.

The stability of domains in a multidomain assembly, whether covalently linked or not, can be described by a model first proposed by Brandts and coworkers [27] and extended by others [23,26], in which the effective stability of a domain in the complex is the sum of its intrinsic stability plus an extrinsic contribution from the interacting partner domain. Recently, based on a series of antibody mutants, scFv fragments were ordered into four classes, depending on the relative stabilities of the domains and the extrinsic energy provided by the assembly [28]. Briefly, (i) one domain may be intrinsically much more stable than the other, even including the extrinsic contribution, leading to an equilibrium unfolding intermediate with one folded and one unfolded domain, (ii) the intrinsic stability of one domain may be only slightly larger than the sum of intrinsic and extrinsic stabilities of the other, leading to conspicuously broad transitions, (iii) the assembly may stabilize both domains above their intrinsic stability, leading to a cooperative unfolding of the scFv fragment, and (iv) reasonably stable native domains may be held together by a very weak interface, leading to an intermediate of dissociated native domains.

In the present study, we were able to conveniently vary the effective interface energy by changing the protein concentration in the Fv fragment, by adding PC or by covalently linking the domains. By this strategy, we could observe the Fv fragment to essentially follow model (iv), until the interface is so stable that the scFv fragment, and most dramatically the PC-bound scFv fragment, clearly follows model (iii). It should be pointed out that model (iv) has not been found so far in scFv fragments. This quantitative comparison shows the enormous influence that the relative mutual stabilization of a domain can provide to stability.

Kinetic unfolding experiments show that the unfolding rate of the domains V_H and V_L is drastically reduced in the scFv fragment. The observed unfolding rate for the scFv fragment is monophasic and much slower than the unfolding rate of either domain at all conditions tested. This implies that the two domains must still interact in the transition state for unfolding. The higher stability of the scFv fragment can therefore be linked to the heterodimeric assembly, demanding a compact, native-like and high-energy transition state for unfolding, with about 94% native contacts estimated to be present.

We believe that the scFv fragment described here is a useful model system for future studies on the stability of multidomain proteins in general, as there is this convenient variation of interface strength. For example, it would be interesting to quantitatively assess the effect of destabilizing mutations in the constitutive domains on the heterodimer. Previously described V_L variants with widely differing stabilities [9] might be very helpful in this respect [20,28]. By combining stable domains and stable interfaces, antibody fragments very resistant to unfolding should become accessible, which will be decisive for the future application of recombinant antibody technology.

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