Folding of the Fab fragment within the intact antibody

Hauke Lilie*

Institut für Biotechnologie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes Strasse 3, 06120 Halle, Germany

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Abstract At present it is not clear to which extent the Fab fragment and the Fc part of an antibody interact in the intact immunoglobulin structure. To determine such potential interactions the unfolding and refolding of an isolated Fab fragment and the respective antibody MAK 33 (κ/IgG1) are compared. It could be shown that the proline independent renaturation kinetics of both an unfolding intermediate and the fully denatured form of both proteins are identical. Upon denaturation, the loss of antigen binding activity occurs with the same rate for both the Fab fragment and the intact antibody. However, the complete structural unfolding of the Fab part of the antibody is significantly slower than that of the isolated Fab fragment. These kinetic data suggest that the structure of the Fab fragment within the intact antibody is stabilized by interactions, presumably with the Fc part, missing in the isolated Fab.

Key words: Antibody; Fab fragment; Denaturation; Renaturation; Folding intermediate

1. Introduction

Antibodies and their fragments belong to the first generation of model proteins used for studying protein folding [1]. Detailed mechanistic work was performed on single antibody domains [2]. An extensive kinetic and structural characterization of a C_L domain demonstrated the importance of the intradomain disulfide bond for the stability and folding of the protein [3,4]. This approach was supplemented by the analysis of the influence of temperature and dynamics of other antibody domains [5–7]. The folding of a light chain could be demonstrated to be composed of the individual folding reaction of V_L and C_L plus an additional reaction, which was probably due to an intramolecular interaction between these two domains [8].

In another set of experiments the interactions between the different antibody chains were characterized by measuring the dissociation constants of light chain and heavy chain and their fragments [9]. Furthermore, the structure of several Fab fragments and one intact antibody were solved by crystallography [10,11].

Despite this huge amount of information on antibody structure and folding only little was known about the relation of domain folding and domain association reactions during the structure formation of a Fab fragment or the intact antibody [12].

Recently we analyzed the folding of a Fab fragment in detail (Fig. 1, [13]). We dissected the folding process in prolyl isomerization dependent and independent reactions and correlated folding and association steps. One of the proposed intermediates, Dc, was kinetically and spectroscopically characterized [14]. Here, the analysis was extended to the complete antibody MAK 33, showing that the isolated Fab fragment and the Fab part within the structural context of the antibody behave in some aspects identical, but differed in the sensitivity towards denaturation. The data suggest that the Fab fragment as part of the antibody is more stable than the isolated Fab.

2. Materials and methods

2.1. Materials

The Fab fragment and the respective antibody MAK 33 (κ/IgG1) [15,16] and all substances used for the ELISA including the biotinylated human creatine kinase (EC 2.7.3.2) were obtained from Boehringer Mannheim GmbH. Tris was from ICN, GdmCl AA grade was obtained from NIGU.

2.2. Methods

2.2.1. Protein concentration. The protein concentrations of Fab and MAK 33 were determined spectroscopically using extinction coefficients at 280 nm of $\varepsilon = 1.6$ and $\varepsilon = 1.5$ for a 1 mg/ml solution in a 1 cm cell [17], respectively.

2.2.2. Denaturation. Denaturation of Fab and MAK 33 was performed in 0.1 M Tris/HCl, pH 7 at different GdmCl concentrations at 10°C. The protein concentration was 10–50 mg/ml for fluorescence and ELISA analysis. In the case of CD measurements a concentration of 1 mg/ml and a light path of 0.1 cm was utilized. The kinetics were monitored using a Hitachi F4500 fluorimeter (excitation: 295 nm; emission 350 nm; band width both 5 nm; light path 1 cm) in a stirred and temperature controlled cell. The CD measurements were performed using a temperature controlled Jasco 720 CD-spectrometer at 222 nm. To follow the kinetics of antigen binding activity a quantitative ELISA was used [18].

2.2.3. Renaturation. Renaturation was achieved by diluting the denatured protein 100-fold in 0.1 M Tris/HCl, pH 7 at 10°C. The final protein concentration was 10 μg/ml.

In the case of renaturation of Dc, Fab and the complete antibody were denatured by incubation in 0.1 M Tris/HCl, pH 7, 3.5 M GdmCl for 50 s at 10°C [14]. The protein concentration was 1 mg/ml.

For renaturation starting from the fully denatured state (Uc), native protein was incubated in 0.1 M Tris/HCl, pH 7, 6 M GdmCl for 10 s at < 10°C. Renaturation was subsequently initiated as described above.

3. Results

3.1. Unfolding kinetics

Recently, it was demonstrated that unfolding of a Fab fragment is a biphasic process. First, the antigen binding activity is lost and subsequently the whole secondary structure brakes down [14]. Thus, unfolding of the intact antibody MAK 33 should also exhibit these two processes. Fig. 2A shows the denaturation kinetics of Fab and MAK 33 in the presence

*Fax: +49 (345) 5527013.

Abbreviations: Fab, Fab fragment of an antibody consisting of the entire light chain and the two N-terminal domains of the heavy chain; Fab′, proteolytically derived antibody fragment consisting of two Fab fragments covalently linked by disulfide bonds within the hinge region; MAK 33, intact murine monoclonal antibody (κ/IgG1); GdmCl, guanidinium chloride

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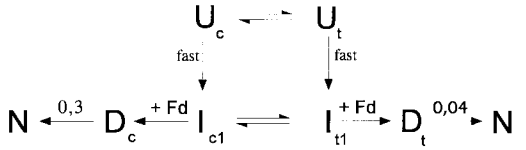

Fig. 1. Kinetic scheme for the folding of MAK 33 Fab fragment. U, denatured protein; I, folding intermediates with non-associated chains; D, folding intermediates after intramolecular association; N, native protein; c, all prolyl residues in the native conformation; t, some prolyl residues in a non-native conformation. The rate constants (min^{-1}) for the rate limitimg steps are indicated (taken from [13]).

of 3 M GdmCl, measured by the loss of antigen binding activity, which monitors the conversion of the native state N to the intermediate Dc (see Fig. 1). Clearly, the denaturation kinetics of both proteins are identical ($k = 0.012\ s^{-1}$). However, monitoring the denaturation kinetics at 3 M GdmCl by far UV CD as a measure of secondary structure reveals different rates ($k_{Fab} = 1.33 \times 10^{-3}\ s^{-1}$, $k_{MAK} = 0.2 \times 10^{-3}\ s^{-1}$) for Fab and MAK 33, respectively (Fig. 2B). Using fluorescence, both kinetic phases of the denaturation process can be observed for Fab and MAK 33 (Fig. 3). It seems that the complete structural denaturation of the Fab portion within the antibody proceeds 3–4-fold slower than in the isolated Fab fragment. Furthermore, the denaturation of the Fab portions and the Fc part seems to be coupled, because the loss of secondary structure of the complete antibody is a monophasic

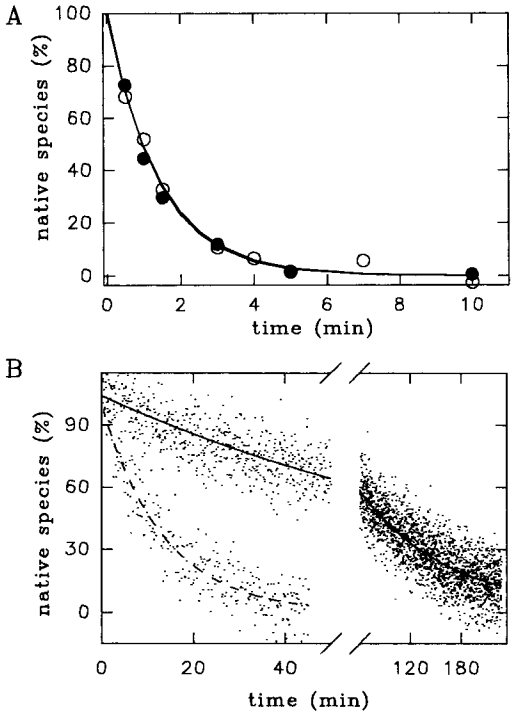

Fig. 2. Denaturation kinetics of Fab and MAK 33. Native protein was diluted into 0.1 M Tris/HCl, pH 7, 3 M GdmCl at 10°C. The lines represent fits to single exponential reactions. The rate constants are given in Fig. 3. A: ELISA: The protein concentration during denaturation was 10 μg/ml. At different time points aliquots were diluted in buffer containing 400 μg/ml trypsin to stop further renaturation and the antigen binding activity was measured by ELISA. MAK 33 (●); Fab (○). B: CD: The protein concentration was 1 mg/ml. The kinetics were monitored at 222 nm. MAK 33 (——); Fab (– – –).

process and no further intermediates during denaturation can be observed.

Although the slow phases of denaturation of Fab and MAK 33 are different, the GdmCl dependence of these reactions is identical within the experimental error (Fig. 3). A molecular interpretation of this phenomenon cannot be given at present.

3.2. Population of Dc during denaturation of MAK 33

The monophasic denaturation kinetic of MAK 33 measured by far UV CD indicates a complete loss of secondary structure – including that of the Fab portions – in one step. In order to confirm that this phase can be attributed to the unfolding of the Fab part the time dependent distribution of Dc during unfolding of the complete antibody was measured. The native protein was denatured in 4 M GdmCl. At different time points of denaturation aliquots were diluted in strong native conditions. After 10 min of refolding the intermediate Dc should be converted completely to the native state (see below). To prevent further reactivation of other intermediates trypsin was added after 10 min of renaturation. The yield of renaturation measured by ELISA represents the concentration of Dc at the respective time of denaturation. Fig. 4 shows the decrease in the amount of fast refolding species as a function of time of denaturation of the complete antibody. This represents the amount of the intermediate Dc in the case of MAK 33. The observed rate for the decrease of the renaturation yield is identical to that observed for denaturation measured by far UV CD. This result confirms directly that part of the amplitude of the monophasic denaturation kinetic measured by CD is due to the complete unfolding of the Fab portion. Therefore, Dc denatures more slowly in MAK 33 than in Fab.

3.3. Renaturation kinetics

The renaturation of the Fab fragment is a complex process comprising domain folding, domain association and prolyl isomerization [13,19]. In order to simplify the kinetic analysis of the reactivation of Fab and MAK 33, the effect of prolyl isomerization was circumvented by short unfolding. Therefore, two kinds of renaturation experiments could be analyzed, either starting from the intermediate Dc (and the analogous structure of MAK 33) or from the fully denatured

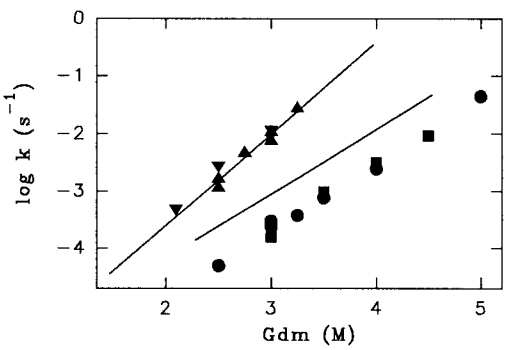

Fig. 3. Guanidinium dependence of denaturation kinetics. The denaturation was performed as described in Fig. 1. In the case of fluorescence measurements a protein concentration of 50 μg/ml was used. The straight lines represent the two denaturation reactions of Fab (taken from [14]). The denaturation of MAK 33 was analyzed by ELISA (▼), CD at 222 nm (■) and tryptophan fluorescence (▲, ●).

protein with all prolyl residues still in the native isomeric form, Uc. Because the rate limiting step of Fab folding is the reaction Dc→N [13], the refolding kinetics starting from the intermediate Dc and from the completely denatured protein, Uc, are identical, if analyzed by ELISA. The rate constant was determined to be $k_{app} = 0.3$ min^{-1} [14].

The comparison of Fab refolding and MAK 33 refolding, starting from the fully denatured protein or the intermediate structure Dc, analyzed by ELISA or fluorescence, respectively, showed no significant difference either (Fig. 5 and data not shown). Thus, despite differences in the denaturation behaviour of Fab and MAK 33, the renaturation of isolated Fab or of Fab within the structural context of the intact antibody was identical.

4. Discussion

Although detailed knowledge exists about folding and stability of isolated antibody domains [2], with these model systems the rate limiting and problematic steps in the structure formation of intact antibodies cannot be analyzed. Only little is known about the folding of complex systems composed of antibody domains like Fab fragments [12,20] or intact antibodies [1,21]. In order to get a more detailed insight into the problem of antibody folding we recently analyzed the renaturation of the oxidized Fab fragment of the monoclonal antibody MAK 33 and of a mutant from it, lacking the interchain disulfide bond [13,14]. Based on these results, the folding and unfolding of the intact antibody was now analyzed and compared to the renaturation of the respective Fab fragment. For this analysis, an experimental approach was chosen allowing to compare only folding or unfolding steps which were not determined by prolyl isomerization.

The kinetic analysis shows that there are no differences between both at the late stage of folding and the early unfolding reactions (Dc⇌N) of the complete antibody and the Fab fragment, respectively. Therefore, the formation of native structure including tight packing of the side chains in the contact sites between domains and the antigen binding site seems to occur in a very similar way in both proteins. However, the Fab fragments isolated or within the structural context of the antibody are clearly not identical. The complete denaturation of the Fab portion is significant slower within the intact antibody, indicating a higher energy barrier for unfolding compared to that of the isolated Fab fragment. This could be achieved by a higher structural stability of the Fab portion in the complete antibody as compared to the isolated Fab fragment.

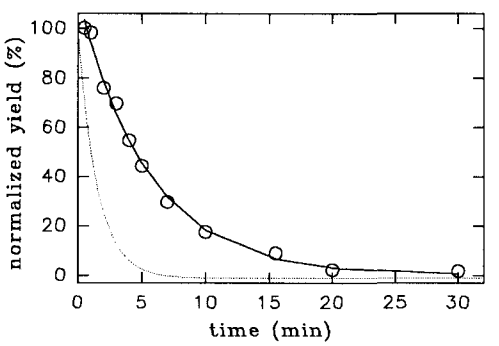

Fig. 4. Time dependent distribution of Dc during MAK 33 denaturation. MAK 33 was denatured in 4 M GdmCl at 1 mg/ml, 10°C. At different time points aliquots were withdrawn and 100-fold diluted into 0.1 M Tris/HCl, pH 7, 10°C to allow renaturation of the denatured protein. After 10 min incubation renaturation was blocked by adding 400 μg/ml trypsin and the amount of native antibody was determined by ELISA (○). The first value after 30 s of denaturation was set to 100%. The observed rate, representing the kinetics of Dc denaturation (see text), was $k = 0.003$ s^{-1}. For comparison, the denaturation kinetic of the intermediate Dc of the isolated Fab fragment is given (dotted line, taken from [14]).

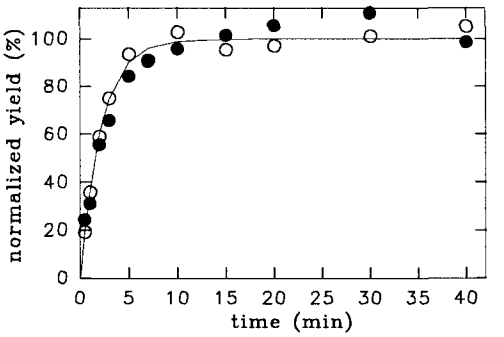

Fig. 5. Renaturation kinetics of the complete antibody. The partially denatured (Dc, ○) and completely denatured (Uc, ●) protein was refolded by a 100-fold dilution into 0.1 M Tris/HCl, pH 7 at 10°C. The final protein concentration was 10 μg/ml. Renaturation was measured by ELISA. Fits of the data yield apparent rate constants between $k = 0.3$ and 0.43 min^{-1} for all refolding reactions.

There is only very limited information on the structure of intact antibodies. The crystal structure of an IgG2a antibody [11] shows two Fab fragments in different angles to the Fc part, indicating the flexibility of the structure. The authors reasonably proposed that this structure might be only one of many possible conformations, probably selected by its ability to crystallize. Analysis in solution using H/D exchange experiments showed different kinetics of H/D exchange reactions for Fab and Fab′ on one hand and the intact antibody (IgG1) on the other hand [22]. This indicates interactions between Fab and Fc in the complete antibody. This interpretation together with the kinetic data presented here suggests an energetic scheme given in Fig. 6. Although folding and unfolding kinetics (N⇌Dc) are identical for Fab and MAK 33, the respective structures N an Dc are more stable in the case of the complete antibody. The difference in stability becomes significant and apparent upon further denaturation of Dc. The intermediate Dc is characterized by native like secondary structural content, but clearly different tertiary/quaternary contacts than the native state [14]. At least one of the variable domains is non-native in this intermediate, because Dc has lost its ability to react in the ELISA system. Probably, also the interaction between the constant domains differs from the tight packing of the native contacts. This is suggested by the fact that the C-terminal interchain disulfide bond between light chain and Fd fragment is essential for population of this intermediate [14], which would not be the case if the intramolecular association of the two chains within Dc would be mediated by the stable interaction of native constant domains. Nevertheless, the kinetic analysis of the complete antibody showed a stabilization of Dc compared to the isolated Fab fragment, presumably by the interaction with the Fc part. In the native context such an interaction was observed between C_H1 and C_H2 [22], suggesting that even in the intermediate, Dc, part of the surface of C_H1 possesses native like features allowing this interaction to occur.

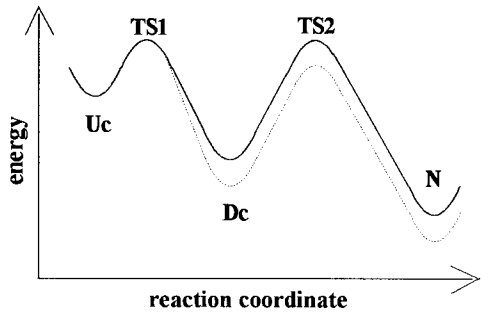

Fig. 6. Possible energetic scheme of Fab and MAK 33 folding. Fab (——) and MAK 33 (dotted line) differ in their stability of the native structure N and the intermediate Dc. The activation energy of the reaction Dc→Uc is different. In this scheme, the reaction Uc→Dc is assumed to be identical for Fab and MAK 33.

The structural unfolding of the complete antibody is a highly cooperative process. The antibody consists of 12 structural domains. However, the denaturation kinetics measured by far UV CD only reveals a single denaturation reaction. Thus, all domains unfold simultaneously. Also Fc seems to be stabilized because the isolated domain C_H3 denatures much faster in the isolated form than in the intact antibody (unpublished data).

It should be noted that Fig. 6 represents the energetics under native conditions. Thus, the denaturation kinetics of MAK 33 must be slower than that of Fab at 0 M GdmCl. Denaturation at 0 M GdmCl cannot be measured directly. However, measuring the kinetics of unfolding at different GdmCl concentrations allows to extrapolate to 0 M GdmCl. As shown in Fig. 3 the slopes of the rate of denaturation versus GdmCl concentration are identical for Fab and MAK 33, respectively. Therefore, at conditions relevant for the energetic scheme (e.g. at 0 M GdmCl, Fig. 6) denaturation of the structured intermediate of MAK 33 is slower than that of Fab.

This interpretation assumes that during folding interaction between the Fab portion and Fc of the complete antibody occurs for the first time at the level of Dc. In principle, it is also possible that this interaction changes the transition state between Dc and less structured conformations (TS1 in Fig. 6). Whether this transition state is identical for Fab and MAK 33 or differs energetically cannot unambiguously be concluded from the data.

Taken together, it was shown that Fab fragments, isolated or within the structural context of an antibody act in some aspects clearly different. The structure of Dc within the intact antibody seems to be stabilized by the additional interactions to the Fc part, resulting in slower denaturation kinetics compared to the isolated Fab. An analysis of the protein stability of Fab and MAK 33 cannot be given because unfolding of both proteins is not completely reversible. However, the kinetic data together with the dynamic information on antibody architecture [22] suggest that the Fab portion is more stable within the intact antibody than in the isolated form.

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