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Mapping and Modification of an Antibody Hapten Binding Site: A Site-Directed Mutagenesis Study of McPC603†

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ABSTRACT: The quantitative contributions of various amino acid residues to hapten binding in the F_v fragment of the antibody McPC603 were investigated by site-directed mutagenesis. The three-dimensional structure of the F_{ab}' fragment of McPC603 is known to atomic resolution. The haptens phosphocholine, choline sulfate, 3-(trimethylammonium)propane-1-sulfonate, 4-(trimethylammonium)butyric acid, and 4-(trimethylammonium)butyric acid methyl ester were tested for binding. It was found that the phosphate group, but not the sulfate and sulfonate groups, interacts with the hydroxyl group of Tyr33(h). The required positive charge for the binding of the phosphate must be contributed by Arg52(h); a lysine at this position or an additional positive charge at position 33(h) abolishes the binding to a phosphocholine affinity column. The interaction between Tyr100(l) and Glu35(h) was found to be essential and could not be functionally replaced by any other pair of residues tested. Binding of the quaternary ammonium ion needs a negative charge; it can reside in either Asp97(l) or Asp101(h), but both together prevent binding to the affinity column. These data may serve as the basis for the development of quantitative treatments of antigen–antibody interactions.

The antigen combining site of an antibody consists of residues from six loops (three contributed by the variable domain of the light chain, V_L,[1] and three by the variable domain of the heavy chain, V_H) [for recent reviews see, e.g., Alzari et al. (1988) and Davies et al. (1990)]. Antigen binding requires the simultaneous interaction of several residues from the loops with appropriate atoms of the antigen. The determination of the three-dimensional structure of antibody–antigen complexes, either with small haptens [see, e.g., Amzel et al. (1974), Segal et al. (1974), and Herron et al. (1989)] or protein antigens [see, e.g., Amit et al. (1986), Sheriff et al. (1987), Padlan et al. (1989), and Colman et al. (1987, 1989)], has led to qualitative and geometrical descriptions of these interactions as well as to quantitative hypotheses of binding interactions (Novotny et al., 1989).

The advent of gene technology now makes it possible to put these interactions to a quantitative test. Particularly, the facile expression of F_v and F_{ab} fragments of an antibody in *Escherichia coli* now allows the rapid experimental determination of binding constants of altered antibodies (Skerra & Plückthun, 1988; Plückthun & Skerra, 1989; Glockshuber et al., 1990; Skerra et al., 1990). While site-directed mutagenesis studies of antibodies have been reported previously [see, e.g., Roberts et al. (1987), Riechmann et al. (1988), and Baldwin and Schultz (1989)], in the present study a direct correlation with the experimental X-ray structure is possible. This allows a more detailed interpretation of the effects of the mutations. The present work also demonstrates the convenience of the current improved *E. coli* expression system (Skerra et al., 1991).

We have chosen the particularly well characterized antibody McPC603 (Potter, 1977; Perlmutter et al., 1984) as a model system. It is an immunoglobulin A of the mouse with a specificity for phosphocholine. The crystal structure of its F_{ab}' fragment with (Segal et al., 1974) and without (Satow et al., 1986) bound phosphocholine has been determined. We have shown previously (Skerra & Plückthun, 1988; Glockshuber et al., 1990) that the F_v fragment of this antibody has the full intrinsic binding affinity to phosphocholine when compared to the whole antibody McPC603 (Metzger et al., 1971), the proteolytic F_{ab}' fragment (Glockshuber et al., 1990), or the recombinant F_{ab} fragment produced in *E. coli* (Skerra et al., 1990). However, the dissociation of the F_v fragment at low concentration requires cross-linking of the V_H and V_L domains for fluorescence measurements (Glockshuber et al., 1990) or high protein concentrations for equilibrium dialysis measurements (Skerra & Plückthun, 1988) to obtain linear Scatchard plots with identical binding constants to the F_{ab} fragment or the whole antibody. Both experimental approaches have been shown to be in quantitative agreement (Glockshuber et al., 1990). This demonstrates that the modification of surface lysines by glutaraldehyde with subsequent reduction to obtain the cross-linked F_V fragment, which preserves the positive charge, does not affect the binding of the hapten. In this paper, we therefore employed fluorescence measurements of glutaraldehyde cross-linked F_v fragments to determine hapten binding constants.

Phosphocholine-binding antibodies have been examined with a variety of haptens and analogues as a model for understanding the genetics and specificity of the immune response [see, e.g., Leon and Young (1971), Padlan et al. (1976), Potter (1977), Young and Leon (1977), Goetze and Richards (1977a,b, 1978), Gearhart et al. (1981), Perlmutter et al. (1984), and Claflin et al. (1985, 1987)]. While a large body of data has been accumulated from these studies, quantitative and structural interpretations are hampered by several prob-

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[1] Abbreviations: PC, phosphocholine; V_H, variable domain of the heavy chain; V_L variable domain of the light chain.

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lems. First, complete sequences of both chains are not known in all cases. Second, binding constants have not been determined directly in all cases. Often, the inhibition of solid-phase binding assays has been employed, making quantitative comparisons between different studies difficult. Third, even in myeloma or hybridoma proteins with known sequence, variations in the length of the loops makes model building exceedingly uncertain [see, e.g., Padlan et al. (1976)]. It was therefore desirable to test some critical interactions in the only phosphocholine-binding antibody with known three-dimensional structure (McPC603) by employing the methods of site-directed mutagenesis and expression in *E. coli*.

Phosphocholine is a very polar charged molecule and the quantitative study of its interactions may provide access to improved understanding of electrostatic forces in proteins. In this paper, we investigate the quantitative effects of a number of amino acid substitutions of residues in direct contact with the hapten as well as some that occur in equivalent positions in related antibodies of the phosphocholine binding family.

Experimental Procedures

Materials. Phosphocholine (**1**) and 4-(trimethylammonium)butyric acid (chloride salt) (**4**) were obtained from Sigma. Other precursors for the syntheses were from Sigma or Aldrich.

Syntheses: *Choline Sulfate* (**2**). This compound was prepared from choline chloride and concentrated sulfuric acid according to Schmidt and Wagner (1904). The product was obtained as a white solid in 45% yield: MS (FAB) *m/e* (%) 184 (57, M + H), 104 (46, M + H − SO_3), 58 [9, $H_2C{=}N^+(CH_3)_3$]; ^{1}H NMR (D_2O) δ 3.33 (s, 9 H, CH_3); 3.84 [m, 2 H, $(CH_3)_3N^+CH_2$), 4.58 (m, 2 H, CH_2O).

3-(Trimethylammonium)propane-1-sulfonate (**3**). To a suspension of 3-aminopropanesulfonic acid (0.695 g, 5.0 mmol) was added diisopropylethylamine (2.56 mL, 5.0 mmol) and methyl iodide (0.940 mL, 15.1 mmol). The mixture was boiled under reflux at 65 °C for 16 h and was left, after cooling, at −20 °C for 24 h. The colorless crystalline precipitate was filtered, washed with methanol, and dried in vacuo. The solid was dissolved in H_2O (4 mL) and loaded onto a mixed-bed ion-exchange column [AG 501-X8 (D); 15 × 2.5 cm]. The product was eluted with 300 mL of H_2O and freeze-dried. A colorless powder (0.144 g, 16% yield) was obtained: MS (FAB) *m/e* (%) 363 (31, 2 M + H), 182 (100, M + H), 100 (13, M + H − H_2SO_3), 58 [30, $H_2C{=}N^+(CH_3)_3$]; ^{1}H NMR (D_2O) δ 2.31 (m, 2 H, $CH_2CH_2SO_3^-$), 3.02 (m, 2 H, $CH_2SO_3^-$), 3.18 (s, 9 H, CH_3), 3.58 [m, 2 H, $(CH_3)_3N^+CH_2$].

4-(Trimethylammonium)butyric Acid Methyl Ester (Iodide Salt) (**5**). This compound was prepared from 4-aminobutyric acid and methyl iodide according to Nozawa et al. (1980). The product was obtained in 12% yield as colorless crystals: ^{1}H NMR (D_2O) δ 2.14 (m, 2 H, $CH_2CH_2CO_2CH_3$), 2.54 (m, 2 H, $CH_2CO_2CH_3$), 3.14 (s, 9 H, N^+CH_3), 3.39 (m, 2 H, $N^+(CH_3)_3CH_2$), 3.70 (s, 3 H, CO_2CH_3).

Recombinant DNA Techniques and Protein Expression. Recombinant DNA techniques were based on those of Maniatis et al. (1982). The antibody fragments were expressed in *E. coli* JM83 (Vieira & Messing, 1982) by using a vector similar to that described (Skerra & Plückthun, 1988), which contained an f1 phage origin (Vieira & Messing, 1987; Skerra et al., 1991). Site-directed mutagenesis was carried out according to Kunkel et al. (1987) and Geisselsoder et al. (1987). The expression of all mutant proteins was verified by Western blotting with an antibody directed against McPC603. In all cases described here, normal expression and processing of both chains was observed (data not shown), even for those mutant proteins that failed to bind to the affinity column (see below).

Protein Purification. The recombinant antibody fragments were purified by phosphocholine affinity chromatography essentially as described previously (Skerra & Plückthun, 1988) except that the bacterial growth was performed at 20 °C and the cells were induced for 3 h before the harvest. The cells were then disrupted in a French pressure cell and the soluble part of the lysate was directly applied onto the affinity column.

Cross-Linking. The cross-linking with glutaraldehyde was carried out as described previously (Glockshuber et al., 1990). The cross-linked F_v fragment was then purified by affinity chromatography as described above.

Hapten Binding. Hapten binding was followed by recording changes in protein fluorescence as described previously (Glockshuber et al., 1990).

Sequence Numbering. We used the simple consecutive numbering of Davies and co-workers in the crystal structure in the Brookhaven Protein Data Bank, entry 2MCP. This differs from the consensus nomenclature used by Kabat et al. (1987) and some of the older literature. Residues in the heavy chain are suffixed with (h) and those of the light chain with (l).

Structure. The description of the structure of McPC603 is based on the published X-ray crystal structure at 3.0-Å resolution (Segal et al., 1974; Satow et al., 1986; Brookhaven Protein Data Bank entry 2MCP). Very recently, the refinement was continued (E. Padlan and D. Davies, personal communication), but the general conclusions about the hapten binding interactions do not change in the new structure. We refer to the published structure in this paper.

Results and Discussion

Description of the Binding Site. Phosphocholine is bound by the antibody McPC603 with moderate affinity (K_{bind} = (1.6 ± 0.4) × 10^5 M^{-1}; Metzger et al., 1971) in a binding pocket of known structure (Segal et al., 1974, Satow et al., 1986) (Figure 1). The positively charged quaternary ammonium group of phosphocholine is bound by electrostatic and van der Waals forces within a cavity in the protein formed by the hypervariable loops of both variable domains. The phosphate group, which carries two negative charges at pH 8 (Goetze & Richards, 1978; Gettins et al., 1982), where the measurements were carried out, and part of the choline carbon backbone are partially exposed to the solvent. The phosphate is bound by both electrostatic interactions and hydrogen bonds. The orientation of the hapten is mainly determined by the binding of the phosphate group as well as the trimethylammonium group. Since probably little orientational discrimination is contributed from binding of the methylene backbone, a hapten possessing no phosphate group may bind in an orientation somewhat different from phosphocholine but with the quaternary ammonium group at the same position.

The trimethylammonium group of phosphocholine is bound in a cavity within the F_v fragment with walls lined by hydrophobic residues making van der Waals contacts to the methyl groups. Looking from the phosphate group along the methylene backbone of phosphocholine toward the trimethylammonium group, these residues are, in clockwise direction, the heavy-chain residues Trp107(h) and Tyr33(h) and the light-chain residue Tyr100(l) (Figure 1). The backbone carbonyl oxygen of Asp97(l) also contributes a van der Waals contact to a methyl group. Trp107(h), Tyr33(h), and Tyr100(l) all have their ring planes oriented more or less parallel to the axis connecting N and P in choline. While Trp107(h) and Tyr100(l) have their ring planes facing the quaternary ammonium group, Tyr33(h), wedged between the

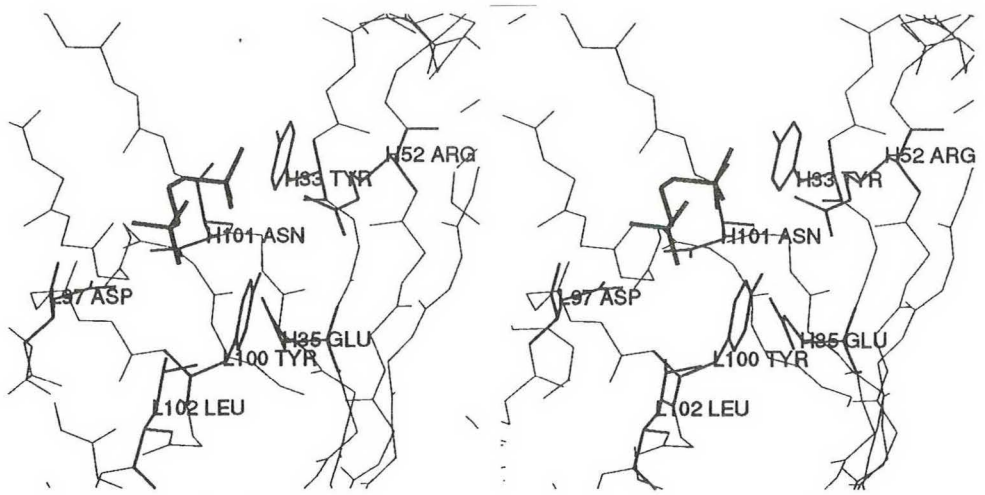

FIGURE 1: Stereoview of the binding pocket of McPC603. Amino acids substituted in this paper are labeled and highlighted by a thicker line. The backbone (N, Cα, C, and O) is shown as thin lines for all other amino acids. The hapten phosphocholine is emphasized by thick lines. The phosphate group lies to the right, the quaternary ammonium group to the left.

Table I: Mutants That Fail To Bind to the PC Affinity Column[a]

light chain	heavy chain
Asp97(l)Leu	Tyr33(h)Arg
Tyr100(l)Phe	Tyr33(h)His
Tyr100(l)Trp	Tyr33(h)Lys
Leu102(l)Tyr	Glu35(h)Gln
Leu102(l)Phe	Arg52(h)Lys
	Asn101(h)Asp

[a] Single mutations were introduced either in the light chain or in the heavy chain.

two, only makes contact to the hapten through one edge of the phenyl ring. The bottom of the binding pocket is formed by the hydrogen-bonded pair Asp97(l) and Asn101(h). The electrostatic interaction of the positively charged quaternary ammonium group with one oxygen of the negatively charged carboxylate group of Asp97(l) (at a distance of 4.8 Å from the choline nitrogen) is probably the strongest force holding the trimethylammonium group in place. A weaker electrostatic interaction may exist to the closest oxygen of the carboxylate group of heavy-chain Glu35(h), 5.8 Å away. Glu35(h) also makes an important hydrogen bond to Tyr100(l), positioning the latter for hydrophobic contact to the trimethylammonium group and defining the wall of the pocket. Van der Waals contacts from a methyl group of Leu102(l) and from the amide nitrogen of Asn101(h) also contribute to the formation of the bottom of the pocket.

Exchange of Amino Acids in Direct Contact with the $N^+(CH_3)_3$ Group. We will now discuss the amino acids in direct contact with the hapten and focus first on those that are thought to be important for binding the trimethylammonium group of phosphocholine (PC). Changing most of the amino acid residues that form the binding pocket for the quaternary ammonium group by mutagenesis abolishes phosphocholine binding (Table I) as defined by the lack of binding to a phosphocholine affinity column. It must be pointed out that the affinity ligand is a phosphodiester (Chesebro & Metzger, 1972) and therefore carries only one negative charge at pH 8.0. Thus, it is possible that some mutations may affect the binding of phosphocholine carrying two negative charges at pH 8.0 (Goetze & Richards, 1978; Gettins et al., 1982)] differently from that of the diester. The most weakly binding mutant protein that can still be purified by phosphocholine affinity chromatography, Tyr33(h)Phe, binds phosphocholine approximately 66-fold less efficiently than the wild type (see below). If column binding and hapten affinity are affected in parallel, this would mean that all mutant proteins that fail to bind to the column must have suffered a more than 66-fold reduction in binding affinity to phosphocholine. We cannot rule out the possibility, however, that some mutants still able to bind phosphocholine fail to bind to the column.

An F_v fragment with Asp97(l) changed to Leu [denoted Asp97(l)Leu] does not bind to the PC column. The simplest explanation for this result is that the electrostatic interaction between the quaternary ammonium group and a negatively charged group is mandatory for PC (**1**) binding. This particular mutation was chosen since leucine occurs at this position in related antibodies that carry the required negative charge in the heavy chain (Perlmutter et al., 1984) at the position corresponding to Asn101(h). We therefore also introduced the mutation Asn101(h)Asp separately. This mutant protein does not bind to the PC column either. A likely explanation for this behavior is the repulsion between the two negative charges in Asp101(h) and Asp97(l). This may lead to a conformational change or even to the failure of V_H and V_L to associate. However, the ability to bind phosphocholine is restored by the double mutation Asp97(l)Leu/Asn101(h)Asp. The binding constant is very similar to that of the wild-type McPC603 (7.8×10^4 M^{-1}, corresponding to a ΔG_{bind} of −27.4 kJ/mol or a reduction of 1.8 kJ/mol compared to the wild type). This clearly shows that the negative charge must be present but may reside either in the light chain (as in McPC603) or in the heavy chain [as encoded in the germ-line heavy-chain gene (Crews et al., 1981) and retained in many antibodies of this class that utilize a different light chain (Perlmutter et al., 1984)].

The mutation Glu35(h)Gln also abolishes binding to the PC column. Gln might in principle be capable of preserving the hydrogen bond required for correctly positioning Tyr100(l), and this mutation would also preserve all steric interactions. The observed loss of binding to PC may be a direct consequence of the removal of the electrostatic interaction of Glu35(h) with the quaternary ammonium group. Alternatively, the uncharged Gln may be unable to position Tyr(l)100 correctly. This would happen if the amide nitrogen and not the carbonyl oxygen of Gln35(h) is positioned closest to the phenolic OH group of Tyr100(l) or if the higher strength of a charged hydrogen bond is required. The importance of the hydrophobic contact between Tyr100(l) and PC is difficult

Table II: Comparison of Wild Type and Tyr33(h)Phe Properties

hapten	McPC603 wild type			Tyr33(h)Phe		
	K_{bind} (10^3 M^{-1})	ΔG (kJ mol^{-1})	ΔF_{max} (%)	K_{bind} (10^3 M^{-1})	ΔG (kJ mol^{-1})	ΔF_{max} (%)
$(CH_3)_3N^+CH_2CH_2OPO_3^{2-}$ (**1**)	160	−29.2	15.0	2.5	−19.1	15.2
$(CH_3)_3N^+CH_2CH_2OSO_3^-$ (**2**)	4.0	−20.2	24.8	6.2	−21.2	20.1
$(CH_3)_3N^+CH_2CH_2CH_2SO_3^-$ (**3**)	7.7	−21.8	27.1	6.4	−21.4	21.4
$(CH_3)_3N^+CH_2CH_2CH_2CO_2^-$ (**4**)	93	−27.9	32.4	19	−24.0	23.5
$(CH_3)_3N^+CH_2CH_2CH_2CO_2CH_3$ (**5**)	1.0	−16.8	32.7	0.85	−16.4	24.5

to separate from its role in aligning Glu35(h) and vice versa. The exact positioning of Tyr100(l) might also be required to keep its hydroxyl group from interacting with Tyr33(h), which would change the position of the latter. Phe in position 100(l), however, abolishes binding to the PC column and would not be expected to interfere with Tyr33(h) even when no longer positioned by Glu35(h). Thus, the relative importance of the hydrophobic interaction to PC from Tyr100(l) and the electrostatic interaction of PC with Glu35(h) cannot be separated by these experiments, since any mutation in these two residues affects both amino acids at the same time. Introducing Trp at position 100(l) does not lead to binding to the PC column, either. All mutations exchanging Tyr100(l) or displacing it, while trying to substitute its hydrophobic bonding function by other aromatic residues (see below), led to nonbinding F_v fragments (Table I). The crucial importance of the interaction between Glu35(h) and Tyr100(l) had also been discussed by Rudikoff et al. (1982), who sequenced a variant of the related myeloma protein S107 that no longer was able to bind PC. The mutant protein was found to have a Glu to Ala substitution at position 35(h).

In an attempt to make new hydrophobic contacts to the quaternary ammonium group while displacing Tyr100(l) such that *it* can now make a hydrogen bond to a phosphate oxygen [in effect sandwiching the tetrahedral phosphate between Tyr33(h) and Tyr100(l)], two further mutant proteins were constructed. In these mutant proteins, Trp and Tyr were substituted for Leu102(l). Neither mutant protein bound to the PC column.

Exchange of Amino Acids in Direct Contact with the Phosphate Group. We will now consider the residues that contribute to phosphate binding. Tyr33(h) makes a hydrogen bond to a phosphate oxygen atom and thus should contribute strongly to PC binding. Accordingly, replacing it with phenylalanine reduces the binding affinity for PC 66-fold, which corresponds to a loss of free energy of binding of 10.1 kJ mol^{-1} (Table II). It was not possible to make up for the binding energy contributed by this hydrogen bond by introducing either arginine or lysine at position 33(h). These substitutions constituted an attempt to improve the binding of the phosphate in PC (**1**), which carries two negative charges. These residues may be thought to provide both electrostatic interactions and hydrogen bonding contributed by the guanidinium or ϵ-ammonium group. Neither mutant protein bound to the PC column. This failure to bind may be due to the repulsion of like charges in Arg52(h) and the newly introduced positively charged residue at position 33(h), leading to a significant conformational change. We must also consider the possibility, however, that the behavior toward the diester ligand may be different than that toward PC. Histidine in position 33(h) did not lead to binding to the PC column, either, although it should not be positively charged at pH 8.0. By model building starting from the known wild-type structure, histidine might be thought to be able to make a hydrogen bond to phosphate, depending on the orientation of the imidazole ring. The specific importance of Tyr33(h) for PC binding in McPC603 as evidenced by mutagenesis is at variance with calculations by Novotny et al. (1989), who predicted a *positive* free energy of binding contributed by this residue.

Arg52(h) makes both a hydrogen bond and an electrostatic contact to phosphate. It cannot be replaced by lysine without loss of binding to the PC column (Table I) although Arg and Lys should both be protonated at pH 8. In the current structure, the electron density seems incompatible with a bidentate binding of two peripheral oxygens of phosphate by the two NH groups of the guanidinium group (E. Padlan and D. Davies, personal communication). The importance of arginine must then lie in a better steric fit or a better orientation imposed on arginine by an interaction with close-by Glu61(h).

Properties of Tyr33(h)Phe. To clarify the nature of interactions of the phosphate group with the protein, binding constants of a range of analogous haptens were determined for both the wild-type F_v fragment and the Tyr33(h)Phe mutant protein (Figure 2). Of the haptens tested (for formulas, see Table II), choline sulfate [henceforth called sulfate (**2**)] and 3-(trimethylammonium)propane-1-sulfonate [sulfonate (**3**)] preserve the tetrahedral geometry of the phosphate group while carrying only one negative charge. The corresponding carboxylic acid group in 4-(trimethylammonium)-butyric acid [henceforth called the acid (**4**)] and its methyl ester (**5**) are planar and carry one or no negative charges, respectively. All haptens contain the positively charged trimethylammonium group.

Of the haptens tested, PC (**1**) carrying two negative charges shows the tightest binding to the wild-type F_v fragment, interestingly followed by (trimethylammonium)butyric acid (**4**), with only one negative charge, displaying a binding energy of only 1.3 kJ mol^{-1} less (Table II). These are also the haptens most strongly affected by removal of the hydrogen bond donor Tyr33(h), with their binding energies lowered by 10.1 and 3.9 kJ mol^{-1}, respectively. With only 1.3 kJ mol^{-1} binding energy difference between the haptens **1** and **4**, carrying one or two negative charges, electrostatic interaction, while important, does not seem to be the decisive factor for the discrimination of the various haptens by McPC603. In the absence of a high-resolution structure of the complex with the carboxylic acid, we must consider the possibility of a bidentate binding of both carboxylate oxygens to the guanidinium NH-groups of Arg52(h) for the carboxylic acid (**4**).

Choline sulfate (**2**) and 3-(trimethylammonium)propane-1-sulfonate (**3**) bind to the Phe33(h) mutant by 9.0 and 7.4 kJ mol^{-1} more weakly than PC (**1**) to the wild-type F_v fragment. They have a geometry almost identical with that of PC and only one negative charge on the sulfate or sulfonate group. Their weaker binding may be due to unfavorable (or even the complete absence of) hydrogen bonding between the slightly larger sulfate and Tyr33(h). In accordance with this hypothesis, Tyr33(h) removal does not much affect sulfate (**2**) or sulfonate (**3**) binding, and sulfate (**2**) binding is even slightly improved by 1.0 kJ mol^{-1}. Modifying the hapten by exchanging a sulfate oxygen for a sulfonate methylene group has very little influence on its binding. (Trimethylammonium)-butyric acid methyl ester (**5**) is uncharged at this site and binds most weakly to F_v (a loss of 12.4 kJ mol^{-1} relative to PC), but

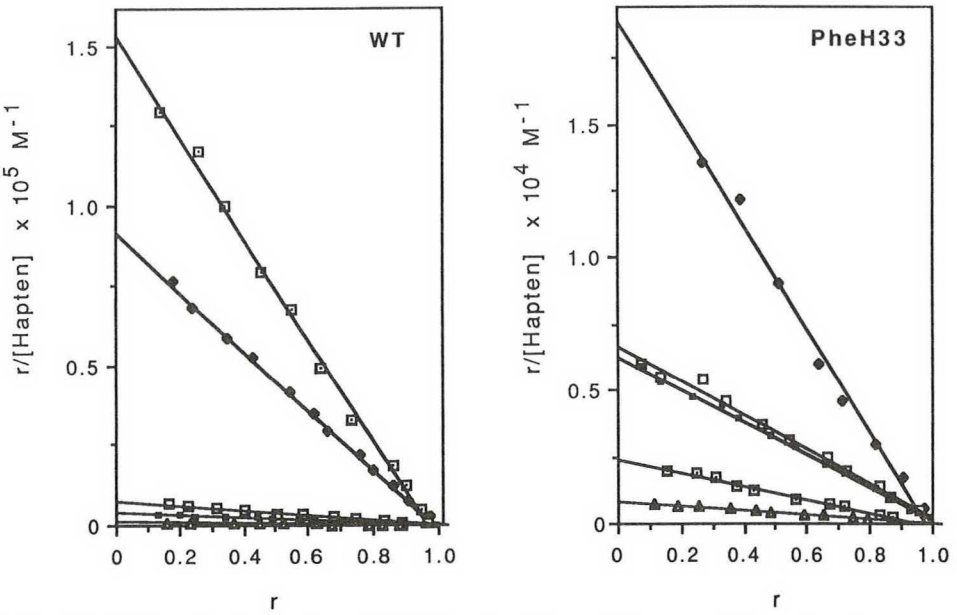

FIGURE 2: Scatchard plots of the binding of various antigens (Table II) to the wild-type F_v fragment (left panel) or the mutant protein Tyr33(h)Phe (right panel) obtained by fluorescence measurements. Note the different scale in the two panels. *r* denotes the fraction of the antibody with a bound ligand, and [hapten] the molar concentration of free hapten. In both panels, ⊡ denotes phosphocholine (**1**), ◆ 4-trimethylammoniumbutyric acid (**4**), □ 3-trimethylammoniumpropane-1-sulfonate (**3**), ■ choline sulfate (**2**), and △ 4-trimethylammoniumbutyric acid methyl ester (**5**).

only 3.4 kJ mol^{-1} more weakly than choline sulfate (**2**). Its binding is also only slightly affected by removal of Tyr33(h). All three haptens that are more weakly bound may be unable to form a hydrogen bond to Tyr33(h) and may be less stably aligned in the binding pocket. One must even consider the possibility that they are bound in an orientation somewhat different from PC (**1**).

We also measured the magnitude of the increase in fluorescence of the F_v–hapten complex compared to the uncomplexed F_v fragment for all five haptens in the wild type and the Tyr33(h)Phe mutant protein. While the fluorescence changes tend to be smaller in the mutant protein, the order of relative fluorescence changes for the various haptens is identical in both the mutant and the wild-type protein. This suggests that Tyr33(h) provides only a small contribution to the observed fluorescence increase. This increase is smallest for PC (**1**), followed by sulfate (**2**) and sulfonate (**3**), and it is largest for acid (**4**) and ester (**5**). As the acid (**4**) and the ester (**5**) both show very similar relative fluorescence changes, the overall polarity of the hapten cannot be the decisive factor for the magnitude of the fluorescence increase. Also, there is no correlation between the binding constant and the magnitude of the fluorescence increase in the F_v–hapten complex.

Conclusions. The binding of haptens in the cavity of the antibody McPC603 is subject to strict geometrical limitations, as seen by the much decreased binding of the slightly larger choline sulfate (**2**) and sulfonate (**3**) when compared to phosphocholine (**1**). The hydrogen-bonding geometry for these haptens to Tyr33(h) is probably rendered unfavorable to the point that this hydrogen bond is no longer formed. The importance of residues Asp97(l) and Tyr100(l) of the light chain and Arg52(h), Tyr33(h), and Glu35(h) of the heavy chain for phosphocholine binding as proposed from the structure of the antibody F_{ab}' fragment is confirmed by mutational analysis. The quaternary ammonium group needs one negative charge at the bottom of the pocket to interact with. This charge may reside in either chain [at 97(l) or 101(h)], but the presence of both negative charges leads to a protein that fails to bind to the affinity column. A more complete understanding of these phenomena will require the determination of the structure of the mutated proteins. The present study should be the first step toward more quantitatively understanding the differences in hapten binding profiles shown by various members of this family of anti-phosphocholine antibodies (Gearhart et al., 1981; Cook et al., 1982; Perlmutter et al., 1984; Claflin et al., 1985, 1987).

The current study begins to elucidate quantitative contributions to hapten binding interactions in the antibody of known structure, McPC603. This antibody (in the form of the F_v fragment expressed in *E. coli*) was shown to catalyze the hydrolysis of *p*-nitrophenyl choline carbonate (Plückthun et al., 1990). The same activity had been demonstrated with the related PC binding myeloma proteins T15 and M167 (Pollack et al., 1986; Pollack & Schultz, 1987). The current binding analysis can now be correlated with a kinetic analysis of some of these mutant proteins (J. Stadlmüller and A. Plückthun, manuscript in preparation) and should provide a deeper insight into the structural requirements of catalytic activity as well as pointing out regions of the protein where changes may be carried out without eliminating binding.

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