Volume 309, number 1, 85–88 FEBS 11483 August 1992

The His-probe method: effects of histidine residues introduced into the complementarity-determining regions of antibodies on antigen–antibody interactions at different pH values

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Received 5 June 1992

We examined the effects of histidine residues that were artificially introduced into complementarity-determining regions of antibodies on antigen–antibody interactions at different pH values. Using a monoclonal antibody specific for hen egg-white lysozyme and three mutant antibodies that contained a histidine residue, we measured binding constants for antibodies and lysozyme at different pH values (pH 5–8). No gross conformational changes were evident over this range of pH values, as determined by analysis of the spectra of circular dichroism. Since the charge on a histidine residue is the most likely factor that can vary over this range of pH values, differences on pH-dependent antigen-binding patterns observed between the wild-type and mutant antibodies should be due mainly to the effects of the charges on the histidine residues. The three mutant antibodies showed different and characteristic patterns of pH-dependent binding to lysozyme, which depended on the location of the artificially introduced histidine residues.

Binding constant; Site-directed mutagenesis

1. INTRODUCTION

In the interaction between an antigen (Ag) and its specific antibody (Ab), physical and structural complementarity of the contact surfaces of both molecules plays a crucial role. The Ag–Ab complex is stabilized through van der Waals, electrostatic and hydrophobic interactions, in addition to hydrogen bond formations (for review see [1]). Since such interactions, which are both attractive and repulsive, can participate simultaneously in Ag–Ab interactions, it is hard to estimate the extent of the contribution of particular interactions to the total binding energy [2].

To estimate effects of charge, utilization of mutant proteins into which histidine residues are introduced would be promising, because the p*K*a values of the side chains of free acidic and basic amino acids are as follows [3]: glutamate 4.3 (4.4–4.6); aspartate 3.9 (4.4–4.6); lysine 10.5 (10.0–10.2); arginine 12.5 (≥12); histidine 6.0 (6.5–7.0); tyrosine 10.1 (9.6–10.0). When these amino acids are embedded in a protein, they can have various p*K*a values depending upon their electrostatic environments, as shown in parentheses [4]. The effects of charges on histidine residues can be evaluated by the pH dependency observed over the range from pH 5 to 8. Sali et al. [5] reported the pH dependency of stability and folding of proteins with histidine residues. In this study, we examined the effects of histidine residues that were introduced artificially into complementarity-determining regions (CDR) of Abs on Ag–Ab interactions at different pH values. The pattern of pH-dependent binding of one mutant Ab was dramatically different from that of the wild-type.

Abbreviations: Ab, antibody; Ag, antigen; CDR, complementarity-determining region; HEL, hen egg-white lysozyme; ELISA, enzyme-linked immunosorbent assay; CD, circular dichroism.

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2. MATERIALS AND METHODS

2.1. *Cloning, sequencing and site-directed mutagenesis*

A monoclonal Ab, Hyb·C1 [6], specific for hen egg-white lysozyme (HEL), and HEL were used in this study. Isolation of the V_H and V_κ genes that encode Hyb·C1 was carried out as described elsewhere [7]. DNA sequencing was carried out by the dideoxy chain-termination method [8]. Mutations were introduced by the published method [9].

2.2. *Expression and purification of antibodies*

The DNA fragments containing the genes for V_H and V_κ were ligated with DNA fragments that contained mouse genes for C_γ and C_κ, and inserted into pSV2gpt and pSV2neo [7], respectively. The DNAs were simultaneously transfected into SP2/0 myeloma cells by electroporation [10]. The transformed cells were selected by their resistance to mycophenolic acid at 5 μg/ml. The efficiency of the transformation was around 300–400 transformants per 2×10^7 cells. Production of Abs was measured by enzyme-linked immunosorbent assay (ELISA) using Ab against mouse IgG (Cappel) [11]. After limiting

dilution, the transformants that secreted Abs were established. Binding activities of the Abs secreted into the medium from the cells were also measured by ELISA. For further analysis, Abs were purified from ascites by precipitation in 50% $(NH_4)_2SO_4$ and chromatography on a lysozyme affinity column.

2.3. *Circular dichroism (CD) spectrum*

CD was measured at 26°C with a Model JASCO J-500. Quartz cells with a path length of 1 mm and a concentration of protein of 0.48 mg/ml in 0.2 M NaCl were used for determination of the CD spectra of Abs between 205 and 260 nm. pH titration was carried out by adding 0.2 N HCl.

2.4. *Measurement of binding constants*

Binding between [^{3}H]HEL and Ab was measured as described elsewhere [6]. [^{3}H]HEL was prepared by labelling HEL (SIGMA) with [^{3}H]acetic anhydride. All the experiments were carried out at 25°C.

3. RESULTS

3.1. *Production of the wild-type and mutant antibodies*

Fig. 1 shows the nucleotide sequences of the V_H and V_K genes that encode Hyb·C1. These sequences are in agreement with the N-terminal amino acid sequences of the V_H and V_K domains of Hyb·C1 that were reported previously [6]. The sequences are different from those of other lysozyme-specific monoclonal Abs, HyHEL5, HyHEL10 and D1.3 [12–14].

Histidine residues were introduced into various sites in the amino acid sequences by site-directed mutagenesis. At twelve positions, the original amino acids were replaced with a histidine residue, to generate twelve mutants: L1A, L1B, L2A, L2B, L3A, H1A, H2A, H2B, H3A, H3B, H3C, H3D (L1A: light chain, CDR1, number A). In addition, His-34 of the L chain was changed to a tyrosine residue, referred to as L1C. All the wild-type and mutant genes were inserted into expression plasmid DNAs. Two fragments of DNA containing genes for H and L chains were transfected into SP2/0 myeloma cells. All the transformant cells that secreted the wild-type and the mutant Abs at 1–10 μg of Ab/ml were established in culture. All the Abs, except for L1C and L3A, bound to HEL in an ELISA assay (data not shown).

To obtain large amounts of Abs, the transformed cells were injected into bellies of BALB/c mice. Only injections of the wild-type, L1C, L2B, L3A, H1A and H3B transformants resulted in ascites forms. The other eight transformants made solid tumors. After recovery of the ascites, the Abs were precipitated with $(NH_4)_2SO_4$ and purified by chromatography on a lysozyme affinity column. L1C and L3A mutant Abs did not bind to the affinity column, as predicted in the ELISA assay. This result suggests the loss of Ag-binding activity. Finally, the wild-type and three mutant Abs, L2B, H1A and H3B, were recovered and used for further analyses. The recovery was 200 μg of Ab/ml ascites.

3.2. *Binding activity at different pH values*

The stability of the Ab structure at various pH values was examined by measuring CD spectra in the short wavelength region (205–260 nm). Fig. 2 shows the results for the wild-type Ab. The patterns observed at pH 7.14, 6.19, 5.13 and 3.92 indicate the presence of a typ-

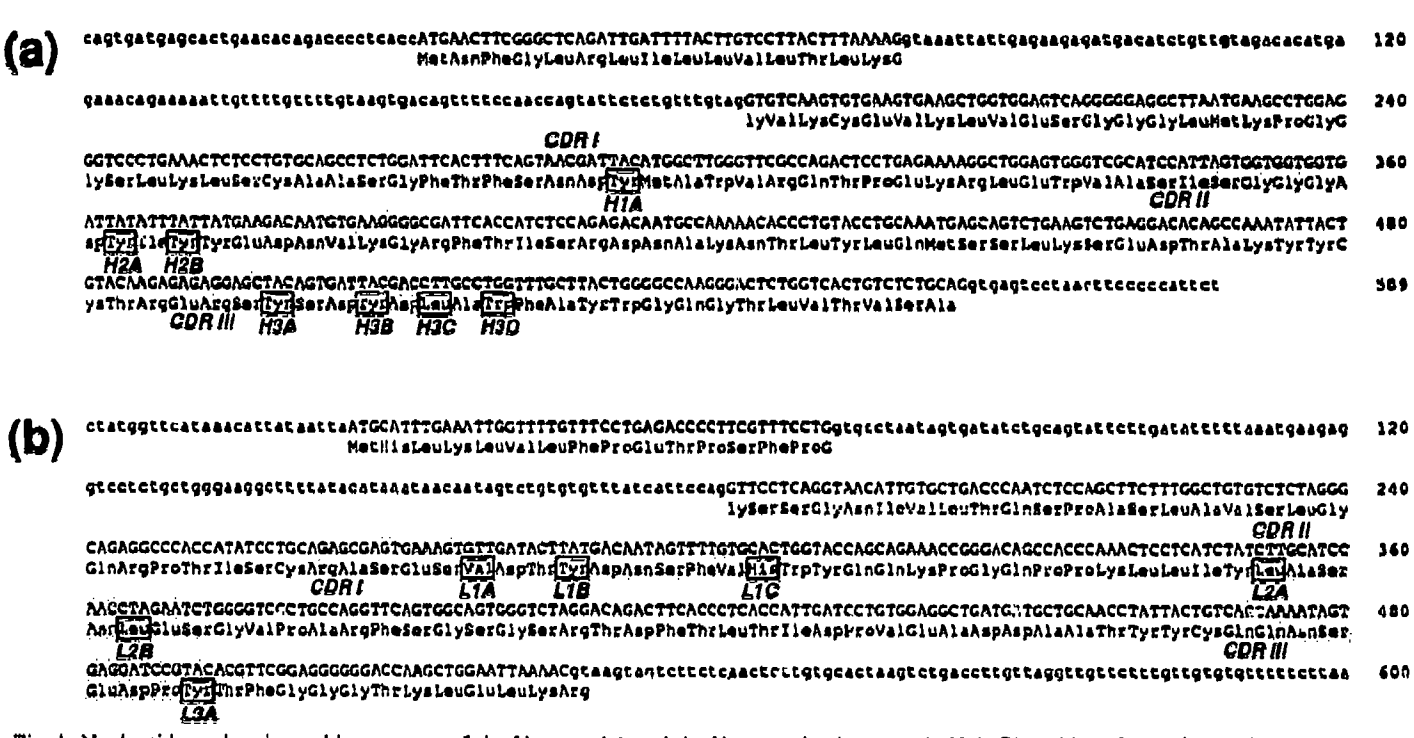

Fig. 1. Nucleotide and amino acid sequences of the V_H gene (a) and the V_L gene (b) that encode Hyb·C1 and locations of mutations. The amino acids that were replaced by histidine residues are boxed. The names of mutants are indicated. In L1C only, a histidine residue was replaced by a tyrosine residue. CDRs are shadowed according to Kabat's definition [17].

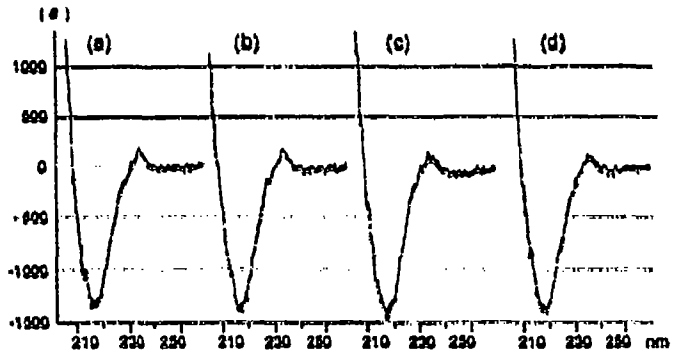

Fig. 2. CD spectra of the wild-type Ab at various pH values. The data are presented in terms of residual molar ellipticities, $[\theta]\lambda$, in deg·cm^2·dmol^{-1}, where $[\theta] = \theta\lambda \times 100/(C \times l)$. Where, l is the path length of the cell in centimeters, C is the molar concentration of the protein residue, and $\theta\lambda$ is the observed ellipticity. Measurement of spectra was made at pH values of (a) 7.14, (b) 6.19, (c) 5.13 and (d) 3.92.

ical immunoglobulin fold, rich in β sheets, with a negative peak around 218 nm [15]. All the patterns were essentially the same as each other. The same results were obtained with three mutant Abs (data not shown). These observations indicated that no gross conformational changes occurred in these Abs over this range of pH values.

We measured binding constants of these Abs with lysozyme at various pH values and at two ionic strengths. Fig. 3A shows logarithmic values (log K) of the binding constants under various conditions. From these data, we drew the following conclusions: (i) Binding constants at low ionic strength (μ=0.10) are higher than those at high ionic strength (μ=0.40) at all pH values tested; (ii) binding constants at higher pH are higher than those at lower pH; (iii) differences between the binding constants at pH 5.2 and those at pH 7.8 at low ionic strength are larger than those at high ionic strength.

Differences (Δlog K) between log K of the wild-type and those of the mutant Abs are plotted in Fig. 3B. In the case of the Abs, H1A and H3B, values of Δlog K were rather constant over this range of pH values. Ab L2B had a characteristic profile of Δlog K. At both low and high ionic strengths, Δlog K of L2B at low pH values was negative and at high pH values it was positive. The difference between Δlog K of L2B at pH 5.2 and that at pH 7.8 is around 0.6 (0.82 kcal·mol^{-1}) at low ionic strength and 0.4 (0.55 kcal·mol^{-1}) at high ionic strength.

4. DISCUSSION

In this study, the wild-type and all the mutant Abs were successfully expressed in SP2/0 myeloma cells and secreted into the medium. This result indicates that the proper folding of the polypeptides was not destroyed by the introduction of histidine residues into the CDRs of the Ab.

The difference between log K of the wild-type at pH 5.2 and that at pH 7.8 is around 2.0 at low ionic strength and 1.4 at high ionic strength, respectively. This difference indicates that electrostatic interactions contribute substantially to the Ag–Ab interaction. The fact that the profiles of the pH-dependent log K did not have clear inflection points but tended to be rather linear (Fig. 3A) may reflect the characteristics of the electrostatic interactions, which were equal to the sum of Coulombic forces derived not only from short-range but also from long-range interactions. At pH 7.8, Δlog K show the values of 0.0 and ×0.3 for H1A, 0.2 and 0.0 for H3B, and 0.4 and 0.1 for L2B at low and high ionic strength, respectively. These results indicate that there are some physical and structural changes in their contact surfaces with HEL, due to substitution of amino acid residues with histidine. The differences in pH dependence observed between H1A/H3B and L2B are noteworthy (Fig. 3B). Only Δlog K of L2B changed in a pH-dependent manner. These differences associated with Δlog K should derive from differences in positions of introduced histidine residues in the Abs. There are two possibilities to explain the effects of the histidine residues. First, charges due to protonation of histidine residues may have directly influenced the electrostatic interactions with lysozyme. Second, these charges may have

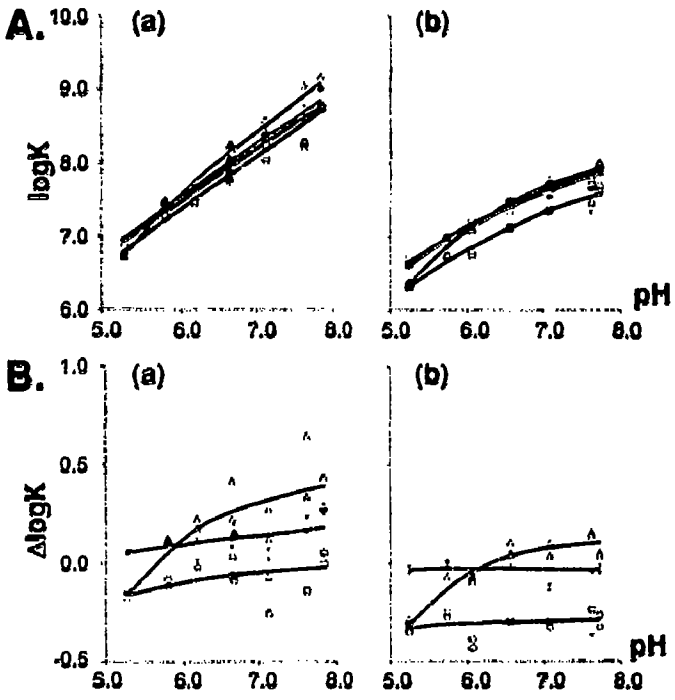

Fig. 3. Binding constants for the wild-type and mutant Abs with HEL at different pH values and ionic strengths. (A) Logarithmic values (log K) of the binding constants at various pH values. Two ionic strengths were used: (a) μ = 0.10; (b) μ = 0.40. □, wild-type, ○, H1A, +, H3B, △, L2B. (B) Δlog K indicates the difference, i.e. log K for the mutant Ab was subtracted by that of the wild-type Ab, using the data shown in A. (a) μ = 0.10; (b) μ = 0.40. ○, H1A, +, H3B, △, L2B.

induced a conformational change in the Ab itself, with a resultant change in the binding constant. Considering the following observations, however, we think that the first explanation is more likely in the case of L2B: there was no remarkable change of CD spectra, the other two mutant Abs, H1A and H3B, showed $\Delta\log K$ profiles with a pH-independent manner, and the difference between $\Delta\log K$ at low pH and that at high pH was smaller at high ionic strength than at low ionic strength.

The strategy described in this paper should be of general use in attempts to examine the effects of charge–charge interactions on macromolecular interactions, and even to measure the absolute values of charge-related forces. Even if one charged amino acid is introduced into a specific site by site-directed mutagenesis, the mutation not only causes a difference in charge but also induces some topological changes [15,16]. Our strategy is based on comparisons between binding constants of the wild-type and those of the mutants at different pH values. As long as the newly introduced histidine residue is located on the surface of the molecule and the changes of $\Delta\log K$ are observed to occur in a pH-dependent manner, as well as being dependent on ionic strength, as in the case of L2B, it is likely that any observed difference was due to charge–charge interactions. Since our method involves measuring the effects induced by protonation and deprotonation of histidine residues at different pH values, we refer to our strategy as the His-Probe method.

Acknowledgements: This research was supported in part by grants from the Ministry of Education, Science and Culture in Japan, and Fujita Health Unviersity.

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