Structural features of the McPC603 F_{ab} fragment not defined in the X-ray structure

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The proteolytic F_{ab} fragment of the well characterized antibody McPC603 was compared to the recombinant F_{ab} fragment, which was obtained in functional form from an *Escherichia coli* expression system [(1989) Methods Enzymol. 178, 497–515]. We found evidence that the proteolytic fragment is glycosylated at Asn H160 in the C_H1 domain, where additional electron density had been observed in the crystal structure [J. Mol. Biol. 190, 593–604]. In addition, its heavy chain is about 30 amino acids longer than visible in the electron density and thus contains the complete hinge region. These structural differences between the recombinant F_{ab} fragment, which had been designed exactly according to the defined electron density, and the proteolytic F_{ab} fragment of McPC603 had no effect on the hapten binding properties of these antigen binding fragments. Yet, it may be important to be aware of these structural features of McPC603 in folding studies and some comparative analyses of antibody structures.

Antibody; *E. coli*, expression; Glycosylation; X-ray crystallography

1. INTRODUCTION

The F_{ab} fragment of the phosphorylcholine-binding myeloma protein McPC603 [1,2] has been one of the first antibody fragments the X-ray structure of which was solved [3]. The structure of both the uncomplexed molecule and the F_{ab} fragment with the hapten phosphorylcholine bound was determined to atomic resolution [4]. The affinities of the antibody McPC603 for phosphorylcholine [5] and various related haptens [6,7] have been determined. McPC603 belongs to a family of genetically related myeloma proteins that all bind the hapten phosphorylcholine (PC) [1,2]. The members of this family MOPC167 and TEPC15 [8] as well as the recombinant F_v fragment of McPC603 [9] were recently shown to possess catalytic activity towards hydrolysis of choline carbonate esters.

We chose the antibody McPC603 as a model system for investigating the fundamentals of hapten binding and transition state stabilization by antibodies as well as the folding behavior of its fragments by protein engineering [9–11]. For the rapid generation and preparation of mutant immunoglobulins we developed a system for the functional expression of both the F_v and the F_{ab} fragment of McPC603 in *E. coli* [12,13]. We have now compared the properties of the recombinant F_{ab} fragment purified from *E. coli* with those of the proteolytic F_{ab} fragment, obtained by limited digestion of the antibody McPC603 with pepsin. This extends our previous work on the physical characterization of recombinant antibody fragments [13,14], as the F_{ab} fragment does not dissociate at high dilution like the F_v fragment [14].

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2. MATERIALS AND METHODS

2.1. *Purification of native antibody and proteolytic F_{ab} fragment*

Native antibody McPC603 [1] was purified from mouse ascites fluid by phosphorylcholine affinity chromatography as described [15]. The F_{ab} fragment was prepared from McPC603 according to Rudikoff et al. [16] by a limited proteolytic digest of the partially reduced antibody with pepsin, followed by gel filtration. The proteolytic F_{ab} fragment was finally purified to homogeneity by phosphorylcholine affinity chromatography as above.

2.2. *Expression and purification of recombinant F_{ab} fragment*

The recombinant F_{ab} fragment was expressed in *E. coli* strain W3110 harboring the plasmid pASK19 [12], which carries the structural genes encoding the light and the heavy chain of the F_{ab} fragment of McPC603 [10]. The recombinant F_{ab} fragment was purified as a functional protein from the periplasmic fraction of the *E. coli* cells by phosphorylcholine affinity chromatography as described previously [15].

2.3. *Deglycosylation*

For the deglycosylation reaction, the proteins were dissolved in 30 μl buffer I (0.2 M borate/NaOH (pH 8.0); 160 mM NaCl; 0.33% SDS (w/v); 1.66% 2-mercaptoethanol (v/v)) at an approximate concentration of 1 mg/ml and denatured by heating to 100°C for 5 min. The samples were then cooled on ice and a solution of both 0.1 U endoglycosidase F and 1.7 to 2.5 U N-glycosidase F (both Boehringer Mannheim) in 20 μl buffer II (0.2 M borate/NaOH (pH 8.0); 160 mM NaCl) was added. The reaction mixtures were incubated at 37°C for 24 h. An aliquot of this solution was directly loaded onto

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an SDS polyacrylamide gel [17]. The proteins were detected by silver staining ('Quick silver' staining kit, Amersham).

2.4. *Hapten binding constants*

Hapten binding constants were measured by fluorescence titration as described previously [14].

2.5. *Amino sugar analysis*

The F_{ab} fragment was hydrolyzed with 4 M HCl for 6 h and amino sugars were detected with ninhydrin on a Beckmann amino acid analyzer 6300 according to the manufacturer's instructions.

2.6. *C-terminal analysis with carboxypeptidase*

The carboxypeptidase digestion of the proteolytic F_{ab} fragment was performed using the method of Oberthür and Lottspeich (Oberthür and Lottspeich, manuscript in preparation). The F_{ab} fragment was repeatedly treated with enzyme and separated after each incubation from cleaved amino acids by a C4 Vydac mini-column. The amino acids were identified on a Beckmann Amino Acid Analyzer 6300.

3. RESULTS AND DISCUSSION

The genes for the recombinant F_{ab} fragment of McPC603 were designed to correspond exactly to the natural light chain and, in the case of the heavy chain, to the sequence visible in the electron density of the X-ray structure of the proteolytic F_{ab} fragment [3,4]. Functional expression was achieved in *E. coli* with a plasmid [12], in which both genes are arranged in an artificial operon under transcriptional control of the IPTG-inducible *lac* promoter, and both chains are fused to bacterial signal sequences. Thus, both chains of the recombinant F_{ab} fragment are secreted to the periplasmic space of *E. coli* where the disulfide bonds form and the functional F_{ab} fragment assembles.

The comparison of the recombinant F_{ab} fragment with the proteolytic F_{ab} fragment by SDS-PAGE (Fig. 1) showed that the light chain had an identical mobility in the whole antibody (lane 1), in the proteolytically prepared fragment (lane 3) and in the recombinant fragment (lane 5). The heavy chain of the proteolytically obtained F_{ab} fragment, on the other hand, did not give rise to a band of the same mobility as in the recombined fragment (lane 5), but appeared to be larger (lane 3).

In order to investigate whether the decreased electrophoretic mobility might be due to an N-linked glycosylation, the proteolytic F_{ab} fragment as well as the intact antibody McPC603 was treated with a preparation of endoglycosidase F that additionally contained N-glycosidase F [18,19]. After the glycosidase treatment, the mobility of the heavy chain of the intact antibody had significantly increased (lane 2) as expected from the glycosylation within the F_c region. Interestingly, the mobility of the heavy chain of the F_{ab} fragment had also increased (lane 4), thus demonstrating an N-glycosylation site within the F_d fragment. However, the mobility was still lower than that of the recombinant protein (Fig. 1).

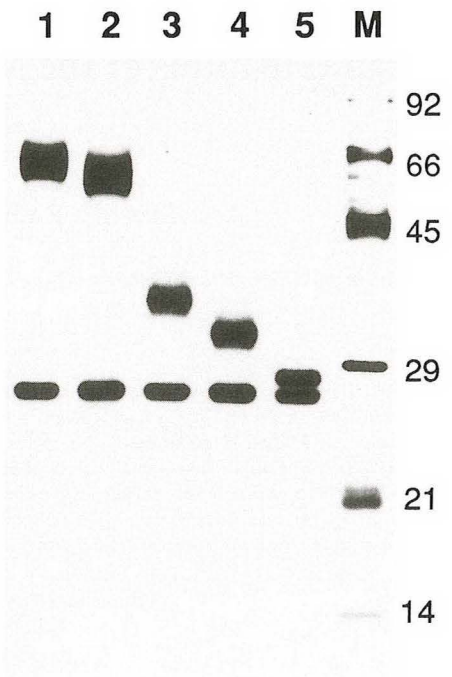

Fig. 1. Comparison of recombinant and proteolytic F_{ab} fragment as well as intact antibody McPC603 by SDS-PAGE (12.5%) under reducing conditions. (Lane 1) Intact antibody McPC603, (lane 2) deglycosylated antibody, (lane 3) proteolytic F_{ab} fragment, (lane 4) deglycosylated proteolytic F_{ab} fragment and (lane 5) recombinant F_{ab} fragment from *E. coli*. *M* denotes the molecular weight standard.

The N-glycosylation site of the F_d fragment is most likely located within the C_H1 domain, since the highly homologous antibody MOPC511, also an IgA from mouse, had been shown to be N-glycosylated at the site corresponding to Asn H160 in McPC603 [20], a typical acceptor site [21]. Furthermore, Satow et al. [4] had noticed some additional electron density around Asn H160 in McPC603 and attributed it to glycosylation at this position. While there is another Asn-X-Thr site at position Asn H127, the middle amino acid X is proline, and this prevents N-glycosylation [22].

To eliminate the possibility of an additional O-linked glycosylation, which had been found in another mouse IgA at the position corresponding to Ser H223 [23], as the cause of the remaining discrepancy between the molecular weight of the recombinant and the N-glycosidase treated F_{ab} fragment, an amino sugar analysis was carried out as described in section 2. Glucosamine, but no galactosamine was detected, indicating the absence of O-linked glycosylation [24,25].

Therefore, pepsin cleavage must give rise to a larger heavy chain fragment than visible in the electron density. This observation is in complete agreement with previous results from pepsin cleavage of human IgA ([26] and references cited there). An analysis of the heavy chain amino acid sequence shows that pepsin, whose preferred cleavage occurs between aromatic or leucine residues [27,28] is likely to predominantly

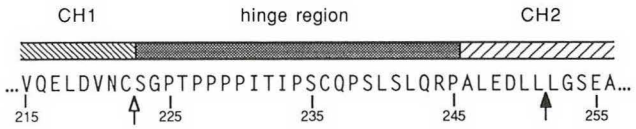

Fig. 2. Predominant cleavage site of pepsin in the heavy chain of McPC603 generating the F_{ab}' fragment (filled arrow). The C-terminus of the recombinant heavy chain is indicated by the open arrow.

cleave about 30 amino acids downstream from the end of the C_H1 domain (Fig. 2). The molecular weight of the corresponding fragment is entirely consistent with the observed mobility of the chains of the heavy chain of the glycosidase-treated F_{ab} fragment on SDS-PAGE. While we cannot rule out that extensive pepsin digestion under different sets of conditions or a protease contaminant might further truncate the F_{ab} fragment, we have obtained no evidence for any shorter fragments.

To confirm this interpretation, a carboxypeptidase digestion of the proteolytic F_{ab} fragment was performed using the method of Oberthür and Lottspeich as described in section 2. The resulting time course of liberated amino acids indicated an immediate appearance of leucine corresponding to about two mol/mol protein. This is consistent with pepsin cleavage as shown in Fig. 2.

To investigate whether the binding function of the F_{ab} fragment depends in any way on these structural differences between recombinant and proteolytically prepared material, we compared the binding constants of the recombinant F_{ab} fragment and the proteolytically prepared glycosylated F_{ab}' fragment for phosphorylcholine by fluorescence titration [14] (Fig. 3). The binding constants so obtained were indistinguishable for the recombinant F_{ab} fragment ($1.62 \times 10^5\ M^{-1}$) and the proteolytic F_{ab} fragment ($1.60 \times 10^5\ M^{-1}$). This demonstrates that the slight differences observed previously between the recombinant F_v fragment and the F_{ab}' fragment or the whole antibody are caused by a partial dissociation of the chains, depending on the protein concentration [14], since these differences disappear completely if the chains are prevented from dissociation, either by the presence of the constant domains as shown here, or by chemically crosslinking the F_v fragment [14].

Since the crystal structure of the F_{ab} fragment of McPC603 has been widely used in many comparative analyses of antibody structures and, because of its extensive characterization, may serve as a model for protein folding, it may be important to be aware of the glycosylation and the C-terminal extension of its heavy chain, neither of which was clearly visible in the electron density map ([4]; E. Padlan and D. Davies, personal communication). We could now show that these two differences between recombinant and proteolytic material have no influence on hapten binding. Our

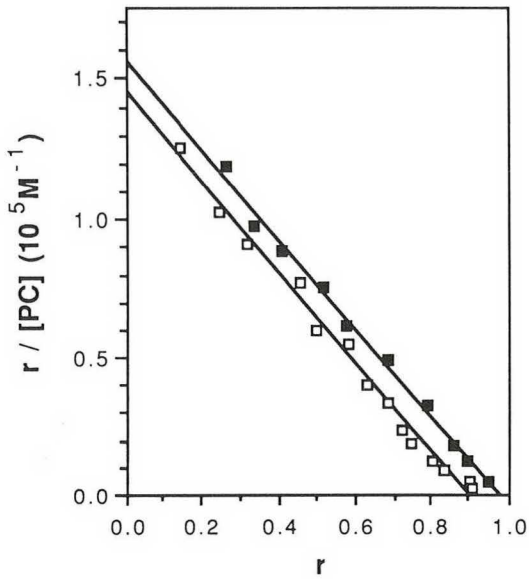

Fig. 3. Scatchard plot of the binding of phosphorylcholine to recombinant (□) or proteolytically obtained F_{ab} fragment (■). *r* denotes the fraction of antibody fragment with bound hapten and [*PC*] denotes the concentration of free phosphorylcholine.

results thus demonstrate that functional F_{ab} fragments can be obtained from an *E. coli* expression system and that the lack of N-glycosylation in this expression host does not influence the hapten-binding properties of this antibody fragment.

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