Characterization of a quaternary-structured folding intermediate of an antibody Fab-fragment

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Abstract

Antibody folding is a complex process comprising folding and association reactions. Although it is usually difficult to characterize kinetic folding intermediates, in the case of the antibody Fab fragment, domain–domain interactions lead to a rate-limiting step of folding, thus accumulating folding intermediates at a late step of folding. Here, we analyzed a late folding intermediate of the Fab fragment of the monoclonal antibody MAK 33 from mouse (κ /IgG1). As a strategy for accumulation of this intermediate we used partial denaturation of the native Fab by guanidinium chloride. This denaturation intermediate, which can be populated to about 90%, is indistinguishable from a late-folding intermediate with respect to denaturation and renaturation kinetics. The spectroscopic analysis reveals a native-like secondary structure of this intermediate with aromatic side chains only slightly more solvent exposed than in the native state. The respective partner domains are weakly associated. From these data we conclude that the intramolecular association of the two chains during folding, with all domains in a native-like structure, follows a two-step mechanism. In this mechanism, presumably hydrophobic interactions are followed by rearrangements leading to the exact complementarity of the contact sites of the respective domains.

Keywords: β -sheet structures; disulfide bonds; protein association; protein folding; protein stability

Characterization of protein folding comprises kinetic analysis and the examination of folding intermediates and transition states. Detailed work on the folding pathways has been performed for a few small proteins like BPTI (Weissman & Kim, 1991; Goldenberg, 1992), lysozyme (Dobson et al., 1994), barnase (Bycroft et al., 1990; Fersht, 1993; Sanz & Fersht, 1993), or single antibody domains (cf. Hamaguchi, 1991). In the case of BPTI, all highly populated folding intermediates during oxidative refolding have a native-like structure (Deisenhofer & Steigemann, 1975; Stassinopoulou et al., 1984; van Mierlo et al., 1991). Also, the two-disulfide intermediates with one non-native disulfide bond possess at least native-like secondary structure and a native-like hydrophobic core (van Mierlo et al., 1994). In contrast, several differently structured intermediates have been observed in the case of lysozyme, suggesting that lysozyme refolds via multiple folding pathways during early stages of fold-

ing (cf. Dobson et al., 1994). Barnase is one of the few proteins for which the transition state has been analyzed. It was shown that in the transition state the α -helix is fully formed, but only few structural constraints have been detected within the region of the β -sheets (Fersht, 1993).

The folding of antibody domains, starting either from the oxidized or reduced form, was extensively analyzed by Goto and Hamaguchi (cf. Hamaguchi, 1991). They detected a folding intermediate with native-like secondary structure in which at least one prolyl residue was not in its native *cis*-configuration (Isenman et al., 1979; Goto & Hamaguchi, 1982a). Based on these data, the process of structure formation for the antibody light chain could be described by the independent folding of the two domains with an additional reaction, which was interpreted as an interaction of the two domains of the light chain (Tsunenaga et al., 1987). In the intact antibody, the light chain is associated with the Fd part of the heavy chain, forming an Fab fragment. The two chains of the Fab fragment are composed of two domains, which are linked via a C-terminal disulfide bridge. Analysis of the association of light chain and heavy chain and of single partner domains (Bigelow et al., 1974; Dorrington, 1978; Klein et al., 1979), together with the crystal structure of several antibody Fab fragments (Matsushima et al., 1978; Marquart & Deisenhofer, 1982; He et al., 1992; Tormo et al., 1992), gave insight into the specificity and energetics of protein–protein interactions (Chothia et al., 1985; Getzoff et al., 1986; Padlan et al., 1986).

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Abbreviations: DTT, dithiothreitol; Fab, antigen-binding antibody fragment consisting of the entire light chain and the two N-terminal domains of the heavy chain with intact interchain disulfide bond; Fab_{alk}, Fab fragments with the intermolecular disulfide bond reduced and irreversibly alkylated; Fd, the two N-terminal domains of the heavy chain; Gdm/Cl, guanidinium chloride; MAK 33, murine monoclonal antibody of subtype κ /IgG1.

However, little is known at which stage during refolding of Fab fragments domain pairing occurs. Two different models were proposed: one of which postulates that the noncovalent association of the two chains takes place while the variable domains are still unfolded (Rowe & Tanford, 1973; Rowe, 1976), the other one that the association occurs after all four domains have folded to a native-like structure (Azuma & Hamaguchi, 1976).

Recently we analyzed the folding of the Fab fragment of the monoclonal antibody MAK 33 (Lilie et al., 1993). The association of the light chain and the Fd fragment of the heavy chain at 10 °C and a protein concentration of 0.2 μ M was shown to be fast compared to the overall folding reaction, with the rate-limiting step occurring after association. This folding step depends on a prolyl isomerization within the Fd chain (Lilie et al., 1995). Figure 1, based on kinetic data, suggests the formation of several folding intermediates with domain-domain contacts already present, differing in prolyl isomerization and covalent linkage of the respective chains.

Here we characterize Dc, one of these Fab folding intermediates in more detail (see Fig. 1A). We show that this intermediate is not only a folding but also an unfolding intermediate, which can be populated to about 90%. The spectroscopic analysis reveals a native-like secondary structure of Dc with aromatic side chains only slightly more solvent-exposed than in the native state. Because the covalent linkage of the two chains via a C-terminal disulfide bond is necessary for accumulation of Dc, the noncovalent domain pairing within this intermediate seems to be very weak. This may be due to the fact that a rearrangement of the contact sites necessary to reach the native state has still not taken place.

Results

It has been established that several intermediates are formed during the folding process of Fab and Fab_{alk}, respectively (Lilie et al., 1995). Because in every pathway more than one intermediate is postulated, but only one rate-limiting step for Fab and two for Fab_{alk} can be detected, it is difficult to obtain detailed information about one of these intermediates from refolding experiments. Therefore, we tried to populate the late-folding intermediate Dc (see Fig. 1A) by denaturation of the native protein (N).

Denaturation kinetics for both Fab and Fab_{alk} were measured using fluorescence, CD spectroscopy, and antigen-binding activity by ELISA. As shown in Figure 2A, the denaturation kinetics observed for Fab were dependent on the method used for monitoring. The loss of antigen binding activity occurred in a reaction with a rate constant of $k_1 = 1.99 \times 10^{-3} \text{ s}^{-1}$. In contrast, the loss of secondary structure as monitored by far-UV CD is a comparably slow process that can be described by a single first-order reaction and a rate constant of $k_2 = 2.51 \times 10^{-4} \text{ s}^{-1}$. Using fluorescence, both reactions could be detected (Fig. 3A). These results imply that the correct quaternary structure disappears first, followed by a loss of secondary structure monitored by CD. Thus, an intermediate could be populated during unfolding that seems to be identical to the folding intermediate Dc (see below).

The two phases obtained were independent of the ionic strength, as measured by urea-induced denaturation in the absence of any salt and in the presence of 1 M NaCl or 0.3 M

Fig. 1. Kinetic scheme for the folding of MAK 33-Fab fragment. U, denatured protein; I, folding intermediate with nonassociated chains; D and D*, folding intermediates after association; N, native Fab and Fab_{alk}, respectively. Rate constants (s⁻¹) of the different reactions are indicated (from Lilie et al., 1995). **A:** Fab. **B:** Fab_{alk}.

Fig. 2. Denaturation kinetics of the Fab fragments. Native Fab fragment was diluted into 0.1 M Tris/HCl, pH 8, 2.5 M Gdm/Cl at 10 °C. Kinetics were followed by ELISA (○) and by CD at 220 nm (dots). Protein concentration was 10 μ g/mL and 1 mg/mL, respectively. Lines represent fits to single exponential reactions. Rate constants are given in Figure 3. **A:** Denaturation of Fab. **B:** Denaturation of Fab_{alk}.

Fig. 3. Guanidinium dependence of Fab denaturation kinetics. Denaturation kinetics of the Fab fragments were measured as described in Figure 2 at different Gdm/Cl concentrations using ELISA (∇), CD at 220 nm ($\square$), and fluorescence ($\bullet$, $\circ$). Rate constants were obtained by fitting the ELISA and CD measurements to a single first-order reaction and the fluorescence kinetics to a serial first-order reaction. **A:** Denaturation of Fab. Rate of denaturation of a short-term refolded (1 min, see text) but not active Fab (Dc) is also shown ($\bullet$). **B:** Denaturation of Fab_{alk} . Fast phase of the Fab denaturation ($\bullet$) is included for comparison.

Mg_2SO_4 (data not shown), and only slightly dependent on the pH in a range between pH 5 and 9.

Under identical conditions, the denaturation kinetics of Fab_{alk} did not depend on the method used for monitoring (Fig. 2B). The rate of denaturation was identical with the fast phase of Fab denaturation. Although an intermediate analogous to Dc, Dc^* , has to be formed during refolding of Fab_{alk} (see Fig. 1B), it could not be detected during denaturation under conditions suitable to populate the analogous intermediate of Fab. Only at low temperatures could this intermediate (Dc^*) be observed (see below).

Guanidinium dependence of denaturation

The rates of the two phases shown in Figure 2A were not sufficiently different to trap the respective folding intermediate. In order to search for appropriate trapping conditions for a denaturation intermediate we varied the Gdm/Cl concentration during denaturation. Determination of the respective rate constants for the unfolding reaction showed that the two reactions were affected by Gdm/Cl in a different way (Fig. 3A). The disruption

of the native quaternary structure is much more dependent on the Gdm/Cl concentration than the disruption of the secondary structure. The Gdm/Cl dependence of the two inactivation steps allows to distinguish the rates of the reactions by a factor of 20 at high Gdm/Cl concentrations (i.e., 3.5 M Gdm/Cl). Thus, Dc could be populated to a high degree. The distribution of native Fab, Dc, and the fully denatured protein during denaturation at 3.5 M Gdm/Cl is shown in Figure 4. The rate constants used for this calculation were taken from Figure 3A. Under these conditions the intermediate is maximally populated after about 60 s. At this time point 3% of the Fab are still native and 7% are fully denatured.

To test whether this intermediate is formed not only during denaturation but also during refolding, we performed triple jump experiments. Fully denatured Fab with all prolyl residues in their native configuration (Uc in Fig. 1A) was refolded (double jump) for 1 min. Under the conditions used, about 20% of the molecules had reached the native state within this time period. The remaining 80% should be present as the folding intermediate Dc (cf. Fig. 1A). The sample was then diluted into 3.5 M Gdm/Cl. The denaturation kinetics observed are identical to the slow phase of Fab inactivation, suggesting that both the refolding and unfolding intermediates were identical.

In the case of Fab_{alk} , denaturation followed only one phase at all Gdm/Cl concentrations. This phase was identical to the fast phase of Fab denaturation. Therefore, the interchain disulfide bond seemed to exhibit a strong stabilizing effect on the folding/unfolding intermediate Dc. Otherwise, the analogous intermediate Dc^* of Fab_{alk} should also be observed.

Dc^* in the case of Fab_{alk} can only be detected at even lower temperatures. When Fab_{alk} was denatured in 3.5 M Gdm/Cl at 3 °C, the renaturation kinetics were strongly dependent on the time of denaturation (Fig. 5). After >40 min of denaturation, the renaturation kinetics were similar to those published previously (Lilie et al., 1995). The rate-limiting steps were characterized previously as a slow-folding step of the light chain and an additional prolyl isomerization. Renaturation of Fab_{alk} after 10 min denaturation showed only one phase, which characterizes the slow folding of the light chain. Whether or not a small fraction of the denatured protein refolds already with a sigmoidal

Fig. 4. Formation of the intermediate Dc during denaturation. Based on the rate constants given in Figure 3A, the kinetics of the native (—), intermediate Dc (---), and completely unfolded Fab (...) at 3.5 M Gdm/Cl, 10 °C were calculated.

Fig. 5. Renaturation of partially denatured Fab_{alk} . Fab_{alk} was incubated in 0.1 M Tris/HCl, pH 8, 3.5 M Gdm/Cl at 3 °C for different periods. Subsequently, it was diluted 50-fold in 0.1 M Tris, pH 8, 10 °C. Final protein concentration was 20 $\mu\text{g}/\text{mL}$, residual Gdm/Cl concentration, 70 mM. At time points indicated, aliquots were withdrawn, supplemented with trypsin, and the concentration of refolded Fab_{alk} was determined by ELISA. Time of denaturation was 25 s ($\circ$), 10.5 min ($\bullet$), and 41 min (∇). Solid lines were obtained by fitting the data to two parallel first-order reactions ($\circ$; $k_1 = 6.7 \times 10^{-3} \text{ s}^{-1}$ and $k_2 = 2.2 \times 10^{-4} \text{ s}^{-1}$), a single first-order reaction ($\bullet$; $k_{\text{app}} = 2.2 \times 10^{-4} \text{ s}^{-1}$) and a serial first-order reaction (∇ ; $k_1 = 2.5 \times 10^{-4} \text{ s}^{-1}$ and $k_2 = 6.7 \times 10^{-4} \text{ s}^{-1}$). See text for explanation.

dal time course cannot be judged from the data. Most interesting, after 25 s denaturation, an additional fast phase during renaturation is observed with a rate constant of $6.7 \times 10^{-3} \text{ s}^{-1}$. This fast phase represents the reaction $\text{Dc}^* \rightarrow \text{N}$ previously determined by another kind of renaturation experiment (Lilie et al., 1995; Fig. 1B). Thus, after short-term denaturation of Fab_{alk} in 3.5 M Gdm/Cl at 3 °C, the intermediate Dc^* is partially populated. But, as can be seen in Figure 5, together with Dc^* , a small fraction of native Fab_{alk} is still present and about 50% of the protein is even more denatured. Although an early unfolding intermediate, presumably Dc^* , can be demonstrated by this experiment, a detailed analysis of this intermediate is difficult because of its low population. Therefore, we concentrated on the characterization of the folding/unfolding intermediate Dc.

Spectroscopic characterization of Dc

To characterize the structural organization of the folding intermediate Dc, fluorescence and CD spectroscopy were employed. In order to obtain spectra of Dc, the native Fab fragment was denatured in 3.5 M Gdm/Cl for 90 s and then shifted to 1.75 M Gdm/Cl. Using this strategy, unfolding of Dc after its accumulation was drastically slowed down. Over a period of 30 min, no significant changes could be observed by either CD or fluorescence (data not shown). The far-UV CD spectra for native Fab and Dc are very similar (Fig. 6A), suggesting that the secondary structure is almost completely preserved in Dc. In contrast near-UV CD spectra of native Fab and Dc are clearly different. Although the fine structure of the spectra is similar between 260 and 285 nm, the overall shape is different (Fig. 6B). Nevertheless, the spectrum of Dc shows that the aromatic side chains are fixed in a defined tertiary structure.

Similar results were obtained by fluorescence spectroscopy. We compared the fluorescence spectra of native Fab, Dc, and

the denatured protein under “native buffer conditions” (see the Materials and methods) because the amplitudes of the fluorescence of both Dc and denatured Fab were dependent on the Gdm/Cl concentration (not shown). As shown in Figure 6C, the amplitude of native Fab was slightly smaller than that of the denatured protein and the emission maximum was shifted from 343 nm in the native state to 352 nm in the unfolded protein. The specific fluorescence of Dc in the absence of Gdm/Cl is higher than that of the denatured protein. With 350 nm, the emission maximum is similar but not identical to that of the fully denatured Fab, suggesting that the aromatic amino acids are not fully solvent exposed.

Structural flexibility of Dc

The fluorescence and near-UV CD spectra suggest the aromatic amino acids to be in a defined environment in Dc different from that of the native and the denatured state. Nevertheless, one would expect that the conformation of the folding intermediate is more flexible than that of the native state. To test this assumption we analyzed (1) acrylamide quenching of tryptophan fluorescence and (2) the effect of DTT on the reactivation of Dc.

Acrylamide is able to quench the fluorescence of denatured Fab to a great extent (Fig. 7). This effect seems not to be linear with the acrylamide concentration used, probably due to slightly different life times of the nine tryptophan residues in the denatured Fab. The fluorescence quench of the native Fab fragment is less pronounced but clearly detectable. This is in accordance with the native protein showing a maximum emission in fluorescence at 344 nm, thus indicating that some aromatic side chains are solvent exposed. The effect of acrylamide on the fluorescence of the intermediate Dc is slightly increased compared to the native Fab fragment, suggesting that more tryptophan residues are solvent exposed or that the structure of Dc is more flexible than the native state.

The latter possibility is confirmed by renaturation experiments of Dc in the presence of DTT. Reactivation of Fab starting from the fully denatured protein with all prolyl residues in the native configuration (Uc in Fig. 1A) cannot be detected in the presence of DTT (data not shown), because the disulfide bonds within the domains were reduced. However, Dc can be reactivated with DTT present in the reactivation buffer (Fig. 8). The kinetics of reactivation are identical to those without DTT; the yield is about 60% compared to the renaturation of Dc in the absence of redox equivalents. Thus, within Dc the intradomain disulfide bonds were to a great extent not accessible for DTT. The reduction of the interchain disulfide bond does not influence the time course of renaturation, because such a noncovalently linked intermediate was detected using Fab_{alk} (Fig. 5; Lilie et al., 1995).

Discussion

Recently we have described the renaturation behavior of the monoclonal MAK 33-Fab fragment. Based on renaturation kinetics, we developed a refolding scheme (Fig. 1) starting from two fully denatured states differing in their prolyl *cis/trans* conformations with several intermediates and rate-limiting steps (Lilie et al., 1995). Whereas previously detailed analyses of the folding of single antibody domains and the isolated light chain were performed (Goto & Hamaguchi, 1982a, 1982b; Tsunenaga et al., 1987; Kawata et al., 1988; Kawata & Hamaguchi, 1991),

Fig. 6. Spectroscopic characterization of the intermediate Dc. Native Fab was incubated in 0.1 M Tris/HCl, pH 8, 3.5 M Gdm/Cl, 10 °C for 60 s. Samples were then diluted to 1.75 M Gdm/Cl at 10 °C and spectra were recorded in less than half an hour in the case of Dc (---). In addition, spectra of the native (—) and completely denatured Fab (····) were measured. **A:** CD spectrum in the peptide region. **B:** CD spectrum in the aromatic region. **C:** Fluorescence spectrum. Amplitudes of the spectra of Dc and the denatured protein were extrapolated to 0 M Gdm/Cl (see the Materials and methods).

little is known about folding intermediates of Fab with domain-domain contacts already present. From equilibrium denaturation transitions and denaturation kinetics, it was concluded that an intermediate of Fab folding/unfolding has to exist with the constant domains associated, but the variable N-terminal domains still denatured (Tanford & Rowe, 1973; Rowe, 1976).

Here we analyzed one of the late-folding intermediates (Dc) of the antibody Fab fragment, proposed in Figure 1A. In order to populate this intermediate, native Fab was partially denatured and the intermediate was trapped by decreasing the Gdm/Cl concentration. Under these conditions, the intermediate is populated to about 90% (Fig. 4) and is kinetically stable over the

time range of the measurements (ca. 30 min). The denaturation intermediate is kinetically identical to the refolding intermediate Dc. This was ensured by denaturation of a renaturation sample 1 min after starting the renaturation. (Dc should be populated, but only minor amounts of the native species are present.) The denaturation kinetics observed are identical to the slow phase of denaturation, starting from native Fab (Fig. 4A). Under identical conditions, an analogous intermediate of Fab_{alk}, Dc*, cannot be detected, strongly suggesting that the noncovalently associated chains of Dc are stabilized by the interchain disulfide bond. This view is supported by the fact that the isolated light chain, whose denaturation should otherwise be detectable, de-

Fig. 7. Acrylamide quenching of tryptophan fluorescence (Stern-Vollmer plot). Quenching of native Fab (●), Dc (▽), and unfolded protein (○) is displayed. Buffer conditions were always 0.1 M Tris/HCl, pH 8, 1.75 M Gdm/Cl, 10 °C. Dc was produced by incubating native protein in 3.5 M Gdm/Cl, 10 °C for 60 s and subsequently diluted to 1.75 M Gdm/Cl. Denatured protein was incubated in 6 M Gdm/Cl for 10 min prior to dilution to 1.75 M. In all cases, a spectrum of the respective sample was measured within 10 min after adjusting the Gdm/Cl concentration. Amplitudes A of the buffer-corrected spectra at 350 nm were normalized to the amplitudes A_0 of the respective protein in the absence of acrylamide.

Fig. 8. Influence of DTT on the refolding of Dc. Reactivation of Dc was performed by diluting the protein 100-fold in 0.1 M Tris/HCl, pH 8, 10 °C in the absence (○) and in the presence (●) of 50 mM DTT in the renaturation buffer. Final protein concentration was 5 $\mu\text{g/mL}$, the residual Gdm/Cl concentration was 35 mM. At time points indicated, further reactivation was blocked by adding trypsin, and the yield of renaturation was determined by ELISA. Solid lines represent single first-order reactions. Dc was produced by incubating native Fab at 3.5 M Gdm/Cl, 10 °C for 30 s. Consequently, almost no completely denatured Fab was present, but about 20% of the protein should still be in the native state.

natures much faster than the fast phase of Fab denaturation (data not shown).

The intermediate Dc is characterized by a native-like CD spectrum in the peptide region. Therefore, in contrast to the previous proposal of a late-folding intermediate of a Fab containing denatured N-terminal domains (Tanford & Rowe, 1973; Rowe, 1976), this late-folding intermediate must exhibit native-like secondary structure in all four domains. An interpretation of the slightly higher ellipticity of Dc compared to native Fab cannot be given because not only secondary structure but also disulfide bonds and aromatic amino acids contribute to the ellipticity in the far-UV region (Neumann & Snatzke, 1990). This result does not exclude the possibility that an intermediate with associated chains but unfolded N-terminal domains exists; however, there is no evidence from the kinetic data presented for such an intermediate, suggesting that it is, at least in the case of MAK 33-Fab, not populated. Differences in the environment of aromatic side chains can clearly be detected by CD spectra in the near UV region. The amplitude of the Dc spectrum is significantly higher than that of the spectrum of the native and denatured Fab, indicating an altered tertiary/quaternary structure compared to the native protein. However, within Dc, the aromatic amino acids are in a defined environment, which is in many aspects similar to that of the native protein, because the fine structure of the CD spectra of Dc and the native Fab is similar in several bands. Nevertheless, the fluorescence spectrum of Dc exhibits an emission maximum at 350 nm compared to 344 nm and 353 nm observed for the native and denatured proteins, respectively. Although the CD spectra of Dc suggest a defined tertiary/quaternary structure, the red shift of the fluorescence maximum implicates an enhanced solvent exposure of aromatic amino acids in Dc. This effect can be explained by either a more flexible structure of Dc compared to native Fab or solvent exposure of some aromatic side chains, which are buried in the native state.

As a third method to monitor aromatic amino acids, we used acrylamide quenching of tryptophan fluorescence. In the native state, only a small effect of acrylamide on the tryptophan fluorescence could be detected, whereas the fluorescence of the denatured protein is strongly quenched. In the case of Dc, quenching is very similar to that of the native protein, indicating that the aromatic side chains are only slightly more exposed than in the native structure. A similar result was obtained when, instead of aromatic amino acids, the accessibility of the intrachain disulfide bonds was monitored. If Uc (see Fig. 1) is renatured in the presence of 50 mM DTT, no activity could be obtained because reduction of the intrachain disulfides leads to molecules incapable of reaching the native structure. Reduction of the interchain disulfide bond does not lead to a total block of renaturation (Azuma & Hamaguchi, 1976; Rowe, 1976; Lilie et al., 1995). In contrast, refolding of Dc in the presence of 50 mM DTT results in 60% reactivation compared to renaturation without DTT. As expected, the kinetics are not influenced by DTT, because reduction of the interchain disulfide bond does not lead to a change in the rate-limiting step ($Dc \rightarrow N$ and $Dc^* \rightarrow N$; cf. Fig. 1). Thus, not only the aromatic side chains but also intrachain disulfide bonds were protected against solvent accessibility. However, in both cases small differences between Dc and native Fab are detectable. This is most likely due to a more flexible structure of Dc compared to the native Fab. Nevertheless, the same kind of interaction seems to stabilize both the native and the in-

termediate structure, because both phases of the denaturation kinetics are affected by ionic strength and pH in a similar way (data not shown). In the native state, the two chains of the Fab fragment are held together mainly by close packing of hydrophobic amino acid side chains within the contact sites of the two domain pairs (Chothia et al., 1985; Getzoff et al., 1986; Padlan et al., 1986). Upon association of the partner domains, structural rearrangements must occur at least in the contact sites (Chothia et al., 1985). Such conformational changes were interpreted as the basis for the rate-limiting step of Fab folding (Azuma & Hamaguchi, 1976; Lilie et al., 1995). Therefore, it is reasonable to assume that the hydrophobic contact between the domains already exists in Dc, but that the close packing of the complementary surfaces characteristic for the native state has not yet been reached.

The analogous intermediate Dc* (see Fig. 1B) for Fab_{alk} cannot be detected by denaturation kinetics because these are only monophasic and identical to the fast phase of denaturation of the disulfide-bridged Fab. Only at very low temperatures, Dc* becomes detectable (Fig. 5), but not highly populated. Therefore the second phase leading to complete denaturation must be significantly faster than the observed first phase. The totally different second phase of denaturation of Fab and Fab_{alk} ($Dc \rightarrow U$ and $Dc^* \rightarrow U$) implies that the interchain disulfide bond does not only destabilize the denatured state. It has been shown that disulfides can stabilize proteins by reducing the conformational entropy of the completely denatured state (Poland & Scheraga, 1965; Pace et al., 1988; Cooper et al., 1992; Betz, 1993), but quantitative analysis of a mutant phage T4 lysozyme using a loop permutation method showed that, in addition to the entropic effect, sequence-specific aspects have to be considered (Zhang et al., 1994). In the case of Fab, the interchain disulfide bond clearly does not only affect the fully denatured state, but also the intermediate and/or the transition state between Dc and U. Otherwise the denaturation kinetics under fully denaturing conditions should not be different for Fab and Fab_{alk}. Similar complex effects of disulfide bonds on protein stability have been described for several proteins (reviewed by Betz, 1993).

The demonstration of the occurrence of Dc and Dc*, independent of the data recently published (Lilie et al., 1995), proves the respective part of the renaturation scheme (Fig. 1A,B). One of these intermediates, Dc, is populated to a high extent during unfolding and refolding. In the latter case, this accumulation is due to a relatively slow folding step after noncovalent association of the two chains. Recently, it was suggested that folding of small monomeric proteins can occur within the millisecond time scale and that slow-folding reactions depend on kinetic traps (Sosnick et al., 1994). Such a kinetic trap has been shown to be based on wrong tertiary interactions of the heme group in denatured cytochrome *c*. Thus, in the case of small monomeric proteins the polypeptide chain seems to have the intrinsic capacity to reach the native state without accumulating folding intermediates (including molten globules/compact denatured states, but excluding alterations of the covalent structure like disulfide bond formation or isomerization of peptide bonds). Nevertheless, during Fab folding, Dc is populated clearly as an obligatory on-pathway intermediate, most likely due to slow packing of the domains. Similar results were obtained previously for other multidomain proteins (Jaenicke, 1987). Thus, whereas folding of single antibody domains can be a very fast process, for the Fab fragment, composed of four fast-folding domains,

the overall rate of renaturation is several orders of magnitude slower than domain folding.

Material and methods

Reagents

MAK 33, a murine antibody of subclass κ /IgG1 directed against dimeric muscle-specific human creatine kinase (CK-MM, EC 2.7.3.2.) (Buckel et al., 1987) and the respective Fab fragment were obtained from Boehringer Mannheim GmbH. The Fab fragment was produced by proteolytic digestion of the intact murine monoclonal antibody as described (Johnstone & Thorpe, 1987). In previous studies using the MAK 33-Fab fragment, spontaneous reactivation was found to be about 30% (Schmidt & Buchner, 1992; Lilie et al., 1993) or, using a different preparation of the Fab fragment, about 70% (Lilie et al., 1995). Despite the difference in yield, the two preparations are indistinguishable in their folding characteristics. The concentration of the Fab fragment was determined spectrophotometrically using the extinction coefficient at 280 nm: $\epsilon_{\text{Fab}} = 80,000 \text{ M}^{-1} \text{ cm}^{-1}$ (Schmidt & Buchner, 1992). Trypsin and iodoacetamide were obtained from Sigma, and ELISA reagents, including biotinylated CK-MM, were from Boehringer Mannheim GmbH.

Preparation of noncovalently linked Fab (Fab_{alk})

Reduction and alkylation of the interchain disulfide bond of Fab was achieved according to Johnstone and Thorpe (1987). Fab (10 mg/mL) dissolved in 0.1 M Tris/HCl, pH 8, was incubated in the presence of 20 mM DTT for 15 min at 25 °C. Subsequently, iodoacetamide to a final concentration of 200 mM was added and further incubated for 30 min at 25 °C. Excess DTT and iodoacetamide were removed using a PD10 column (Pharmacia) equilibrated with the starting buffer.

Unfolding and refolding of the Fab fragment

Renaturation was performed in 0.1 M Tris/HCl, pH 7, at 10 °C with protein concentrations varying between 5 and 10 $\mu\text{g/mL}$. In the case of Fab_{alk} , the protein was denatured in 3.5 M Gdm/Cl at 3 °C for various periods of time (see figure legends). Uc was populated by denaturing Fab in 6 M Gdm/Cl at 2 °C for 15–20 s. Formation of the intermediate Dc was performed by incubating native Fab in 3.5 M Gdm/Cl at 10 °C for 40 s.

Denaturation of the Fab fragment was performed in 0.1 M Tris/HCl, pH 7, 10 °C at various Gdm/Cl concentrations. The protein concentration was 10 $\mu\text{g/mL}$ in the case of fluorescence and ELISA measurements and 1 mg/mL and 0.5 mg/mL for far- and near-UV CD, respectively. The light path used was 1 cm for fluorescence and near-UV CD and 0.1 cm in the case of far-UV CD. Fluorescence measurements were performed with a Spex Fluoromax, using a bandwidth of 4.25 nm for excitation and emission. A wavelength of 280 nm was used for excitation; the emission was recorded at 350 nm. In the case of slow kinetics, the shutter was closed during the measurement to avoid photobleaching.

For CD measurements, a computer-equipped Jasco 500A CD spectrometer was utilized. The kinetics were recorded at a wavelength of 220 and 279 nm in a 0.1×1 -cm thermostatted cuvette. Because of manual mixing, the recording of the kinetics was

started 30 s after initiating the denaturation process. The time constant used was 1 s.

The ELISA assay was carried out as described previously (Buchner & Rudolph, 1991; Schmidt & Buchner, 1992). In short, CK-MM was attached to tubes using the biotin/streptavidin system. At times indicated, aliquots of the unfolding or refolding Fab fragment were diluted in ELISA buffer containing 30 mM sodium phosphate supplemented with 0.3 M NaCl, pH 7.5. To prevent further reactivation of the Fab fragment, the samples were kept on ice, and trypsin to a final concentration of 400 $\mu\text{g/mL}$ was added. Binding of functionally refolded Fab fragments to CK-MM was detected by using peroxidase-coupled anti-mouse-IgG-antiserum. The amount of functionally reactivated protein was quantitated by comparison with standard curves obtained with the authentic Fab fragment.

Spectral analysis

All spectra of the intermediate Dc were performed by denaturing native Fab in 3.5 M Gdm/Cl for 90 s. Then the Gdm/Cl concentration was adjusted to 1.75 M. The spectra were recorded in thermostatted cells with a light path of 1 mm in the far-UV CD and 1 cm for near-UV CD and fluorescence. Spectra of the native and denatured Fab were recorded in 0.1 M Tris/HCl, pH 7, and 0.1 M Tris/HCl, pH 7, 6 M Gdm/Cl, respectively. The protein concentration was 0.5 mg/mL and 1 mg/mL (far-UV CD), 0.5 mg/mL (near-UV CD), and 10 $\mu\text{g/mL}$ (fluorescence). In the case of fluorescence spectra, it was observed that the specific fluorescence of the intermediate Dc and the denatured protein was dependent on the Gdm/Cl concentration. Therefore, the amplitudes were measured at different Gdm/Cl concentrations and extrapolated to 0 M Gdm/Cl (native conditions).

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