Domain interactions stabilize the *alternatively folded state* of an antibody Fab fragment

Hauke Lilie, Johannes Buchner*

Institut für Biophysik und Biophysikalische Chemie, Universität Regensburg, 93040 Regensburg, Germany

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Abstract The structure of the Fab fragment of the monoclonal antibody MAK 33 (κ/IgG1) at pH 2 was characterized. Spectroscopic and kinetic analysis revealed a molten globule-like state, characterized by elements of secondary structure but less defined tertiary contacts than in the native state. However, some aromatic side chains are in an asymmetrical environment. This structure was not detected using the isolated light chain or a Fab fragment lacking the covalent linkage of the light chain and Fd via the C-terminal disulfide bond. Therefore, interactions between the two chains, stabilized by the interchain disulfide within the Fab fragment, are essential for formation of the *alternatively folded state*.

Key words: Denaturation; Molten globule; Tertiary structure; Fab fragment

1. Introduction

Denaturation of proteins often leads to conformations with residual structure, depending on the denaturant used [1]. An acidic environment can result in protein denaturation by repulsion of intramolecular charges [2], but in most cases denaturation is not complete; secondary structure and some unspecific tertiary contacts were found in a number of proteins [3–5]. These structures have been termed *molten globule*. Under appropiate conditions the molten globule is in equilibrium with the completely denatured or native state and, therefore, was proposed to be the third themodynamic state of a protein [6]. Furthermore, it is a universal kinetic intermediate formed early during protein folding [4,7–10].

However, the structure of a protein, obtained under partially denaturing conditions, must be investigated carefully, before deciding whether or not it fits to the molten globule model. Recently, we investigated the structure of an intact antibody at low pH. Although some of the characteristics of a molten globule could be observed, the antibody exhibited a high stability against gdm/Cl and temperature, indicating a cooperative denaturation of a at least partially defined tertiary structure which we, therefore, termed *alternatively folded state* [11]. In this study we have extended the investigations to the Fab fragment of the respective antibody. The Fab fragment in which the light chain and the Fd chain were covalently linked by a natural disulfide bond showed spectroscopic characteristics similar to that of the intact antibody. However, neither the isolated light chain nor a Fab fragment lacking the covalent linkage of the two chains (Fab_{alk}) retained any residual structure under acidic conditions. Therefore, the stability of the *alternatively folded state* for the intact antibody and the Fab fragment is not a property of the antibody domains, but strongly depends on interchain interactions, stabilized by the disulfide between the two chains. These interactions are presumably the molecular basis for the unusual thermodynamic characteristics of the *alternatively folded state* of the intact antibody.

*Corresponding author. Fax: (49) (941) 943 2813.

Abbreviations: Fab_{alk}, Fab fragment with the interchain disulfide reduced and alkylated; DTT, dithiothreitol; CD, circular dichroism; gdm/Cl, guanidinium hydrochloride; FKBP, FK506 binding protein; Fd, proteolytically derived fragment of the heavy chain, containing the two N-terminal domains.

2. Materials and methods

2.1. Materials

The Fab fragment [12] and all reagents used for the ELISA including biotinylated creatine kinase were obtained from Boehringer Mannheim GmbH. Tris and DTT were from ICN and acrylamide was from Serva.

2.2. Incubation of Fab at pH 2

To achieve a rapid pH shift a stock solution of the Fab fragment was diluted 100-fold in 10 mM HCl. To remove residual salt this protein solution was dialyzed (>10 h, 4°C) against a 1000-fold volume of 10 mM HCl. After dialysis the protein concentration was adjusted to 1 mg/ml using an extinction coefficient $\varepsilon_{Fab} = 80{,}000\ M^{-1} \cdot cm^{-1}$.

2.3. Preparation of a Fab fragment lacking the interchain disulfide (Fab_{alk})

The reduction and irreversible alkylation of the interchain disulfide bond was performed as described previously [13].

2.4. Spectral analysis

CD: The CD spectra were recorded with a computer-equipped Jasco 500A CD spectrometer. The protein concentration used was 1 mg/ml for far UV CD in a 0.1 mm cell and 0.5 mg/ml for near UV CD in a cell of 1 cm light path.

Fluorescence: The spectra of the Fab fragments at different buffer conditions were measured in a 1 cm thermostated cell (25°C) with a SpexFluoromax fluorimeter. The protein concentration was always 10 μg/ml. The excitation wavelength was set to 280 nm. In the case of acrylamide quench it was 295 nm. The slits for both excitation and emission were adjusted to 4.25 nm.

2.5. ELISA measurements

To follow the regain of antigen binding activity upon renaturation a quantitative ELISA was used as described previously [14].

3. Results and discussion

3.1. Comparable 'alternatively folded states' are achieved for the Fab fragment and the intact antibody upon incubation at acidic pH

Recently, we described the structure of the monoclonal antibody MAK 33 (κ/IgG1) at pH 2 and low ionic strength. Under these conditions the antibody adopted a defined structure different from the native and denatured state, termed *alternatively folded state* [11]. To characterize this structure in more detail,

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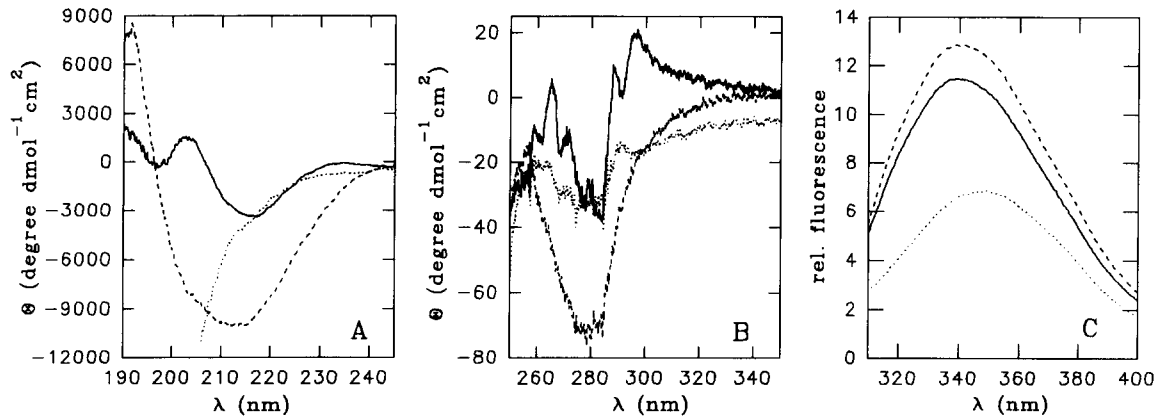

Fig. 1. Spectroscopic properties of different Fab conformations. The spectra of the Fab fragment in the native state (—), the denatured state (...) and at pH 2 (- -) are displayed. The buffer conditions were 10 mM potassium phosphate, pH 7; 10 mM HCl, 6 M urea and 10 mM HCl, respectively. (A) CD spectra in the peptide region; (B) CD spectra in the aromatic region; (C) Fluorescence spectra.

we investigated a less complex system, the Fab fragment of the respective antibody at low pH. In Fig. 1 the spectroscopic characteristics of the native, denatured and *alternatively folded state* of the Fab fragment are shown. With all methods used, differences between the three states could be detected. Most interestingly, the higher amplitude of the far UV CD spectrum of the *alternatively folded state* compared to the native protein might indicate significant changes in secondary structure, that are accompanied by changes in tertiary structure as monitored by near UV CD and fluorescence. Similar results were previously obtained for the intact antibody [11], strongly suggesting that the principal aspects of this conformational change occurring at incubation at low pH are the same for the antibody and the respective Fab fragment.

The *alternatively folded state* of the intact antibody was previously analyzed with respect to thermodynamic parameters. It was shown that the antibody can be reversibly denatured by gdm/Cl in a cooperative transition and transition enthalpies were calculated from calorimetric measurements [11]. Although the spectroscopic data suggest that at low pH the Fab fragment has a structure similar to that of the intact antibody, it differs significantly in stability. Whereas the *alternatively folded state* of the antibody was stable up to 2.5 M gdm/Cl [11], the Fab fragment at pH 2 was fully denatured at 1.8 M gdm/Cl (data not shown) or 3 M urea (Fig. 2). Thermodynamic parameters cannot be obtained for the Fab fragment because the denaturation is not fully reversible (Fig. 2) due to partial aggregation during renaturation (data not shown).

3.2. *Conformational probes for analyzing the structure of the 'alternatively folded state'*

The spectroscopic characterization of the Fab fragment at low pH proved that it adopts the *alternatively folded state*. However, an interpretation of these spectra with respect to secondary and tertiary structure of the protein is very difficult. Therefore, we used two different probes, specific for tertiary/quaternary structure, to evaluate the properties of the *alternatively folded state*: (i) conformation of prolyl peptide bonds and (ii) solvent exposure of tryptophan residues.

(i) In the native state the Fab fragment contains four prolyl residues in the *cis* conformation as judged by comparison with antibodies of known structure [15]. The isomerization of these prolyl residues is rate determining during refolding from the gdm/Cl denatured state [16, 17]. If the prolyl residues in the *alternatively folded state* maintain their native conformation, the refolding to the native state at neutral pH should not depend on a slow *cis/trans* isomerization reaction. In Fig. 3 renaturation starting from the *alternatively folded state* is shown. This folding reaction is a slow process, characterized by a rate constant $k_{app} = 0.03\ min^{-1}$, which is identical to the proline-determined refolding of the gdm/Cl denatured state [16,17]. This reactivation reaction could be catalyzed by cyclophilin and FKBP in the same way as known for the refolding of the gdm/Cl denatured state. Thus, none of the *cis* prolyl peptide bonds is maintained in the *alternatively folded state.*

(ii) The other probe used to analyze tertiary structure was quenching of tryptophane fluorescence by acrylamide. This quenching is based on an energy transfer from the fluorophore to acrylamide molecules by collision and can therefore be used as a measure of solvent exposure of the respective fluorophores. In Fig. 4 acrylamide quenching of the fluorescence of the native Fab, the gdm/Cl denatured protein and the *alternatively folded state* are compared. In the native state only a small effect of acrylamide can be seen because most of the tryptophanes are buried in the interior of the protein. However, in the completely denatured and the *alternatively folded state* the quench effect is much higher. Therefore, all tryptophane residues of the Fab

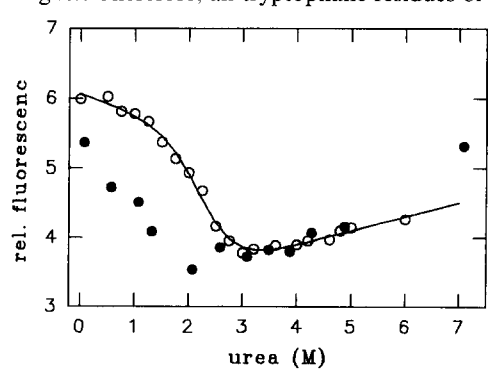

Fig. 2. Urea induced denaturation of the *alternatively folded state* of Fab. The Fab fragment (10 μg/ml) was incubated in 10 mM HCl at various concentrations of urea/HCl, pH 2 for 45 h at 20°C, either starting from 0 M (○) or 7 M (●) urea. The fluorescence at 350 nm (excitation 280 nm) was measured.

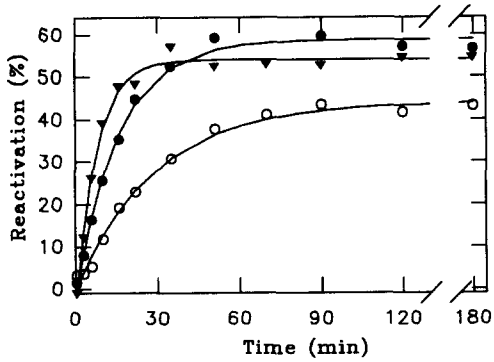

Fig. 3. Renaturation kinetics of Fab starting from the *alternatively folded state*. Renaturatin of the *alternatively folded state* of Fab was initiated by diluting it 100 fold in 0.1 M Tris-HCl, pH 8. The final Fab concentration was 0.2 μM. At time points indicated further reactivation was blocked by adding trypsin to a final concentration of 400 μg/ml. The amount of native Fab was analyzed by ELISA. The kinetics were performed in the absence of other factors (○), in the presence of 6 μM FKBP (●) and in the presence of 6 μM Cyp (▼). The solid lines represent fits to a single first order reaction with rate constants of $k_{(○)} = 0.035$ min^{-1}, $k_{(●)} = 0.06$ min^{-1} and $k_{(▼)} = 0.11$ min^{-1}.

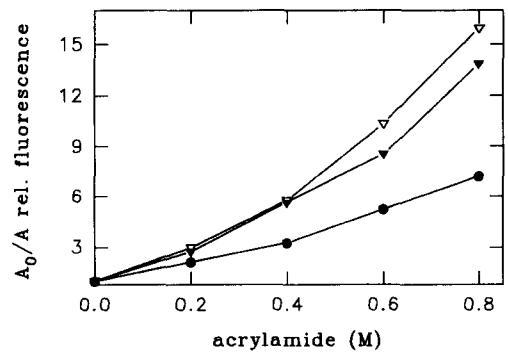

Fig. 4. Acrylamide quenching of tryptophane fluorescence Quenching of the native Fab (●), the *alternatively folded state* (▽) and the fully denatured protein (▼) are shown. The buffer conditions are the same as given in Fig. 1. The amplitudes A of the buffer corrected spectra at 350 nm were normalized to the amplitudes A_0 of the respective protein in the absence of acrylamide.

fragment seem to be solvent-exposed at pH 2, although some aromatic side chains adopt defined positions within this structure as judged from the near UV CD spectra (Fig. 1).

3.3. Fab_{alk} does not exhibit the 'alternatively folded state'

In order to investigate whether this *alternatively folded state* is an intrinsic property of the isolated antibody chains the conformation of the isolated light chain at pH 2, pH 7 and in gdm/Cl was characterized spectroscopically. Under the conditions used no differences in the far- and near UV CD spectra of the protein at pH 2 and pH 2, 4 M gdm/Cl could be observed, while the spectrum at pH 7 was typical for that of an immunoglobulin (data not shown). These results show that, in contrast to the Fab fragment and the intact antibody, the isolated light chain is not structured at low pH. The *alternatively folded state* may thus either not be a property of the isolated immunoglobulin domains or the Fd chain may be responsible for the structure at low pH. A third possibility is that quaternary contacts between the light chain and Fd, which stabilize the otherwise unstructured chains may be necessary. In the latter case destabilization of the quaternary structure should result in a destabilization or complete loss of the *alternatively folded state*, in the former cases no differences would be expected. To test this we used an Fab fragment, which lacks the covalent linkage between the light chain and Fd via the C-terminal disulfide bond (Fab_{alk}). Analysis of Fab_{alk} under conditions identical to those for Fab revealed that Fab_{alk} did not form an *alternatively folded state* at low pH (Fig. 5). It should be noted that in the case of Fab_{alk} differences in the amplitudes between the gdm/Cl- and acid-denatured forms could be detected by fluorescence. This difference cannot be explained at present. However, in both near-and far-UV CD, no differences could be observed for the acid- and gdm/Cl-denatured forms of Fab_{alk}. These results clearly contradict the hypothesis that isolated Fd may be responsible for the *alternatively folded state*. Instead, the quaternary structure of the Fab fragment, strongly stabilized by the covalent linkage of the two chains seems to be essential for stabilizing the *alternatively folded state*.

Taken together, most characteristics of the Fab fragment at low pH resemble that of a molten globule: cis prolyl residues were not stabilized by tertiary structure; all parts of the molecule detectable (tryptophane residues) were accessible to solvent which fits to the model of a 'wet' molten globule [10]. Even the necessity of quaternary interactions does not contradict such a classification; a tertiary fold has been proposed to be a feature of the molten globule [18] and a trimeric [19] and a tetrameric molten globule [20] have been described.

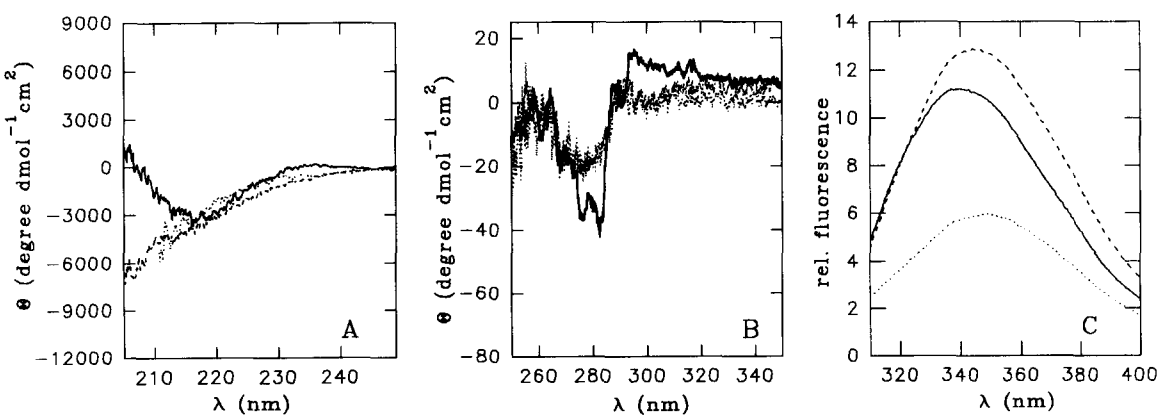

Fig. 5. Spectroscopic analysis of Fab_{alk} under different solvent conditions. The spectra were obtained identical to that for Fab; for notation see Fig. 1.

However, the near UV CD spectrum of Fab at pH 2 distinguishes between the molten globule state and the Fab structure at pH 2. Comparison of the spectroscopic analysis of the Fab fragment and the intact antibody which had been previously characterized and termed *alternatively folded state* [11] revealed that the Fab fragment adopted a similar structure at low pH. Thus, the Fab fragment could be used to analyze the molecular basis of the properties of the *alternatively folded state*. The *alternatively folde state* was only observed with the disulfide-brigded Fab fragment, but not with Fab_{alk} or the isolated light chain under the conditions used. Thus, quaternary contacts, most likely involving some aromatic side chains (see near UV CD spectrum), stabilized by the interchain disulfide bond, seem to be responsible for the thermodynamic properties of the *alternatively folded state* of the antibody molecule.

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References

[1] Shortle, D. (1993) Current Opinion Struct. Biol. 3, 66–74.
[2] Dill, K.A. (1990) Biochemistry 29, 7133–7155.
[3] Bolgikh, D.A., Gilmanshin, R.J., Brazhnikov, E.V., Bychkova, V.E., Semisotnov, G.V., Venyaminov, S.Y. and Ptitsyn, O.B. (1981) FEBS Lett. 136, 311–315.
[4] Kuwajima, K. (1989) Proteins: Stucture, Function and Genetics 6, 87–103.
[5] Fink, A.L., Calciano, L.J., Goto, Y., Kurotsu, T. and Palleros, D.R. (1994) Biochemistry 33, 12504–12511.
[6] Ptitsyn, O.B. and Uversky, V.N. (1994) FEBS Lett. 341, 15–18.
[7] Dobson, C.M. (1992) Current Opinion Struct. Biol. 2, 6–12.
[8] Baldwin, R.L. (1993) Current Opinion Struct. Biol. 3, 84–91.
[9] Ptitsyn, O.B. (1992) in: Protein Folding (T.E. Creighton, Ed.) pp. 243–300, Freeman, New York.
[10] Finkelstein, A.L. and Shakhnovich, E.I. (1989) Biopolymers 28, 1681–1694.
[11] Buchner, J., Renner, M., Lilie, H., Hinz, H.-J., Jaenicke, R., Kiefhaber, T. and Rudolph, R. (1991) Biochemistry 30, 6922–6929.
[12] Buckel, R., Hübner-Parajsz, L., Mattes, R., Lenz, H., Haug, H. and Beaucamp, K. (1987) Gene (Amst.) 51, 13–19.
[13] Lilie, H., Jaenicke, R. and Buchner, J. (1995) Prot. Sci., submitted.
[14] Buchner, J. and Rudolph, R. (1991) Bio/Technology 9, 157–162.
[15] He, X., Rueker, F., Casale, E. and Carter, D.C. (1992) Proc. Nat. Acad. Sci. USA 89, 7154–7159.
[16] Lilie, H., Lang, K., Rudolph, R. and Buchner, J. (1993) Prot. Sci. 2, 1490–1496.
[17] Lilie, H., Rudolph, R. and Buchner, J. (1995) J. Mol. Biol., in press.
[18] Peng, Z.Y. and Kim, P.S. (1994) Biochemistry 33, 2136–2141.
[19] Hlodan, R, and Pain, R.H. (1994) FEBS Lett. 343, 256–260.
[20] Schumann, J. and Jaenicke, R. (1993) Eur. J. Biochem. 213, 1225–1233.