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Characterization of the Combining Site of Mouse Myeloma Protein M315

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ABSTRACT: The interaction of M315 with 2,4-dinitrophenyl haptens was studied. 2,4-Dinitroaniline (DNP-NH_2) showed maximum affinity to M315 at about pH 4. The pH dependence of the association constant of DNP-NH_2 to M315 showed three transitions at pH 4.7, at pH 7.2, and below pH 9, respectively. Since the DNP-NH_2 molecule has no charged group in this pH range, the transitions were explained in terms of amino acid residues with ionizable side chains in M315. Judging from the pK values and the effect of succinylation, these transitions were concluded to be related to ionizations of carboxyl, imidazole, and phenol groups, respectively. Measurement of the fluorescence of affinity-labeled M315 suggested that the transition at pH 4.7 reflected an equilibrium between two forms of M315 with different conformations of the combining site. The contribution of the amino acid sequence on the light (L) chain to the interaction with haptens was studied by use of antibodies (Abs) reconstituted from the heavy chain of M315 (H^{315}) and either a homologous or a heterologous L chain. The reconstituted heterologous Ab ($H^{315}L^{952}$) showed similar pH dependence of binding to DNP-NH_2 to that of the homologous Ab ($H^{315}L^{315}$). Moreover, the two Abs showed no appreciable difference in binding to DNP-haptens of different sizes. These results suggested that the difference in the amino acid sequences of L^{315} and L^{952}, which originated by a somatic hypermutation, has little effect on the ligand binding. The inability of the somatic hypermutation to increase the affinity of M315 to DNP-haptens, which are artificial antigens, was explained in terms of lack of stimulation and selection by DNP, because M315 is a myeloma protein secreted by naturally occurring plasmacytoma, MOPC-315.

The interaction of the mouse myeloma protein M315 with haptens has been studied in detail (Haselkorn et al., 1974). The three-dimensional structure of M315 has not yet been reported, so Padlan et al. (1976) constructed a model of its combining site with hapten on the basis of results of chemical modification and kinetic studies on the interaction of M315 and DNP-haptens,[1] assuming that it has the same backbone

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[1] Abbreviations: DNP, 2,4-dinitrophenyl; DNP-NH_2, 2,4-dinitroaniline; DNP-Gly, (2,4-dinitrophenyl)glycine; DNP-Ala, (2,4-dinitrophenyl)-β-alanine; DNP-But, γ-[(2,4-dinitrophenyl)amino]butyric acid; DNP-Cap, ϵ-[(2,4-dinitrophenyl)amino]caproic acid; CDR, complementarity-determining region; CD, circular dichroism; Q_{obsd}, observed quenching; Q_{max}, maximum quenching; K, association constant; H^{315}, heavy chain of M315; L^{315}, light chain of M315; L^{952}, light chain of T952.

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conformation as M603, for which X-ray crystallographic data were available (Davies et al., 1975). Dwek and co-workers (Dwek, 1977) refined this model on the basis of NMR and ESR studies.

Azuma et al. (1984) studied the effect of amino acid substitution in the L chains on the affinity of M315 to DNP-haptens by preparing reconstituted antibodies (Abs) from H^{315} and various λ chains. They showed that replacement of an amino acid residue at the boundary of the Vλ2–Jλ2 gene segments (position 98) greatly affected the ligand-binding activity: replacement of Phe by Tyr or Trp resulted in a more than 1000-fold decrease in DNP-binding activity. This strict requirement for a particular amino acid residue at position 98 was nott predicted from the previous model. Thus, more information on the hapten-binding site is needed to understand the specificity and affinity of Abs at a molecular level.

In this paper, we first studied the interaction of M315 with DNP-NH_2 to determine the contribution of ionizable amino acid residues to the hapten binding. This information was essential for understanding the microenvironment of the combining site. The important role of the amino acid residue at position 98 is firmly established, but the importance of other residues in the third complementarity-determining region (CDR) is unknown. Therefore, we next examined the contribution of other amino acid residues than residue 98 in the third CDR to the ligand-binding activity. For this purpose we prepared two reconstituted Abs using H^{315} and L^{315} or L^{952} and examined their abilities to bind haptens. The sequences of these λ2 chains differ in five positions, most of which are around the third CDR. Since three of four amino acid substitutions of L^{315} by somatic hypermutation are clustered in the third CDR, these experiments also clarified the effect of somatic hypermutation on the ligand-binding activity of M315.

Materials and Methods

Proteins. The mouse myeloma proteins M315 and T952 were purified as described by Cotner et al. (1981). The H and L chains were isolated and reconstituted into Abs as described previously (Azuma et al., 1981, 1984).

Reagents. DNP-glycine (DNP-Gly), DNP-β-alanine (DNP-Ala), and γ-(DNP-amino)butyric acid (DNP-But) were prepared as described previously (Haselkorn et al., 1974). ε-(DNP-amino)caproic acid (DNP-Cap) and DNP-NH_2 were obtained from Sigma Chemical Co. (St. Louis, MO). 2,4-Dinitrophenyl 1-azide was synthesized as described by Yoshioka et al. 1973). The molar extinction coefficients used to determine the concentrations of haptens were as follows: 17 800 for DNP-Cap, 15 890 for DNP-Gly, 17 940 for DNP-Ala, 17 200 for DNP-But, and 13 600 for DNP-NH_2.

Photoaffinity Labeling. A sample of 5 mL of M315 (1 mg/mL) in 0.2 M Tris-HCl, at pH 8, was treated with a 20 molar excess of 2,4-dinitrophenyl 1-azide with respect to the concentration of the binding site. The reaction mixture was irradiated from 750-W tungsten lamp for 90 min at 25 °C. The temperature of the reaction vessel was controlled with a water jacket. After the reaction, affinity-labeled M315 was purified by Sephadex G-25 and ε-DNP-lysine–Sepharose 4B column chromatographies. About 2 mol of DNP was attached per M315 molecule as estimated by spectrophotometry.

Succinylation. M315 was treated with a 20 molar excess of succinic anhydride over Lys residues of M315 in 0.2 M $NaHCO_3$ at pH 9 (Habeeb et al., 1958). Portions of solid reagent were added at 20-min intervals, and the solution was maintained at about pH 9 by addition of 0.1 N NaOH. The reaction mixture was then treated with 1 M NH_2OH for 1 h. The extent of succinylation was determined by titration of free Lys with 2,4,6-trinitrobenzenesulfonic acid. Under these conditions, 63–67% Lys residues were succinylated.

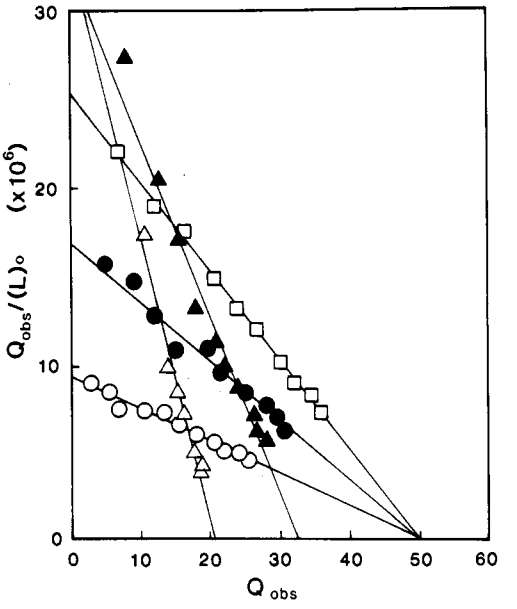

FIGURE 1: Scatchard plots of data on fluorescence quenching according to eq 1 on binding of DNP-NH_2 to M315 at various pH values at 25 °C (see text for details). Δ, pH 4.47; ▲, pH 4.72; □, pH 5.46; ●, pH 6.70; ○, pH 7.95.

Circular Dichroism (CD). CD measurements were carried out in a Model J-20 spectropolarimeter equipped with a CD attachment (Japan Spectroscopic Co., Tokyo) at 25 °C. Results are expressed as observed molar ellipticities, $[\theta]_{obsd}$. Measurements were made with change in pH at constant concentrations of protein and DNP-NH_2 (13 and 36 μM, respectively).

Fluorescence Measurements and Ligand-Binding Assay. The fluorescence quenching method was employed to measure the affinity of intact M315 and reconstituted Abs to DNP-haptens (Eisen & McGuigan, 1971). The quenching of protein fluorescence as a result of binding of DNP was monitored with a Hitachi-MB4 spectrofluorometer (excitation, 280 nm; emission, 340 nm). Measurements were made at protein concentrations of less than 50 nM at 25 °C.

The binding equilibrium of a ligand and a combining site of Ab was analyzed by the Scatchard equation

$$Q_{obsd}/(L) = K(Q_{max} - Q_{obsd}) \quad (1)$$

where Q_{obsd} and Q_{max} are the observed and maximum fluorescence quenching, K is an association constant, and (L) is the free ligand concentration. When the concentration of the ligand–Ab complex is much smaller than the total ligand concentration, $(L)_0$, we can use $(L)_0$ instead of (L) in eq 1.

Results

Figure 1 shows plots of $Q_{obsd}/(L)_0$ vs Q_{obsd} according to eq 1 for data on the interaction of M315 with DNP-NH_2 at selected pH values. Since plots at each pH fall on straight lines, it is clear that the assumption that $(L) = (L)_0$ is valid. These plots gave the values shown in Figure 2 for K and Q_{max} at the repective pH values. It is clear that M315 shows maximum affinity for DNP-NH_2 at about pH 4 and that on an increase in the pH the affinity decreased. Three transitions in the pH–log K profile are evident, but the end of the transition in the alkaline pH region is not clear due to overlap of alkaline denaturation. The midpoints of these transitions are at about pH 4.7, 7.2, and below 9. The values of K for DNP-Ala and DNP-Cap changed with pH similarly to that of DNP-NH_2, although they were larger than that of DN-

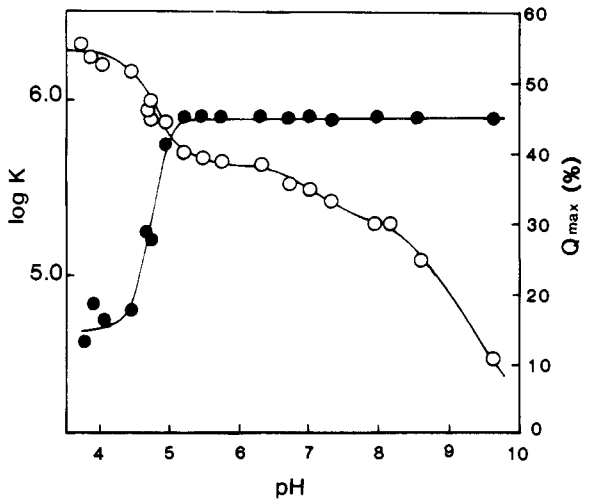

FIGURE 2: pH dependence of K and Q_{max} in the interaction of M315 with DNP-NH_2. ○, K; ●, Q_{max}. Data on fluorescence quenching were analyzed by eq 1.

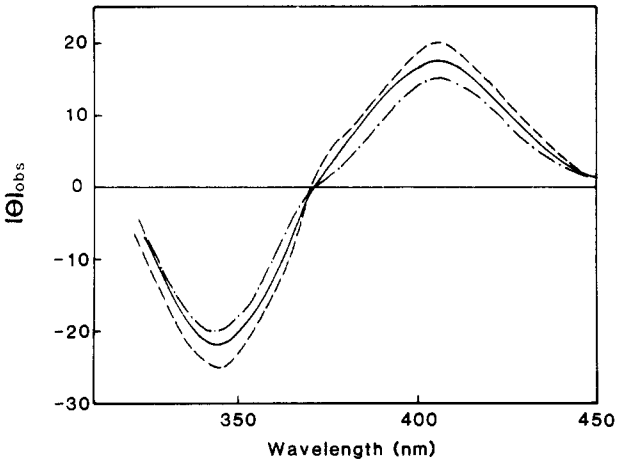

FIGURE 3: CD spectra of the complex of M315 with DNP-NH_2 at pH 4.2 (---). The inset shows first-order plots of the change of fluorescence on shift from pH 8 to pH 4 (●) and from pH 4 to pH 8 (○).

P-NH_2 over the pH range studied (data not shown). Thus, it is clear that the site that accommodates the dinitroanilino group of these haptens determines the pH dependence of the interactions.

The values of Q_{max} give additional information on the interaction. As shown in Figure 2, only the transition at pH 4.7 was associated with change of Q_{max}. Since we measured the protein fluorescence originating from a Trp residue(s), change of Q_{max} may reflect alteration of some special relation between DNP and Trp and suggests that the transition at pH 4.7 reflects conformational change of the combining site.

To test whether these changes in the combining site could be detected by other methods than fluorescence quenching, we first measured the CD spectra of the mixture of M315 and DNP-NH_2. Neither free DNP-NH_2 nor M315 showed any detectable CD at wavelengths of above 330 nm, but the mixture showed CD bands with a positive maximum at about 410 nm and a negative maximum at about 350 nm (Figure 3). These CD bands were known as an extrinsic Cotton effect and are explained in terms of binding of the optically inactive DNP-NH_2 molecule to an asymmetric environment in the Ab. The shape, position, and crossover point of the CD bands of all pH values were similar, suggesting similarity in the environment around the bound hapten at different pH values. The variation in the magnitude is a reflection of change in the amount of M315–DNP-NH_2 complex due to the pH dependence of the association constant. These facts suggest that the change in the microenvironment of the combining site associated with the transition at pH 4.7 may not be sufficiently large to detect by CD. We were also unable to detect change of protein fluorescence corresponding to the transition at pH 4.7 (data not shown), so the conformational change of the combining site with pH can probably be detected only by the fluorescence quenching method.

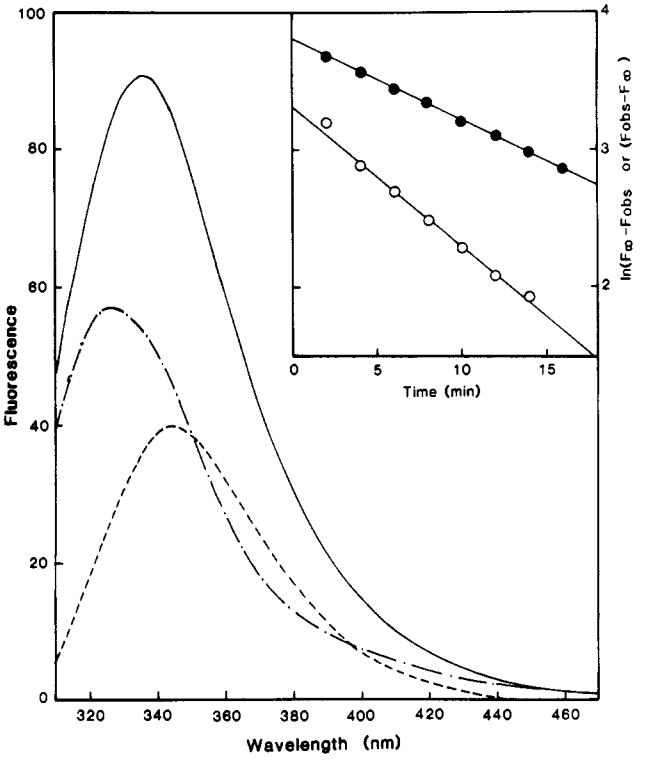

FIGURE 4: Fluorescence spectra of the M315–DNP complex at pH 4 (—) and pH 8 (-·-) and their difference spectrum (---). The inset shows first-order plots of the change of fluorescence on shift from pH 8 to pH 4 (●) and from pH 4 to pH 8 (○).

For further examination of the transition at pH 4.7, DNP was conjugated covalently to the combining site of M315 by the photoaffinity labeling method, and fluorescence spectra at various pH values were measured. In this case the DNP group was expected to act as a reporter of conformational change. Between pH 5 and pH 9 slight variation with pH was seen, but the spectra were essentially similar and showed no time-dependent change with a shift of pH. However, below pH 5 the spectra changed markedly (data not shown). Figure 4 shows the fluorescence spectra at pH 4 and 8 and their difference spectrum. The difference spectrum was similar to the fluorescence spectrum of tryptophan, which has a peak at 343 nm. Upon the shift from pH 8 to pH 4 or vice versa, the fluorescence at 343 nm changed slowly. This change was reversible and followed first-order kinetics, with rate constants of 9.8×10^{-4}/s (pH 8 → pH 4) and 1.7×10^{-3}/s (pH 4 → pH 8). As expected from the pH dependence of Q_{max}, the fluorescence change of the M315–DNP complex on shift of the pH was observed only around pH 4.7 corresponding to the transition point.

To obtain information about amino acid residues participating in the transition below 9, we succinylated M315 and examined the interaction of the derivative with DNP-Cap (Figure 5). Since succinylated M315, M315 (Suc), was unstable in alkali, only limited information could be obtained, but results showed that succinylation did not seriously affect the interaction with DNP-Cap. Thus amino acid residues other than Lys are probably responsible for this transition.

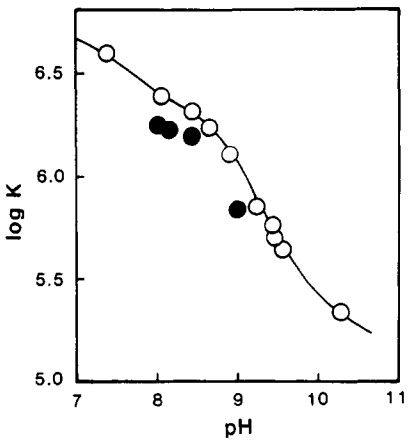

FIGURE 5: pH dependence of K for the interaction of M315 (○) and M315(Suc) (●) with DNP-Cap at 25 °C.

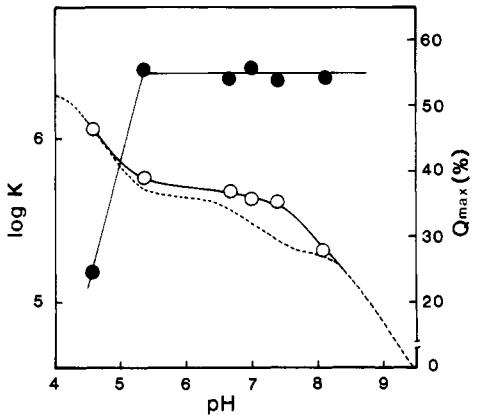

FIGURE 6: pH dependence of K (○) and Q_{max} (●) for the interaction of $H^{315}L^{952}$ with DNP-NH_2. The corresponding profile of K for M315 is shown by the broken line.

Next we examined amino acid substitution of L chains affected the ligand-binding activity. For this we reconstituted homologous and heterologous Abs ($H^{315}L^{315}$ and $H^{315}L^{952}$, respectively). L^{952} and L^{315} are λ_2 subtypes with the same Vλ and Jλ gene segments. Compared with the germ line sequence, L^{952} has a mutation at position 99 while L^{315} has mutations at positions 38, 94, 95, and 96 (Dugan et al., 1973; Elliott et al., 1984). Since H^{315} is common to both reconstituted Abs, results provided information about the effect of amino acid substitution in L chains on the function. As reported previously (Azuma et al., 1984), $H^{315}L^{315}$ showed the same ligand-binding activity as intact M315 (data not shown). The pH dependence of the interaction of $H^{315}L^{952}$ with DNP-NH_2 is shown in Figure 6. The profile of pH–log K was very similar to that of M315, except that the small transition at about pH 7.2 was not seen. This suggests that the transitions at about pH 4.7 and 9 are not related with the amino acids that are different in the two L chains. Judging from its pH value, the transition at pH 7.2 seems to reflect ionization of His. But none of the substitutions in L^{952} is of a His residue. Thus, it is probable that another (other) substitution(s) affects (affect) the microenvironment of a His residue and that this causes a small difference in the pH dependence of K.

Table I shows the dependence of the association constants and Q_{max} values of the reconstituted Abs on the length of the aliphatic side chains of DNP-haptens. DNP-Gly showed lower affinity and a smaller Q_{max} than DNP-NH_2. Thus, the side chain of DNP-Gly contributed negatively to the binding. This negative effect could be overcome by increase in the number of methylene groups from DNP-Gly to DNA-Ala. A further lengthening of the side chain from DNP-Ala to DNP-But

Table I: Dependence of Association Constants and Q_{max} Values of Reconstituted Abs on the Lengths of the Side Chains of DNP-haptens

hapten	assocn const ($\times 10^5$ M^{-1})		Q_{max} (%)	
	$H^{315}L^{315}$	$H^{315}L^{952}$	$H^{315}L^{315}$	$H^{315}L^{952}$
DNP-NH_2	1.9	3.2	50	55
DNP-Gly	1.6	2.1	35	36
DNP-Ala	17	21	65	65
DNP-But	39	47	65	67
DNP-Cap	39	47	64	65

resulted in a small increase in K but not in Q_{max}. Hydrophobic interaction of the methylene group thus seems to increase the affinity. $H^{315}L^{952}$ showed slightly higher affinity to all haptens than $H^{315}L^{315}$, reflecting the difference in pH dependence at about pH 7.2.

Discussion

In this work, studies on the pH dependence of hapten binding to M315 provided some information about the amino acid residues with ionizable side chains that affect the binding of haptens. It is rather surprising that DNP-NH_2, a small, simple hapten with no charged group, showed a complicated pH dependence of interaction with M315. These were at least three transitions in the pH–log K profile, indicating that the combining site involves several amino acid residues with ionizable side chains and that change of the hydrogen ion equilibrium of a few amino acid residues results in a large change in affinity to hapten. M315 showed maximum affinity to DNP-NH_2 at pH 4, which was ten times that at pH 7. Rao et al. (1982) reported that a human monoclonal IgM specific for *Klebsiella* with pyruvylated galactose showed optimum precipitation with *Klebsiella* K21 antigens at pH 4. This may be due to an increase in the affinity at lower pH values. It is therefore likely that the higher Ab affinity at acidic pH than at neutral pH is not unique to M315. The biological significance of this phenomenon in vivo is unknown.

The amino acid residues responsible for the transition at pH 4.7 may be Asp or Glu. Since this transition was seen in the pH–log K profile of $H^{315}L^{952}$, it should be due to amino acid residues common to $H^{315}L^{315}$ and $H^{315}L^{952}$. Asp101H is one candidate (Dwek, 1977). This transition was different from other transitions in that it was associated with a change of Q_{max}. This suggests a change of special orientation on DNP and a Trp residue at about this pH. In other words, the transition may be related to an equilibrium between M315 molecules in which the combining sites have different conformations. The fluorescence change of the M315–DNP complex on shift of pH followed first-order kinetics with slow rate constants. Since hydrogen equilibrium should be rapid, a step subsequent to protonation or deprotonation may be rate determining. Probably protonation of a carboxyl group induced conformational change of the combining site with a slow rate of reaction, which resulted in higher affinity at acidic pH. A similar slow rate of reaction of the Fv fragment of M315 with lanthanides was reported by Dwek et al. (1976). They also suggested the presence of two conformers with different affinities to lanthanides and slow rates of interconversion. The slow pH-dependent interconversion can also be interpreted in terms of isomerization of two conformers, though a retarding effect of covalent attachment of DNP to the combining site on the rate of reaction cannot be ruled out.

The transition at pH 7.2 may be due to a His residue judging from its pH value. Wain-Hobson et al. (1977) reported that one of three His residues with a pK value of 6.9 was perturbed by binding of DNP-haptens. There is no dif-

ference in the locations of His residues in L^{315} and L^{952}, so the absence of a transition at pH 7.2 in the pH–log K profile of $H^{315}L^{952}$ could be interpreted in terms of a secondary effect of amino acid substitution on the environment around a His residue. Wain-Hobson et al. (1977) concluded that this His was 102H, but we consider that it is His97L because most of the differences between the amino acids in L^{315} and L^{952} are in positions around this residue (94, 95, 96, and 99). Previous findings by Azuma et al. (1984) provide further evidence for the present assignment. They prepared reconstituted Abs from H^{315} and λ_3 chains (L^{5-8} and L^{c-49}). These λ_3 chains differed in amino acid sequences only at position 97 (His in L^{5-8} and Asn in L^{c-49}) (Reilly et al., 1984). Both $H^{315}L^{5-8}$ and $H^{315}L^{c-49}$ showed ability to bind to ϵ-DNP-lysine, but the affinity of $H^{315}L^{c-49}$ was about half that of $H^{315}L^{5-8}$, indicating that His97L is important, but not essential, for ligand binding.

The transition below pH 9 was seen in the profiles of both $H^{315}L^{315}$ and $H^{315}L^{952}$. The ionizable group responsible for this transition could be Tyr or Lys. Haimovich et al. (1972) reported that N^{α}-(bromoacetyl)-N^{ϵ}-DNP-L-lysine and N-(bromoacetyl)-N'-DNP-ethylenediamine reacted with Lys52H and Tyr34L, respectively. On the other hand, Klostergaard et al. (1977) showed by maleoylation of M315 that Lys52H did not contribute directly to ligand-binding activity. The present results support this finding of Klostergaard et al. (1977) because succinylated M315 was similar to intact M315 in terms of affinity and pH dependence of K. On the other hand, discrepant results have been obtained on the role of Tyr34L in hapten binding. Klostergaard et al. (1978) reported that iodination of Tyr caused a marked decrease in affinity to DNP-lysine, whereas Gavish et al. (1979) observed no effect of nitration of Tyr34L on the ligand-binding activity. Since the contribution of Lys could be excluded, a Tyr other than Tyr34L may be responsible for DNP-binding activity. In this case, Tyr may act as a hydrogen donor to the nitro group of DNP for hydrogen bonding, because an increase of pH decreased the affinity.

The difference in the ligand-binding activities of $H^{315}L^{315}$ and $H^{315}L^{952}$ was small and was seen only marginally in the pH–log K profile. In addition, there was probably no difference in the sizes of their combining sites, judging from binding studies using DNP-haptens with different lengths of side chains (Table I). Thus the different amino acids in the sequences of L^{315} andd L^{952} probably do not contribute appreciably to the ligand-binding activity. The substitutions of amino acid residues from the germ line sequence occurred at position 99 in L^{952} and at positions 34, 94, 95, and 96 in L^{315}. They were probably generated by somatic hypermutation. Although substitutions were seen in or near the third CDR region, their contribution to the ligand-binding activity was extremely small. This is in contrast to substitution at the Vλ–Jλ boundary (position 98), where replacement of Phe by Tyr resulted in an about 1000-fold decrease in affinity to DNP-Cap (Azuma et al., 1984). These facts suggest that relative contributions of amino acid residues to ligand-binding activity differ greatly even when these residues are present in the same CDR region. In this context, the reported contribution of Arg96L to ligand binding (Padlan et al., 1976) is suspicious, since L^{952} has Asn at this position instead of Arg.

The fact that amino acid substitutions generated by somatic hypermutation had no apparent effect on the ligand-binding activity of M315 does not necessarily mean that the somatic hypermutation mechanism is ineffective for increasing Ab affinity in general. There is evidence suggesting a significant role of somatic mutation in the immune response (Tonegawa, 1983; Griffiths et al., 1984; Azuma et al., 1987). It is clear, however, that not all amino acid substitutions contribute to Ab functions. Some of them, such as those in L^{315}, must be functionally silent.

ACKNOWLEDGMENTS

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Registry No. DNP-Gly, 1084-76-0; DNP-Ala, 3185-97-5; DNP-But, 10466-75-8; DNP-Cap, 10466-72-5; DNP-NH_2, 97-02-9.

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