Functional antibody lacking a variable-region disulfide bridge

(immunoglobulin structure/cysteine residue/antigen binding)

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ABSTRACT In 1981, Auffray et al. [Auffray, C., Sikorav, J. L., Ollo, R. & Rougeon, F. (1981) Ann. Immunol. (Inst. Pasteur) 132D, 77-88] reported a partial cDNA sequence of the heavy chain from the ABPC48 plasmacytoma whose protein product had previously been shown to bind bacterial and grass levan. In the cDNA sequence the second half-cystine of the heretofore invariant disulfide bridge had been replaced by a tyrosine. Since the presence of invariant variable-region disulfide bridges has been considered a basic structural feature of the antibody molecule necessary for proper folding and function, we have analyzed the heavy chain protein produced by ABPC48. Our results indicate that heavy chains from ABPC48 quantitatively express tyrosine in place of the normally occurring second half-cystine in the variable region. Furthermore, this antibody population is capable of both binding antigen and subsequent precipitation. Thus, the presence of a disulfide bridge in the heavy-chain variable region does not appear necessary for proper function of this antibody and may not be obligatory for antibody function in general, as has been assumed previously.

The nature of the antibody molecule has been extensively analyzed at both the primary (1) and three-dimensional structural levels (2). These studies have defined a number of structural features that appear to be invariant and that have been presumed to be essential for proper function (i.e., folding and subsequent antigen binding). Among these are, for example, the presence of cysteines at intervals of 65-70 amino acids which form disulfide bridges in each globular domain. It has been thought that these disulfide bridges are critical to the proper folding of the mature protein. To date, no immunoglobulins have been described that lack these properties and still demonstrate normal antibody functions.

In 1981, Auffray et al. (3) published a partial cDNA sequence of the antibody heavy chain derived from the ABPC48 mouse plasmacytoma.* This antibody had previously been shown to specifically bind bacterial and grass levan (4). The unusual feature of this sequence was that the second cysteine in the heavy chain variable region had been replaced by a tyrosine. The authors, however, did not address the question of whether a protein was produced by the line from which their cDNA was generated and, if so, whether the protein still bound antigen. Thus, the possibility existed that the cysteine-tyrosine change represented a mutation that had occurred during propagation of the line, and that any protein produced could not fold properly or bind antigen. Since the presence of variable region disulfides has been considered such a hallmark of the antibody molecule, we have undertaken analysis of the ABPC48 protein to determine whether molecules produced by this line lack a disulfide bridge and, if so, whether these molecules bind antigen, raising the question of the presumed obligatory nature of these bridges for proper antibody folding and function.

MATERIALS AND METHODS

Sequence Determination. CNBr fragments were prepared and sequenced as described (5). Sequencing experiments were performed on an Applied Biosystems model 470A protein/peptide sequencer.

Precipitation Assay. Duplicate tubes containing 0.29 mg of ABPC48 protein in 0.15 M NaCl/0.06 M phosphate, pH 7.4, were incubated with varying concentrations of Aerobacter levan (kindly provided by C. P. J. Glaudemans, National Institutes of Health, Bethesda, MD). Reaction solutions were incubated at 4°C for 4 days. Precipitates were collected by centrifugation, washed three times with 1.0 ml of buffer, and dissolved in 1 mM NaOH. Tryptophan fluorescence was monitored at 340 nm following excitation at 295 nm in a Perkin-Elmer fluorometer as described by Jolley et al. (6).

RESULTS AND DISCUSSION

Antibody from the ABPC48 line, which was shown to bind antigen by Ouchterlony precipitation reaction, was purified from ascites fluid by sequential chromatography on DEAE-Sephadex A-25, Sephadex G-150, and Sephadex G-200 columns (7). Following separation of heavy and light chains, heavy chains were cleaved with CNBr and chromatographed on a Sephadex G-100 column in 5 M guanidinium chloride/0.2 M NH4HCO3 as described (5). Peptides in isolated peaks were then completely reduced, alkylated with iodo[14C]acetylamine to label cysteine residues, and rechromatographed. The peptide normally containing the second heavy-chain cysteine begins at amino acid position 82A following CNBr cleavage at the preceding methionine (position 82). This peptide was sequenced three times, resulting in sequence determination from position 82A into the constant region. The sequence obtained is presented in Fig. 1 and corresponds exactly to the cDNA reported by Auffray et al. (3). Analysis of amino acid recoveries from one of the sequencer runs is also presented in Fig. 1 and indicates that the tyrosine at position 13 in the peptide (position 92 in the heavy chain) was recovered in quantitative yield. The out-of-phase degradation or carryover, based on the recovery of leucine from cycle 10 at cycle 11, was 17%, indicating that the yield of tyrosine at position 13 could not be accounted for by carryover from the preceding cycles. Furthermore, no radioactivity, indicative of radioalkylated cysteine, was observed at this position. Similar results were obtained in all sequencing experiments. We consider, based on previous experience, that the potential error in such analyses is likely to be no more than 10%. Thus, more than 90% of the antibody molecules in this

*We have used the nomenclature of Potter (4) for this plasmacytoma. The clone analyzed by Auffray et al. (3) (designated ABE48) is from this same line.
Preparation contain a tyrosine at position 92 in place of the normally occurring second cysteine.

We next considered the possibility that the ABPC48 antibody population was a mixture of species more than 90% of which had tyrosine at position 92 and 10% or less of which had, in fact, cysteine at position 92 that was not detected in the sequencing experiments. The antigen-binding activity associated with the ABPC48 line might conceivably be due to such a minor population. To test this hypothesis, a series of precipitin assays were performed. It was reasoned that, if antigen binding was associated with a minor antibody population, no more than 10% of the antibody should be precipitable in such experiments. Results of two such studies are presented in Fig. 2. In the first assay, a maximum of 52% antibody was precipitated from a partially purified preparation of polymeric IgA. Densitometry tracing of this material (Fig. 3, lane A) indicated that the preparation contained 52% immunoglobulin. This preparation was subsequently rechromatographed on a Bio-Gel P-300 column to remove additional contaminants and monomeric IgA, which might inhibit precipitation (8). Assay of this material resulted in a maximum of 85% antibody precipitated and densitometric scanning of the starting material (lane B) indicated 85% purity. To rule out the unlikely possibility that precipitation was due to non-antibody contaminants, supernatants from the precipitin assay using the 85% pure preparation were subjected to NaDodSO4/polyacrylamide gel electrophoresis (Fig. 3). As can be seen, the antibody heavy and light chains decreased with increasing precipitation. The concomitant decrease in the band at ≈66 kDa, presumed to be albumin, is suggested to result from nonspecific sticking. It thus appears from the

![Fig. 1](image1.png)

**Fig. 1.** (Upper). Amino acid sequence (standard one-letter abbreviations) of the ABPC48 heavy-chain peptide spanning the region in which the second half-cystine of the variable-region disulfide bridge normally occurs; the peptide includes the carboxyl-terminal 20 residues of the variable (VH) region, the joining region (JH), and a portion of the first constant domain (not shown). Upper numbering indicates the amino acid residue position in the heavy chain according to Kabat et al. (1), and lower numbering, position in the sequenced peptide. Asterisk designates position of the cysteine→tyrosine substitution. (Lower). Sequence analysis of the ABPC48 VH peptide containing the cysteine→tyrosine substitution. Individual amino acids at each position are represented by a + or o. Amino acids identified by + [including lysine (K), valine (V), alanine (A), leucine (L), tyrosine (Y), and glycine (G)] are normally recovered and identified quantitatively. These amino acids were used to generate a theoretical regression plot indicated by dots. Other amino acids (o) were identified qualitatively. Data points were corrected for background at the preceding cycle but not for out-of-phase degradation.

![Fig. 2](image2.png)

**Fig. 2.** Precipitation of ABPC48 by *Aerobacter* levan. Open boxes represent precipitation with a partially purified protein preparation containing 52% immunoglobulin, and triangles, a preparation containing 85% immunoglobulin.
above results that >90% of this antibody population has a tyrosine in position 92 of the heavy chain and that these molecules are capable of antigen binding and precipitation. Crystallographic analyses (9, 10) of immunoglobulin Fab fragments have indicated that the variable or antigen-binding domains consist of two layers of antiparallel β-pleated sheets. The bends that join the anti-parallel sheets at one end of the molecule comprise the hypervariable, or complementarity-determining regions, which interact with antigen, whereas the remainder of the polypeptide segments constitute the framework portion of the variable region. The two cysteines at amino acid positions 22 and 92 which form the heavy-chain variable-region intradomain disulfide bridge are centrally located in each of the layers of β-pleated sheets. It is likely that formation of the disulfide bridge is one of the last events in protein folding and that this bridge serves to stabilize interactions between the two polypeptide layers. An examination of heavy-chain sequences from a variety of species (1) reveals that, excluding the present case, only three positions are completely invariant. These are the two cysteines at positions 22 and 92 and the tryptophan at position 36. Thus, substitutions at these positions are either extremely rare or strongly selected against.

We have assessed the effect of the cysteine→tyrosine change in the three-dimensional structure of the Fab fragment from McPC603 (Fig. 4). It should be cautioned that this assessment is based on the assumption of similarity between the ABPC48 and McPC603 structures. While homologous structure would clearly not be expected in complementarity-determining regions, comparative analysis of independently determined crystallographic structures (11) has revealed that, at the level of α-carbon backbone structure, framework segments of variable domains are essentially interchangeable even between species. Thus, the probability of similar structures between ABPC48 and McPC603, especially in the region of the disulfide bridge, would appear to be high. Despite the large difference in side-chain volume between cysteine and tyrosine (on the order of 100 Å³), the tyrosine can be accommodated with only minor perturbation of the structure. As can be seen, rotation of the side chains of cysteine-22, leucine-4, and methionine-34 provides adequate space for the substituted moiety. Leucine at amino acid position 4 and methionine at 34 are identical in ABPC48 and McPC603, so that the suggested repositioning is likely to be similar in the actual ABPC48 structure. Furthermore, the side chain of amino acid 24 is proximal to the substituted aromatic ring of tyrosine. In ABPC48, position 24 is alanine as opposed to threonine in McPC603. The smaller alanine side chain would facilitate the replacement by tyrosine 92 but is probably not essential for this accommodation, as a slight displacement of the threonine in McPC603 would also result in adequate space.

The above results indicate that the presence of a disulfide bridge in the heavy-chain variable region is not critical to the proper folding and function (antigen binding) of the ABPC48 variable region. This further raises the general question as to the requirement of variable-region disulfide bridges in antibodies or in other molecules, such as the major histocompatibility antigens and T-cell antigen-receptor polypeptides, which show domain homology to immunoglobulins. It is surprising that substitutions have not previously been observed at these positions if the bridges are not critical to antibody function, especially considering the frequency of conservative replacements observed in antibody framework regions (1). The cysteine→tyrosine substitution is extremely nonconservative in nature, and the previous invariance at this

Fig. 3. NaDodSO₄/polyacrylamide gel electrophoresis of starting material and supernatants from precipitin assay in Fig. 2. Lanes: A, starting material for precipitin experiment (open boxes in Fig. 2); B, starting material for precipitin experiment (triangles in Fig. 2); B1–B9, 2-μl aliquots of supernatants from the B precipitin reaction representing increasing antigen concentration. Standards in lane at left are 93, 66, 45, 31, and 22 kDa in descending order. Positions of immunoglobulin heavy (H) and light (L) chains are indicated.

Fig. 4. Stereo drawing of the McPC603 heavy-chain variable region including the cysteine→tyrosine substitution at position 92. Light lines indicate position of bonds in the McPC603 structure, and heavy lines indicate repositioning necessary to accommodate the substitution. The disulfide bridge in McPC603 is indicated by a dumbbell. Alanine found at position 24 in the ABPC48 sequence has been substituted for threonine in the McPC603 structure, which facilitates the accommodation of tyrosine-92.
position may indicate that, in fact, the disulfide bridges are critical to many antibody molecules, although this presumed requirement may now need to be reconsidered.

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