Synthetic Peptides $V_H(27–68)$ and $V_H(16–68)$ of the Myeloma Immunoglobulin M603 Heavy Chain and Their Association with the Natural Light Chain To Form an Antigen Binding Site†

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ABSTRACT: A 53-residue peptide corresponding to the variable region 16–68 of the heavy chain of phosphocholine binding mouse myeloma M603 protein was synthesized by a solid-phase fragment strategy. The homogeneity of the $V_H(16–68)$ peptide was confirmed by high-performance liquid chromatography, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, amino acid analysis, and mass spectrometry. Synthetic $V_H(16–68)$ associated with the M603 light chain, and about 27% of the recombination mixture bound to phosphocholine immobilized on Sepharose as compared to a 28% binding yield obtained for the recombined natural light and heavy chains under the same conditions. The binding yield for the recombinant of the light chain with previously prepared $V_H(27–68)$ fragment was about 11%. These semisynthetic antibodies $V_H(27–68)$ and $V_H(16–68)$ light chain recombinants are forerunners of structural variants designed to study the antigen binding pocket of the M603 immunoglobulin.

The antigen binding site of the phosphocholine-binding IgA[1] mouse myeloma M603 protein (Potter & Leon, 1968; Rudikoff & Potter, 1974) is located on the surface of the immunoglobulin molecule in a cleft between the hypervariable regions of its light (V_L) and heavy (V_H) chains (Padlan et al., 1973, 1985; Segal et al., 1974).

We have undertaken the synthesis of a 120 amino acid residue V_H domain of the M603 protein as an ultimate goal toward preparation of a semisynthetic antibody. Our strategy is to combine, either covalently or noncovalently, two large synthetic fragments corresponding to residues $V_H(1–68)$ and $V_H(69–120)$ and associate this protein with natural light chain or V_L derived from it to constitute the binding cavity of M603.

The concept of the chemical synthesis of an antigen combining site has also been explored in other laboratories. Burton et al. (1977) undertook stepwise solid-phase synthesis of a 112-residue variable domain of the light chain, V_L, of rabbit antibody 3368 specific for pneumococcal type II polysaccharide, but they encountered large losses of chains and severe chain termination side reactions.

Givol et al. (1977) and Gavish et al. (1978) also used the stepwise solid-phase method for the preparation of a 115 amino acid residue protein with the sequence of V_L of mouse myeloma IgA, MOPC 315. It was found that 1.7% of the synthetic V_L was able to associate with V_H to reconstitute a *p*-nitrophenol combining site (Gavish et al., 1978).

For the M603 $V_H(1–68)$ fragment we have chosen a solid-phase fragment assembly approach partly as a result of the synthetic problems just mentioned but mainly because the hypervariable regions can be localized in individual fragments; therefore, the synthesis of structural analogues required for the binding studies can be simplified (Tjoeng et al., 1979; Voss et al., 1983; Whitney et al., 1983, 1984).

Figure 1 shows protected peptides for the fragment synthesis of the V_H half-domain (residues 1–68) on a multidetachable 2-[[4-(oxymethyl)phenyl]acetoxy]propionylcopoly(styrene–divinylbenzene) resin (–OCH_2–Pop-resin) (Tam et al., 1980). All the fragments have glycine at the C-terminus to avoid racemization during subsequent activation and coupling of the fragments, and only one aminoacyl resin, Boc-Gly-OCH_2-Pop-resin, had to be prepared for the synthesis of the fragments.

Recently, we reported a new base-catalyzed reaction employing the hindered, nonnucleophilic base tetramethylguanidine (TMG) for the removal of protected peptides from the Pop-resin (Whitney et al., 1984). Such protected peptides having only a free C-terminal carboxyl group were used for the preparation of the $V_H(27–68)$ domain of M603 (Whitney et al., 1983) by solid-phase fragment condensation.

In this paper, we describe the synthesis and purification of the $V_H(16–68)$ fragment by the same approach. The $V_H(16–68)$ and $V_H(27–68)$ contain seven and nine amino acids of the heavy chain involved in phosphocholine binding. These are Tyr-33 and Arg-52, which are closely in contact with the antigen, and Glu-35, Glu-61, Lys-54, Lys-57, and Lys-67 [the last four amino acids are also numbered 58, 52b, 54, and 64, respectively, according to the numbering system of Kabat et al. (1983)], whose charged side groups appear to play an important role in the binding of phosphocholine (Padlan et al., 1985).

It was of interest to find out whether there would be any phosphocholine (PC) binding upon association of the synthetic fragments $V_H(16–68)$ and $V_H(27–68)$ with the M603 light chain. Affinity chromatography with the PC immobilized on Sepharose revealed that the recombinants of the light chain with $V_H(16–68)$ monomer, $V_H(16–68)$ dimer, and $V_H(27–68)$ all bound phosphocholine.

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[1] Abbreviations: BBS (borate buffer–saline), 0.05 M sodium borate buffer with 0.16 M NaCl, pH 8.2; Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; Bzl, benzyl; Cl_2-Bzl, 2,6-dichlorobenzyl; 2-Cl-Z, 2-chlorobenzyloxycarbonyl; cHex, cyclohexyl; CL, cardiolipin; DIEA, diisopropylethylamine; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; For, formyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; H, heavy chain; Ig, immunoglobulin; L, light chain; NMP, 1-methyl-2-pyrrolidinone; PC, phosphocholine; PC–Sepharose, glycyltyrosine-3,4′-azophenylphosphocholine attached to Sepharose 4B; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TMG, tetramethylguanidine; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)aminomethane; V_H and V_L, variable region of the heavy and light chains, respectively.

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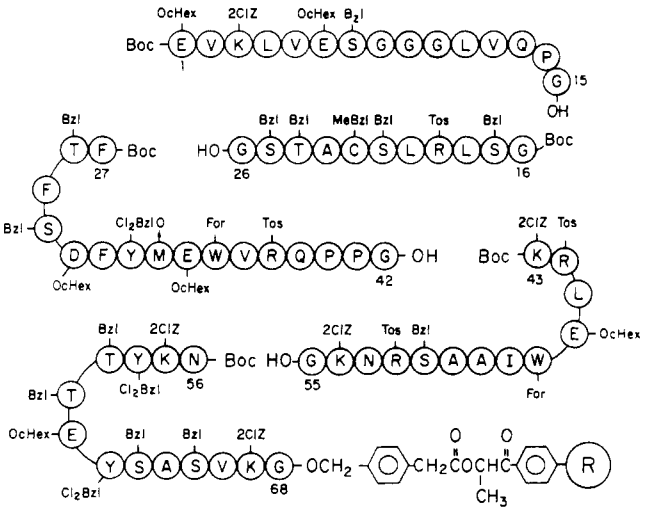

FIGURE 1: Protected peptides for the fragment synthesis of the V_H half-domain (residues 1–68) on a multidetachable 2-[[4-(oxymethyl)phenyl]acetoxy]propionylcopoly(styrene–divinylbenzene) resin (-OCH_2-Pop-resin).

Experimental Procedures

Materials. Commercial protected amino acids were obtained from Peninsula Laboratories (San Carlos, CA). Phosphocholine chloride (calcium salt) was from Sigma (St. Louis, MO), and tetramethylguanidine, *N*-methyl-2-pyrrolidinone (gold label), 1,4-dithiothreitol, and ethylenediaminetetraacetic acid were from Aldrich (Milwaukee, WI). Ultrapure urea was purchased from Schwarz/Mann Biotechnology (Division of ICN Biomedicals, Cleveland, OH), and guanidine hydrochloride was from Whittaker (Heico Division, PA). Other reagents were as described before (Merrifield et al., 1982).

Methods. Reversed-phase HPLC was performed on a Vydac 218 TP column (5 μm, 4.6 × 250 mm, Hesperia, CA) with a Shimadzu instrument. The flow rate was 1 mL/min and detection at 220 nm. Solution A was 5% acetonitrile in water (v/v) containing 0.45 mL of TFA/L, and solution B was 70% acetonitrile in water (v/v) with 0.385 mL of TFA/L.

TLC was performed on silica gel GF plates (Analtech, Newark, DE), and spots were visualized with Cl_2 gas/KI/starch spray (Stewart & Young, 1984).

SDS–polyacrylamide electrophoresis was done on a Phast System instrument (Pharmacia, Piscataway, NJ) using Phast gel gradient media (gel gradient 8–25%, 2% cross-linking, buffer system 0.112 M acetate, 0.112 M Tris, pH 6.4) and Phast gel SDS buffer strips (0.24 M Tricine, 0.2 M Tris, 0.55% SDS). A low molecular weight standard (Bethesda Research Laboratories, Gaithersburg, MD), insulin, A and B chain (apparent M_r 3000), bovine trypsin inhibitor (M_r 6200), lysozyme (M_r 14 300), β-lactoglobulin (M_r 18 400), α-chymotrypsinogen (M_r 25 700), and ovalbumin (M_r 43 000). The gels were stained with 0.25% Coomassie brilliant blue R in methanol/acetic acid/water (3:1:6 v/v) for 30 min and destained with methanol/acetic acid/water (3:1:6 v/v).

Mass spectra were obtained by californium-252 fission fragment ionization mass spectrometry (Macfarlane & Torgerson, 1976; Chait et al., 1982) at the Rockefeller Laboratory. A sample of V_H(1–68) was applied to a nitrocellulose film in 20% acetic acid and processed as described by Chait and Field (1986).

M603 isolation and separation of its light (L) and heavy (H) chains were performed on the M603 myeloma tumor line, a gift from Dr. Michael Potter at the National Cancer Institute. The tumor line was maintained by serial subcutaneous transplantation in Balb/c mice. The myeloma protein was produced by injection of minced tumor into mice that had been injected 3 weeks previously with 0.5 mL of pristane. The M603 protein (Potter & Leon, 1968; Rudikoff & Potter, 1974) was purified from ascites fluid by mild reduction, followed by alkylation and affinity chromatography on glycyltyrosine-3,4′-azophenylphosphocholine-derivatized Sepharose CL-4B (Chesboro & Metzger, 1972). Heavy and light chain separation was achieved by Sephadex G-100 chromatography in 6 M urea/10% acetic acid after mild reduction and alkylation of the affinity-purified antibody (Bridges & Little, 1971).

For chain recombination, L and H were mixed at the H/L absorbance ratio of 2:1 at 280 nm and diluted with BBS to a final concentration of (0.4–2) × 10^{-5} M based on the light chain monomer. Protein concentrations were determined spectrophotometrically at 280 nm. Extinction coefficients ($A^{0.1\%}_{1cm}$) for the M603 and its light chain were found to be 1.4 and 1.0, respectively, as calculated on the dry weight of samples that had been exhaustively dialyzed against 1 M acetic acid, lyophilized, and redissolved in 1 M acetic acid and 6 M urea for the UV measurements. Bovine serum albumin was added (0.2–0.4 mg/mL) in some experiments as protection against chain denaturation. The mixture was saturated with urea and placed in a dialysis bag (Spectra/Por 6, molecular weight cutoff 1000, 18-mm flat width) closed tightly with new closures (Spectrum Medical Industries, Los Angeles) and dialyzed at 4 °C against 6 M urea/2 mM EDTA/0.1 M Tris, pH 8.2 (16 h), 3 M urea/0.1 M Tris, pH 8.2 (5–6 h), and BBS (16–24 h).

Results

Synthesis of Protected V_H(16–26). The protecting groups for the functional side chains of the amino acids were benzyl ether for threonine and serine, tosyl for arginine, and 4-methylbenzyl for cysteine. The Boc-Gly-OCH_2-Pop-resin (2 g) at a substitution of 0.56 mmol/g (0.68 mmol/g of polystyrene) was prepared as described by Whitney et al. (1984). The solid-phase synthesis was performed on a Beckman 990 Model synthesizer that had been modified for computer-assisted operation. The double DCC coupling in CH_2Cl_2 was used with 2.5 equiv of protected amino acid. Essentially, the protocol of Merrifield et al. (1982) was followed. The reaction vessel with the photolabile Pop-resin was protected from the light with aluminum foil throughout the synthesis. At the end of the synthesis, 3.5 g of protected peptide resin was obtained (93.6% yield by weight). Hydrolysis of the peptide resin and subsequent amino acid analysis gave a substitution of 0.64 mmol/g of polystyrene, indicating that the assembly of peptide was completed with an overall yield of 94.1% (99.6%/step), which is in good agreement with the yield calculated by the weight gain.

The fully protected V_H(16–26) peptide was cleaved from the resin by the base-catalyzed elimination reaction with tetramethylguanidine (Whitney et al., 1984). The crude peptide was purified on Sephadex LH-20 with DMF as the eluant. Small aliquots were reacted with Folin–Lowry reagent (Lowry et al., 1959) to monitor separation. The main peak material was collected, and DMF was evaporated in vacuo at 35–40 °C. The residue was redissolved in a very small volume of DMF and cooled to 0 °C, the peptide was precipitated with cold water, and the precipitate was collected by centrifugation. The cleavage yield was in the range 67–84% as calculated from the quantitative ninhydrin test (Sarin et al., 1981) performed on the TMG-treated peptide resin, which had been washed extensively with DMF and methylene chloride prior to the ninhydrin analysis. The total yield of the protected V_H(16–26)

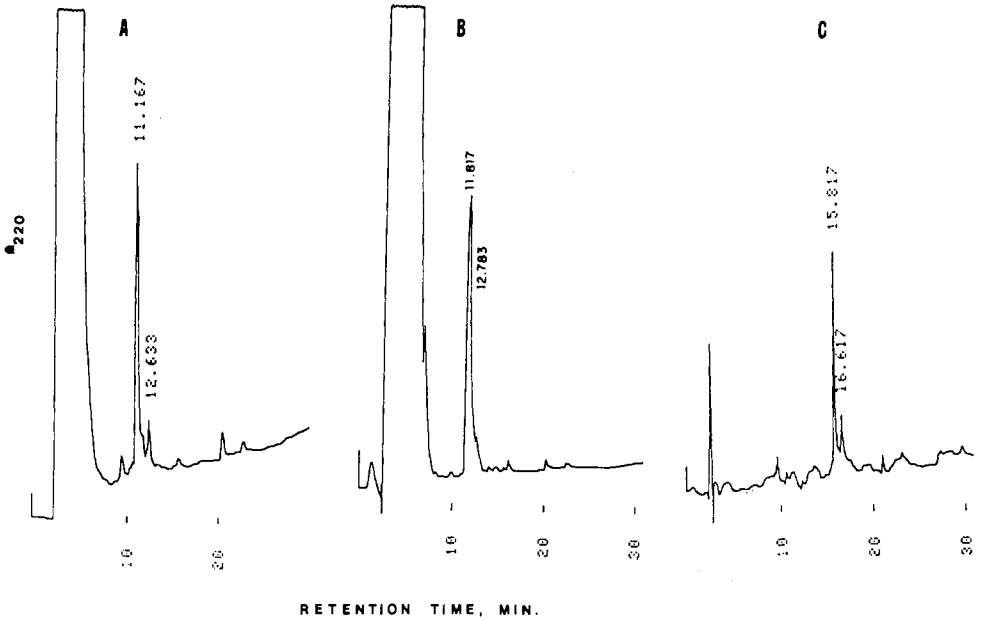

FIGURE 2: Reversed-phase HPLC on Vydac 218 TP with a 30-min linear gradient from 10 to 50% B into solution A. (A) $V_H(16-26)$ monomer obtained from $V_H(16-26)$-OCH_2-OCH_2-Pop-resin after HF cleavage. The crude product was passed through Sephadex G-10 in 10% acetic acid, and the lyophilized peptide fraction was reduced with 0.2 M DTT in 6 M urea/0.1 M Tris, pH 8.0 (10 mg/mL, 2 h) and acidified with acetic acid prior to the HPLC run. 0.064 absorbance full scale. (B) $V_H(16-26)$ monomer after TMG cleavage, followed by HF deprotection. The workup after HF was as in (A). 0.128 absorbance full scale. (C) $V_H(16-26)$ S–S dimer. A small aliquot of product A after gel filtration was adjusted to pH 8.0 with NH_3(aq) and air oxidized overnight. 0.016 absorbance full scale.

obtained after chromatography and precipitation was 30–35%. TLC of the precipitated peptide revealed a single spot in the following solvent systems: chloroform/methanol/acetic acid (80:15:5 v/v), $R_f^1 = 0.72$; chloroform/methanol/acetic acid (85:10:5 v/v), $R_f^2 = 0.50$; 2-propanol/NH_3 (aq) (2:1 v/v) $R_f^3 = 0.75$.

A low/high HF cleavage (Tam et al., 1983) was performed on the N^{α}-deprotected $V_H(16-26)$-OCH_2-Pop-resin and in parallel on the TMG-cleaved and purified protected peptide. The samples were treated with HF/Me_2S/*p*-cresol/*p*-thiocresol (25:65:8:2 v/v, 0 °C, 2 h) followed by HF/*p*-cresol/*p*-thiocresol (95:4:1 v/v, 0 °C, 1 h) (Tam et al., 1983). After the HF was removed, the residue was washed with diethyl ether to remove organic contaminants, and the peptides were extracted with 10% and 50% acetic acid. The HPLC profiles of the HF-cleaved and reduced or oxidized peptides are shown in Figure 2. The TMG-cleaved peptide (Figure 2B) was purer than the peptide directly cleaved by HF (Figure 2A), although the former still contained some contaminants present along with the main deprotected product. It is hard to judge whether the impurities were present in the protected $V_H(16-26)$ or whether they were generated as a result of side reaction(s) during the HF cleavage. The HPLC profiles do indicate that there was no major amount of deletion or termination during the course of the synthesis. The amino acid composition of the TMG-cleaved protected $V_H(16-26)$ as well as that of HF-deprotected peptide was consistent with the expected sequence (Table I).

Table I: Amino Acid Composition[a]

amino acid	$V_H(16-26)$ TMG-cleaved, protected	$V_H(16-26)$ deprotected dimer	$V_H(16-68)$ purified monomer
Asp			3.0 (3)
Thr[b]	1.0 (1)	1.09 (1)	3.85 (4)
Ser[b]	2.76 (3)	2.77 (3)	6.32 (7)
Glu			4.22 (4)
Pro			2.17 (2)
Gly	2.08 (2)	2.12 (2)	4.99 (5)
Ala	1.09 (1)	1.1 (1)	4.15 (4)
Cys[c]	ND (1)	1.04 (1)	0.97
Val			2.11 (2)
Met			0.85 (1)
Ile			1.09 (1)
Leu	2.08 (2)	2.13 (2)	3.16 (3)
Tyr			2.98 (3)
Phe			2.87 (3)
Lys			3.96 (4)
Arg	1.0 (1)	0.95 (1)	3.87 (4)
Trp[d]	ND (2)		ND (2)

[a] Hydrolyses were performed in sealed, evaculated tubes with 5.7 M HCl, 110 °C, 24 h. Values in parentheses are theoretical. ND, not determined. [b] Thr and Ser were not corrected for losses during hydrolysis. [c] Cysteine determined as cysteic acid after performic acid oxidation. [d] Trp was destroyed during HCl hydrolysis and not determined.

Protected $V_H(27-68)$-OCH_2-Pop-Resin. The resin-supported, fully protected 42-residue peptide $V_H(27-68)$-[[(oxymethyl)phenyl]acetoxy]propionylcopoly(styrene–divinylbenzene) resin was synthesized previously (Whitney et al., 1983). It has been shown, by cleavage of a small sample, to be relatively homogeneous by HPLC on a μBondapak C_{18} column. New work with a Vydac 218TP analytical HPLC system showed, however, that the 27–68 peptide still contained additional peptide components. The free peptide needed for binding studies could be purified to homogeneity by an additional HPLC step, but of course the protected resin-bound peptide could not, and further purification had to be done at the free peptide stage after final condensation with the 16–26 fragment and cleavage from the resin.

Coupling of the Protected $V_H(16-26)$ to $V_H(27-68)$-OCH_2-Pop-Resin and Cleavage and Deprotection of $V_H(16-68)$ Resin with HF. The Boc group was removed from the protected $V_H(27-68)$-OCH_2-Pop-resin with 50% TFA/CH_2Cl_2 followed by 5% DIEA/CH_2Cl_2 neutralization. To this was added a preformed HOBt ester of protected $V_H(16-26)$ [2.5 equiv in 3 mL of *N*-methylpyrrolidinone (NMP)], followed by an additional 1 mL of NMP. Reaction time was 72 h at 4 °C. The excess of unreacted reagent was filtered off, and the resin was extensively washed with DMF and neutralized with 5% DIEA/CH_2Cl_2. The coupling yield was 50% based

on the peptide resin hydrolysis. The remaining amino groups were acetylated with acetic anhydride/pyridine. Finally, the N^{α}-Boc group was removed with 50% TFA/CH_2Cl_2, the peptide resin was neutralized with 5% DIEA/CH_2Cl_2, and the protected peptide resin was subjected to a low/high HF treatment.

Purification of V_H(16–68). The crude HF product was extracted into 10% HOAc and lyophilized. It was dissolved in 6 M urea/Tris/EDTA, pH 8.2, and reduced with dithiothreitol because the V_H(16–68) sequence contained one cysteine residue, and some higher molecular weight mixed disulfide material was expected at this stage. Indeed, there was a polymeric fraction that did not dissolve even in the 6 M urea buffer, and it was removed by centrifugation. Also, some more aggregates or polymers were removed by gel filtration on Sephadex G-50 (Figure 3A, pools 1 and 2). Denaturation and reduction of this material did not produce any significant additional amount of the monomeric V_H(16–68). The partially purified reduced peptide (pool 3, Figure 3A) was not well separated from the lower molecular weight fragments; therefore, for further purification it was air oxidized to a dimer that was isolated by Sephadex G-50 chromatography (Figure 3B, pool I). The oxidation at pH 8.2 had to be carried out in the presence of 4 M guanidine hydrochloride, which was needed to dissolve the reduced and lyophilized material. This has been a difficult peptide to work with, and mainly because of low solubility and adsorption, the yields have been low. Use of polyethylene and silanized glass vessels did not improve recoveries significantly. From 70 mg of the peptide resin, 33 mg of the crude peptide was obtained after the HF cleavage, and of this only 6.7 mg (20%) of the reduced V_H(16–68) (pool 3, Figure 3A) was recovered. After a second Sephadex G-50 chromatography, 1.4 mg of the dimer was isolated from 5 mg of the oxidized V_H(16–68) (~28%). The best recoveries of lyophilized peptides were obtained by dissolving in buffers at pH 8 containing 6 M urea. The V_H(16–68) dimer was reduced again, and V_H(16–68) monomer was isolated by reversed-phase HPLC (Figure 4A, R_t = 14.5 min). Under these conditions, recovery from the HPLC columns was usually less than 5% as determined by amino acid analysis of the HPLC fraction collected directly into a pyrrolized hydrolysis tube.

Characterization of V_H(16–68). The synthetic V_H(16–68) was analyzed by Cf-252 fission fragment ionization mass spectrometry (Macfarlane & Torgerson, 1976; Chait et al., 1982) and gave a measured average molecular weight of 5983.5 versus a calculated mean molecular weight 5980.9. The mass difference of 2.6 is consistent with the mass uncertainty of the measurement since the mass spectrum was rather weak.

The purified monomer and dimer gave single Coomassie blue bands after sodium dodecyl sulfate gel electrophoresis, which migrated with approximately the expected molecular weights of 6000 for the monomer and 12 000 for the dimer. Figure 5 shows the electrophoresis on a commercial gel 3.2 cm long from Pharmacia. Similar results were obtained on a 15 cm long 15% polyacrylamide gel run according to Laemmli (1970) (data not shown).

The HPLC-purified peptide monomer was rerun on the Vydac column, showing a single peak with the retention time of 14.6 min (Figure 4B). Amino acid analysis was consistent with the expected sequence (Table I).

Additional Purification of Fully Deprotected V_H(27–68) for Binding Studies. The V_H(27–68) fragment previously described (Whitney et al., 1983) showed a single component on a μBondapak C_{18} column, but on a new Vydac 218 TP HPLC column it was found still to contain a mixture of

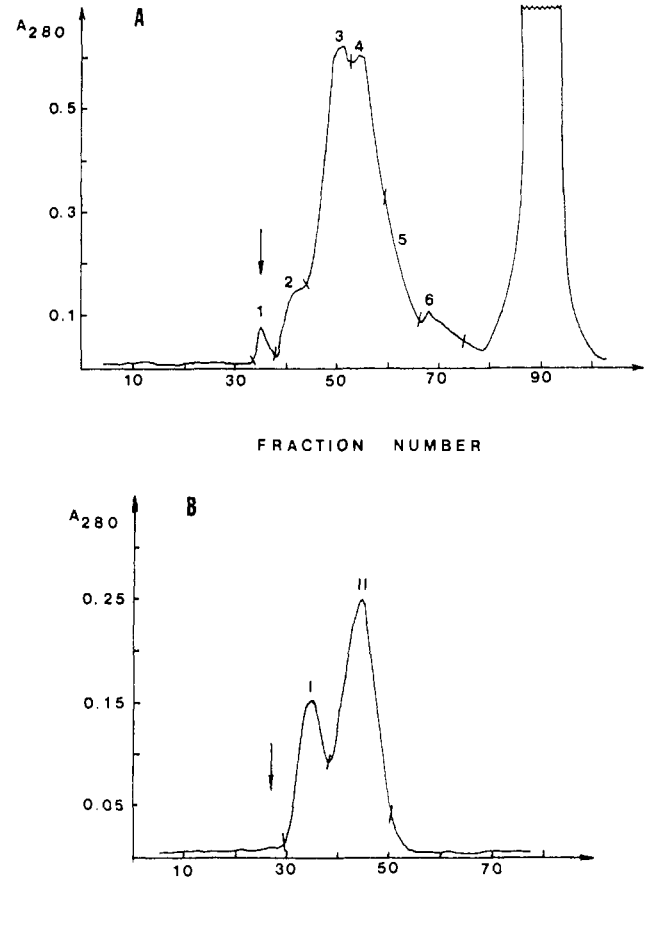

FIGURE 3: Sephadex G-50 chromatography. Column 1.5 × 80 cm in 10% acetic acid. Arrows show exclusion volume. (A) Crude V_H(16–68) peptide mixture (33 mg) after the HF cleavage was reduced with 40 mM dithiothreitol in 6 M urea/0.1 M Tris/2 mM EDTA at pH 8.2 (3 mL) under nitrogen at room temperature for 16 h. The sample was acidified with acetic acid and centrifuged to remove insoluble materials. The supernatant was divided into two equal portions, and each was chromatographed on Sephadex G-50. Fraction size was 2 mL, and flow rate was 30 mL/h. Pools 1 and 2, polymers (1.5 mg); pool 3, partially purified reduced V_H(16–68) (3.3 mg); pools 4–6, lower molecular weight fragments (~5 mg). (B) Pool 3 material (5 mg) was air oxidized in 4 M guanidine hydrochloride and 0.1 M Tris, pH 8.0, at a concentration of 1.5 mg/mL for 64 h in the presence of 0.1% NaN_3. The acidified mixture was saturated with guanidine hydrochloride and purified over Sephadex G-50 at a flow rate of 20 mL/h; fraction size 2.6 mL. Pool I, partially purified V_H(16–68) dimer (1.4 mg); pool II, mixture of V_H(16–68) monomer and lower molecular weight fragments (3.7 mg).

peptides (Figure 4C). Material isolated from the main peak was then found to be homogeneous on a further analytical HPLC column (Figure 4D). This V_H(27–68) preparation was subsequently used for the binding studies.

Chain Recombination and Binding Studies. The natural light and heavy chains (L and H) of the M603 protein were used in model studies in order to find the best conditions for the subsequent recombination of natural L chain with synthetic fragments of V_H.

Both L and H were additionally purified by affinity chromatography on PC–Sepharose to remove any residual natural material that would bind phosphocholine. L and H were each homogeneous on SDS–polyacrylamide gel electrophoresis, and their molecular weights were found to be 27 800 and 58 200, respectively (Weber & Osborn, 1969) (data not shown).

The recombination of the chains was achieved by dialysis against 6 M urea/Tris/EDTA buffer, pH 8.2, 3 M urea/Tris, pH 8.2, 3 M urea/Tris, pH 8.2, and finally, BBS, pH 8.2. The

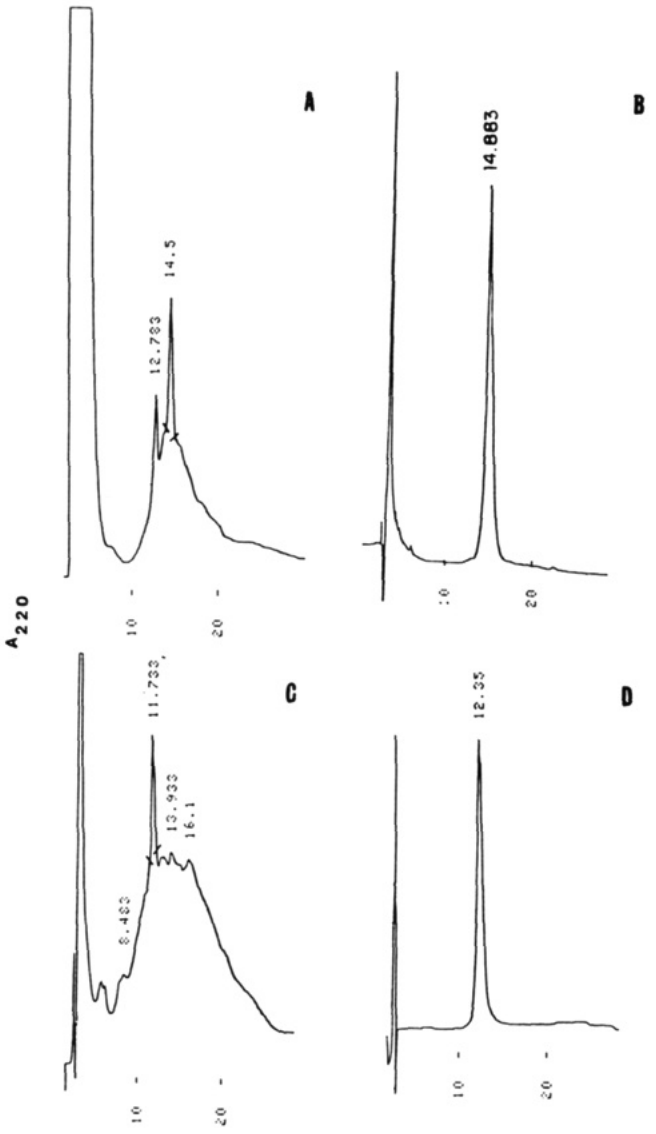

FIGURE 4: Reversed-phase HPLC on Vydac 218 TP with a 30-min linear gradient from 30 to 60% B into solution A. (A) Reduced $V_H(16-68)$. Pool I from Figure 3B (0.2 mg) was reduced with 0.1 M DTT/6 M urea/2 mM EDTA/0.1 M Tris, pH 8.5 (150 μL) for 2 h and acidified with acetic acid prior to HPLC run. R_t = 14.5 min peak material was collected as indicated. 0.256 absorbance full scale. (B) Reduced $V_H(16-68)$ from (A) was rechromatographed. 0.016 absorbance full scale. (C) $V_H(27-68)$ after Sephadex G-50 chromatography. Material with R_t = 11.73 min was collected as indicated. 0.128 absorbance full scale. (D) Rechromatographed $V_H(27-68)$ from (C). 0.032 absorbance full scale.

material inside the dialysis bag was passed through a PC–Sepharose column, and unbound material was washed out with BBS. Finally, bound protein was displaced with 1 mM phosphocholine in BBS.

The reconstitution yield was estimated from the 280-nm absorbance of the product that had bound to the phosphocholine–Sepharose column versus total absorbance of the bound and unbound material. In the case where BSA was present along with L and H chains, a correction was made for its contribution to the total absorbance.

Generally, the L and H chains recombined better at lower concentrations. For example, the recombination yield was 50–60% at a concentration of each chain of 6×10^{-6} M as compared to 39% at 1.5×10^{-5} M. The 6 M urea/Tris, pH

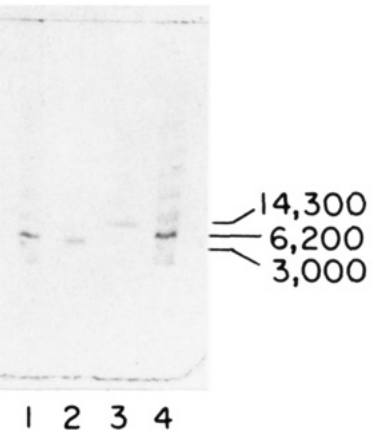

FIGURE 5: SDS–polyacrylamide gel electrophoresis using a Pharmacia Phast system. Lanes 1 and 4, low molecular weight markers; lane 2, $V_H(16-68)$ monomer; lane 3, $V_H(16-68)$ S–S dimer under nonreducing conditions.

8.2, dialysis step was essential for the recombination, and whenever it was omitted, the recombination yield dropped drastically. It seems that L and H self-aggregate on storage, especially in BBS, and with time the aggregation becomes partially irreversible. When the same L and H preparations were used for recombination as a control after they had been stored at −20 °C for several weeks, the recombination yield dropped 2–3-fold. In a final series of recombination and binding experiments between light chain (5.4×10^{-6} M) and the synthetic V_H fragments, a control recombination yield for the native L and H chains was found to be 28% (Figure 6A).

Figure 6B shows the PC–Sepharose chromatogram of the recombined light chain with the synthetic $V_H(16-68)$ monomer. The latter contains a single cysteine residue in its sequence, and therefore, the recombination was carried out with the buffers that had been degassed and saturated with nitrogen in order to protect the peptide from oxidation and dimer formation prior to recombination with the light chain. About 27% of the $V_H(16-68)$ recombined with the light chain and retained on the PC column and was subsequently eluted with 1 mM phosphocholine in BBS.

Similar affinity chromatography of the recombined light chain with a partially purified $V_H(16-68)$ dimer preparation showed less protein bound to the column in this case than for the recombined highly purified $V_H(16-68)$ monomer. The recombination and binding yield was estimated to be about 13–14% from the peak areas (absorbance at 280 mm) of the material released by phosphocholine from the affinity column (Figure 6C).

The shorter $V_H(27-68)$ peptide contains the same number of aromatic residues as does $V_H(16-68)$. Therefore, the binding yield of about 11% (Figure 6D) could be determined by UV absorbance as described for the $L/V_H(16-68)$ dimer recombinant. To exclude the possibility of a nonspecific protein binding to the immobilized phosphocholine, the following controls were used under the conditions used for the recombination and affinity chromatography: (1) light chain alone; (2) BSA; (3) light chain + BSA; (4) $V_H(27-68)$ alone. No binding was found in any of these experiments. Also, the PC column was extensively washed with 2 mM PC in BBS to remove any residual material after each experiment and reequilibrated with BBS.

DISCUSSION

Purity of Protected $V_H(16-26)$. In general, high purity of protected fragments to be coupled to a growing peptide at-

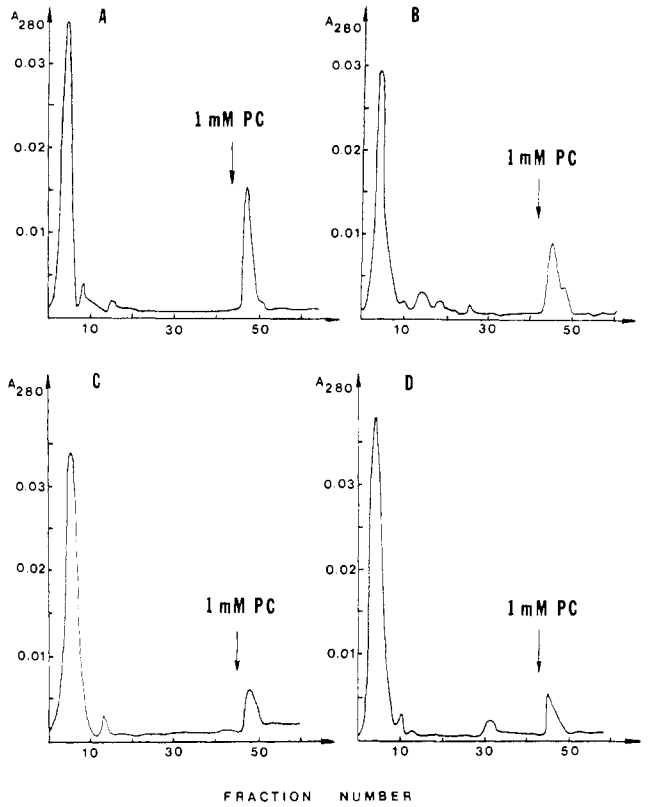

FIGURE 6: Affinity chromatography on phosphocholine–Sepharose. Column (0.65 × 3 cm, 1 mL of swollen gel) was first eluted with BBS (~45 mL) followed by 1 mM phosphocholine in BBS as indicated by arrows. Final light chain concentrations in the recombination mixtures was 5.4×10^{-6} M and BSA 0.22 mg/mL. (A) Natural light chain (1.2 nmol) recombined with an equimolar amount of natural heavy chain (absorbance ratio at 280 nm for L/H 1:2). (B) Light chain (1.2 nmol) recombined with an equimolar amount of V_H(16–68) monomer. Recombination was performed with oxygen-free buffers saturated with N_2 to prevent peptide oxidation. (C) Light chain (1.2 nmol) recombined with partially purified V_H(16–68) dimer after gel filtration (90 μg), Figure 3B, pool I. (D) Light chain (1.2 nmol) recombined with V_H(27–68) (3 nmol).

tached to the resin is essential for a successful fragment assembly of a peptide chain, just as it is in a classical solution synthesis. So far, all the protected fragments prepared by the TMG-cleavage procedure (Whitney et al., 1984; Voss et al., 1983) were purified by Sephadex LH-20 chromatography, followed by precipitation. The same method was used for the protected V_H(16–26), and its purity was checked by thin-layer chromatography. A single spot was observed in three different solvent systems with Cl_2 and KI/starch spray. This, however, was not a very sensitive system, since the protected peptides were difficult to visualize and some hydrophobic contaminants could have been present without detection. The HPLC analysis of the V_H(16–26) obtained after full deprotection of the purified material by HF showed a major synthetic product with some minor impurities (Figure 2), which might have been present in the protected peptide before the HF treatment or might have been generated during the deprotection step.

This synthesis illustrates a general problem with the fragment approach to the synthesis of large peptides. To make full benefit of the advantages of this approach, it is essential for the intermediates to be completely pure. Since it is often much more difficult ot purify protected peptides than free peptides, high purity has not always been achieved. In that case the purification has to be done at the end of the synthesis after full deprotection. In the fragment synthesis of V_H(16–68) four fully protected peptides have been synthesized containing 11, 13, 13, and 16 amino acid residues and after purification have been coupled sequentially to a Pop-resin support. The observed impurities in the final cleaved and deprotected 53-residue peptide are undoubtedly due in large measure to incomplete coupling and capping by acetylation at each step but also to impurities remaining in each of the purified peptide fragments. Fortunately, this peptide could be purified to homogeneity, but with only a low overall yield. The final product had the correct amino acid analysis and molecular weight by mass spectroscopy and by SDS–polyacrylamide gel electrophoresis.

Coupling Yields during Fragment Assembly. The following yields were obtained: 85% for the coupling of the protected V_H(43–55) to the N^{α}-deprotected V_H(56–68)-OCH_2-Pop-resin (Whitney et al., 1983); 75% for the attachment of V_H(27–42) to V_H(43–68)-resin (Whitney et al., 1983); 50% for the coupling of the protected V_H(16–26) to V_H(27–68)-resin as shown in this paper. The coupling yield decreased with increasing chain length of the peptide assembled on the resin, which makes final product purification more and more difficult.

Chain Association and Phosphocholine Binding. The ultimate goal of our long-term project has been to prepare a semisynthetic antibody consisting of its light chain (or the V_L derived from it) and synthetic V_H domain(s). The native L and H chains do not recombine easily. The best reconstitution yield obtained was 50–60% with freshly made preparations. However, both the L and H chains seemed to deteriorate on storage at −20 °C, probably due to self-aggregation and/or denaturation, which led to their decreasing ability to recombine properly in terms of efficient antigen binding.

We used affinity chromatography on a mini phosphocholine–Sepharose column (1 mL of swollen gel) to detect binding activity upon chain association. Due to rather limited quantities of the highly purified synthetic peptides, we were unable to perform equilibrium dialysis assays for more detailed binding studies and binding quantitation (K_A determination). Serious losses of the highly purified V_H(16–68) were experienced due to a very strong interaction with the glass, polyethylene, and column packing materials. Despite that, we were able to demonstrate that the reduced V_H(16–68) fragment recombines with the native light chain to form an active phosphocholine binding site. Since the sequence of V_H(16–68) contains a single cysteine residue, the recombination was first performed with the deaerated and nitrogen-saturated buffers to prevent oxidation of the synthetic peptide and dimer formation prior to the recombination with the light chain. The phosphocholine binding yield was 27% and was comparable with that of the natural L/H recombinant (28%) as judged by the PC-replaced material on the affinity column (Figure 6A,B). It was shown that the S–S dimer of V_H(16–68) also associated with the L chain and bound the antigen, but the binding was about half that found for the monomer. In this case, however, a partially purified synthetic material was used and the lower antigen binding might have been due to a relatively lower content of the proper V_H(16–68) sequence in the peptide mixture.

A question may be raised about nonspecific protein adsorption to the column. The light chain, BSA, light chain plus BSA, and synthetic V_H(27–68) were subjected to the same recombination/binding conditions, and no significant binding was observed. Therefore, it may be claimed that the binding activity was associated with the light and heavy chain recombination products.

The antigen binding site of M603 is a continuous surface formed by hypervariable regions of both the light and heavy chains (Segal et al., 1974). Tyr-33, Arg-52, Asn-95, and

Trp-100a from the heavy chain [the numbering system after Kabat et al. (1983)] and Asp-91, and Tyr-94, and Leu-96 from the light chain are in direct contact with phosphocholine (Padlan et al., 1985). The interactions between hapten and these residues involve extensive van der Waals contacts, hydrogen bonds, and electrostatic interactions. The hydroxyl group of Tyr-33 is hydrogen bonded to one oxygen of the PC phosphate. The guanidinium group of Arg-52 is also within hydrogen-bonding distance of phosphate oxygens. The positive charge of the choline moiety is to some extent neutralized by the side groups of Asp-91 of the light chain. In addition to the residues that are in direct contact with the hapten, there are other charged side groups also involved in the binding of phosphocholine. For example, Glu-35 of the heavy chain partially neutralizes the positive charge of the choline and fixes the position of Tyr-94 of the light chain through a hydrogen bond. Also, the charges on the heavy chain Glu-58 and Lys-52b, -54, and -64 (Glu-61, Lys-54, -57, -67 as marked in Figure 1) could be influential in aligning the hapten as it approaches the cavity (Padlan et al., 1985, and references cited therein).

In our synthetic fragments $V_H(27-68)$ and $V_H(16-68)$ there are seven of the nine amino acids of the heavy chain that are either directly or indirectly involved in phosphocholine binding. The fact that the antigen was found to bind to the $L/V_H(16-68)$ and the $L/V_H(27-68)$ recombinants indicates that the constituent amino acid residues of the synthetic heavy chain fragments properly recognize the light chain residues involved in phosphocholine binding. The yield of recombinant of $V_H(27-68)$ with light chain to give phosphocholine binding semisynthetic antibody was about half that obtained with $V_H(16-68)$ despite the fact that all seven important binding residues are present there as they are in the $V_H(16-68)$. This means that the remainder of the polypeptide segments, which constitute the framework portion of the variable region, are important for the light–heavy recombination.

The two cysteines at amino acid positions 22 and 92 [the numbering by Kabat et al. (1983)] that form the heavy chain variable region intradomain disulfide bridge were among only three positions that were completely invariant in heavy chain sequences from a variety of species (Kabat et al., 1983). This indicated that the disulfide bridge in the heavy chain variable region is critical to the proper folding and antigen binding. However, recently Rudikoff and Pumphrey (1986) reported that the ABPC 48 mouse plasmacytoma protein, which has the second half-cystine replaced by Tyr-92, is still 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 had been assumed previously (Rudikoff & Pumphrey, 1986).

Our finding that the $V_H(16-68)$ fragment, which contains only one half-cystine at position 22, was able to bind phosphocholine upon association with the light chain seems to confirm that idea. However, more detailed quantitative studies will be required to solve the problem of the entire $V_H(1-120)$ domain participation in the antigen binding, as well as that of half domains $V_H(1-68)$ and $V_H(69-120)$.

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Registry No. $V_H(16-68)$, 110717-74-3; protected $V_H(16-26)$, 110697-98-8.

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