The Affinity Maturation of Anti-4-hydroxy-3-nitrophenylacetyl Mouse Monoclonal Antibody

A CALORIMETRIC STUDY OF THE ANTIGEN-ANTIBODY INTERACTION*

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To understand the mechanism of affinity maturation, we examined the antigen-antibody interactions between 4-hydroxy-3-nitrophenylacetyl (NP) caproic acid and the Fab fragments of three anti-NP antibodies, N1G9, 3B44, and 3B62, by isothermal titration calorimetry. The analyses have revealed that all of these interactions are mainly driven by negative changes in enthalpy. The enthalpy changes decreased linearly with temperature in the range of 25–45 °C, producing negative changes in heat capacity. On the basis of the dependence of binding constants on the sodium chloride concentration, we have shown that, during the affinity maturation of the anti-NP antibody, the electrostatic effect does not significantly contribute to the increase in the binding affinity. We have found that, as the logarithm of the binding constant increases during the affinity maturation of the anti-NP antibody, the magnitudes of the corresponding enthalpy, heat capacity, and unitary entropy changes increase almost linearly. On the basis of this correlation, we have concluded that, during the affinity maturation of the anti-NP antibody, a better surface complementarity is attained in the specific complex in order to obtain a higher binding affinity.

In affinity maturation of the immune response, the average affinity of the immunized serum generally increases with time after immunization (1). To investigate the relationship between the primary sequence diversity of antibodies and the progressive change in binding affinity, extensive analyses of antibodies have been carried out by using several haptenes, 4-hydroxy-3-nitrophenylacetyl (NP) (2–5), p-azophenylarsonate (6), phosphorylcholine (7), and 2-phenyl-5-oxazolone (8).

A series of anti-NP mouse monoclonal IgG antibodies used in the present study were produced by the immune response of C57BL/6 mice against NP coupled to T cell-dependent carrier, chicken γ-globulin (9, 10). The variable regions of the primary response anti-NP antibodies show low affinity for NP and carry few, if any, somatic mutations (2, 4), whereas those of the secondary response anti-NP antibodies usually exhibit increased affinity for NP and are somatically mutated (5). The secondary response antibodies are divided into two groups by carrying or lacking a somatic Trp → Leu exchange at position 33 in the variable region of the heavy chain (11, 12). In the present study we compare N1G9, a primary response anti-NP antibody, with 3B44 and 3B62, which are secondary response anti-NP antibodies with and without Trp → Leu exchange, respectively.

Thermodynamic aspect of antigen-antibody association is essential in order to understand the mechanism of the high affinity and specificity of antigen-antibody interaction. Thermodynamic parameters such as Gibbs free energy change, ΔG, enthalpy change, ΔH, entropy change, ΔS, and heat capacity change, ΔCp, can provide useful information to identify fundamental forces involved in the antigen-antibody interaction. For instance, the magnitude of ΔCp is usually related to the contribution of the hydrophobic effect to molecular association (13–16).

With the recent improvement in the sensitivity and reliability of the calorimeter (17, 18), isothermal titration calorimetry (ITC) has become a powerful tool for the direct measurement of thermodynamic parameters in various biological interactions, such as protein–protein interactions (19–22), oligosaccharide–lectin associations (23–25), and ligand binding to proteins (26, 27). Recently this method has been applied to the quantitative thermodynamic analyses of antibody binding to various types of antigens, e.g. haptenes (28–31), oligosaccharides (32–34), and proteins (35–44). However, no calorimetric studies have been reported on the antigen-antibody interaction in affinity maturation.

In the present study we show the ITC analyses of the antigen-antibody associations in affinity maturation. We examined the interactions between 4-hydroxy-3-nitrophenylacetyl caproic acid (NP-Cap) antigen and the Fab fragments of three anti-NP antibodies, N1G9, 3B44, and 3B62. On the basis of the obtained thermodynamic data, we have found that the binding constants, Ka, for NP-Cap correlate with each of the ΔH, ΔCp, and ΔS values. As the logarithm of the Ka values increases in the course of affinity maturation, the magnitudes of the corresponding ΔH, ΔCp, and ΔS values increase almost linearly. Although the interactions between a series of monoclonal antibodies and their same antigen have been investigated in several cases (35–37, 39, 43–45), the linear relationship between log Ka and each of ΔH, ΔCp, and ΔS shown in the
The area under each peak was integrated, and the thermic peak was equivalent to the heat of dilution measured molar ratio \((\text{NP-Cap})/(\text{Fab}(3B62))\). The data were fitted using a nonlinear least-squares method. The binding constant, \(K_a\), and the enthalpy change, \(\Delta H\), were obtained from the fitted curve. Further, the Gibbs free energy change, \(\Delta G\), and the entropy change, \(\Delta S\), were calculated from the equation, \(\Delta G = -RT \ln K_a = \Delta H - \Delta S\). The thermodynamic parameters obtained for Fab(3B62) are the following: \(K_a = 8.0 \pm 0.7 \times 10^6 \text{ M}^{-1}\), \(\Delta H = -20.3 \pm 0.1 \text{ kcal mol}^{-1}\), \(\Delta S = -35.5 \pm 0.5 \text{ cal mol}^{-1} \text{ K}^{-1}\), and \(\Delta G = -9.6 \pm 0.1 \text{ kcal mol}^{-1}\).

The magnitudes of the \(\Delta S\) and \(\Delta G\) values are dependent on the concentration units for the standard state. In order to obtain unitary entropy change, \(\Delta S\), and unitary Gibbs free energy change, \(\Delta G\), which are independent of the concentration units chosen for the standard state since, in essence, solute concentrations are measured in molar fraction units, we used the following equations (48):

\[
\Delta S_u = -RT \ln X_m - \Delta S - 7.98 \text{ (cal mol}^{-1} \text{ K}^{-1})
\]

\[
\Delta G_u = -7.98 \times 10^{-3} \text{T (kcal mol}^{-1})
\]

The cratic contribution to the entropy change, \(R \ln X_m\), is \(R \ln(1/5.56) = -7.98 \text{ cal mol}^{-1} \text{ K}^{-1}\), where 55.6 is the concentration of water in dilute aqueous solution (13). In the case of Fig. 1 \(\Delta S_u\) and \(\Delta G_u\) are \(-27.5 \pm 0.5 \text{ cal mol}^{-1} \text{ K}^{-1}\) and \(-12.0 \pm 0.1 \text{ kcal mol}^{-1}\), respectively. The thermodynamic parameters for the interaction between NP-Cap and each of Fab(N1G9) and Fab(3B44) were obtained in the same way.

**Temperature Dependence of the Antigen-Antibody Interaction**—As a function of temperature between 25 and 45 °C, we analyzed the interaction between NP-Cap and each of Fab(N1G9), Fab(3B44), and Fab(3B62) by ITC. The thermodynamic parameters for these interactions are summarized in Table I. The thermodynamic parameters, \(\Delta G\), \(\Delta H\), and \(\Delta S\) values for NP-Cap correlate with the \(\Delta G\) values for the interaction between each of Fab(N1G9), Fab(3B44), and Fab(3B62), respectively (41, 44, 45).

The associations between NP-Cap and the three anti-NPFab are mainly driven by favorable negative changes in \(\Delta H\). The negative \(\Delta H\) values decrease with increasing temperature and show linear dependence on temperature in the range of 25–45 °C (Fig. 2). The \(\Delta C_p\) value for each interaction can be determined from the slope of the temperature dependence of \(\Delta H\). The negative \(\Delta C_p\) values of \(-309 \pm 27\), \(-363 \pm 12\), and \(-415 \pm 12 \text{ cal mol}^{-1} \text{ K}^{-1}\) are observed for Fab(N1G9), Fab(3B44), and Fab(3B62), respectively (Table I).

Fig. 3 shows that the \(K_a\) values for NP-Cap correlate with each of the \(\Delta H\), \(\Delta C_p\), and \(\Delta S_u\) values. As the logarithm of the \(K_a\) values increases in the order of Fab(N1G9), Fab(3B44), and Fab(3B62), the magnitudes of the corresponding \(\Delta H\), \(\Delta C_p\), and \(\Delta S_u\) values increase almost linearly.
chloride concentration between 20 mM and 400 mM. As the logarithm of the sodium chloride concentration increases, the logarithm of the $K_a$ values of Fab(N1G9), Fab(3B44), and Fab(3B62) for NP-Cap decreases linearly as shown in Fig. 4. The slopes of the regression lines in Fig. 4 are $-0.57 \pm 0.09$, $-0.59 \pm 0.10$, and $-0.48 \pm 0.07$ for Fab(N1G9), Fab(3B44), and Fab(3B62), respectively. Thus, the dependence of the $K_a$ values of Fab(N1G9), Fab(3B44), and Fab(3B62) on the sodium chloride concentration is similar within experimental errors.

**DISCUSSION**

In the present study, we have carried out the ITC analyses of the antigen-antibody associations in affinity maturation, in order to understand the mechanism of affinity maturation. The present results reveal that all of the associations between NP-Cap and the three Fab are mainly driven by favorable negative changes in $\Delta H$. Van der Waals interactions and hydrogen bondings are usually considered to be the major potential sources of the negative $\Delta H$ values (40, 43, 50). Thus, we suggest that van der Waals interactions and hydrogen bondings play a fundamental role in the interactions between NP-Cap and the three Fab. Also, the increase in the magnitude of the negative $\Delta H$ with the increase in $\log K_a$ (Fig. 3a) suggests that, in the course of the affinity maturation, the increase in the van der Waals interactions and hydrogen bondings promotes the increase in the binding affinity of the anti-NP antibody.

The negative $\Delta C_p$ values in Table I are within the range of $-100$ to $-650 \text{ kcal mol}^{-1} \text{ K}^{-1}$, which were previously reported for various antigen-antibody complexes (28, 29, 35, 37, 38, 40, 41, 43, 45). In general, the negative $\Delta C_p$ values for protein folding and protein-ligand interactions are proportional to the reduction in water-accessible nonpolar surface areas of the molecules, and related to the contribution of hydrophobic effect to molecular association (13-16, 51). In order to interpret our data quantitatively, the empirical method of Sturtevant (52) was used to estimate the hydrophobic and intramolecular vibrational contribution to $\Delta C_p$ (Table I). For all the three Fab, the calculated hydrophobic contribution to $\Delta C_p$ is larger than the calculated vibrational contribution. Therefore, we suggest that the observed negative change in $\Delta C_p$ may primarily result from the hydrophobic effect, that is, the decrease in solvent exposure of both the aromatic antigen and the nonpolar groups in the binding site of the three Fab caused by the antigen-antibody association. Furthermore, the increase in the magnitude of the negative $\Delta C_p$ with the increase in $\log K_a$ (Fig. 3b) suggests that, in the course of the affinity maturation, the increase in the hydrophobic effect contributes to the increase in the binding affinity of the anti-NP antibody. This is consistent with the previous NMR result that the binding site of NP-Cap is located in a similar position, but the combining site of Fab(3B62) with higher affinity for NP-Cap is composed of more Tyr residues than that of Fab(N1G9) with lower affinity for NP-Cap (46).

The hydrophobic effect, which drives the association of nonpolar surfaces of molecules by excluding water from the interface, would contribute to the positive change in $\Delta S_u$. However, we observed the unfavorable negative $\Delta S_u$ values in the range of 25–45 °C, as shown in Table I. Negative $\Delta S_u$ has been observed previously for other antigen-antibody interactions (29–32, 35, 36, 39–44). Consequently, we conclude that some other factors should counteract the hydrophobic effect and make larger contributions to the negative $\Delta S_u$. Such effect to the negative $\Delta S_u$ can be produced by the following factors: 1) the constraint of intramolecular vibrational flexibility of Fab due to the antigen binding (52); 2) the reduction in the translational and overall rotational degrees of freedom upon the complex formation (53, 54); and 3) the conformational freezing of the amino acid residues of Fab caused by the antigen binding (55). The previously reported estimate of the factor 2 was almost constant for different antigen-antibody complexes ($T \Delta S_{TR} = 7–11 \text{ kcal mol}^{-1}$, where $\Delta S_{TR}$ is an amount of translational and overall rotational entropy change) (53-55). The estimation of the factor 3 was previously reported in the interactions between lysozyme and a few anti-lysozyme monoclonal antibodies (55). We applied the empirical method of Sturtevant (52) in order to estimate the hydrophobic and intramolecular vibrational contribution (described above as the factor 1) to $\Delta S_u$ (Table I). For all three Fab, the sign of the calculated hydrophobic contribution is positive, but that of the calculated vibrational contribution is indeed negative.

The obtained $K_a$ values shown in Table I are within the range of 25–45 °C. The $K_a$ values of Fab(N1G9), Fab(3B44), and Fab(3B62), which is consistent with the previously reported results (2, 4, 5). As the logarithm of the sodium chloride concentration increases, the logarithm of the $K_a$ values of the three Fab for NP-Cap decrease linearly. The dependence of the $K_a$ values on the sodium chloride concentration is similar for the three Fab (Fig. 4). These results suggest that the electrostatic effect is involved in the antigen-antibody associations, but the proportion of the elec-
trostatic effect to the NP-Cap binding is similar for the three Fab. We conclude that, in the course of the affinity maturation, the electrostatic effect does not significantly contribute to the increase in the binding affinity of the anti-NP antibody.

We have found a linear correlation between log $K_a$ and each of $\Delta H$, $\Delta C_p$, and $\Delta S_u$, as shown in Fig. 3. As the logarithm of the $K_a$ values increases in the course of affinity maturation, the magnitudes of the corresponding $\Delta H$, $\Delta C_p$, and $\Delta S_u$ values increase almost linearly. Although the interactions between a series of monoclonal antibodies and their same antigen have been investigated in several cases (35–37, 39, 43–45), the linear relationship between log $K_a$ and each of $\Delta H$, $\Delta C_p$, and $\Delta S_u$ shown in the present study has not been observed yet.

This linear relation of log $K_a$, $\Delta H$, and $\Delta C_p$ (Fig. 3, a and b)
implies that the surface complementarity of the anti-NP antibody with the antigen increases in the course of the affinity maturation, which may reflect increased van der Waals interactions and hydrogen bondings between specific functional groups. The number of electrostatic interactions involved in the interface remains unchanged with maturation (Fig. 4) suggests that the increase in the surface complementarity. On the other hand, we observed more pronounced effect of unfavorable negative ΔSu in the course of the affinity maturation (Fig. 3c). This apparently contradicting effect seems feasible, because more enhanced surface complementarity would make more restraint in the intramolecular vibrations of the complex.

We conclude that, in the course of the affinity maturation of the anti-NP antibody, a better surface complementarity is attained in the specific complex in order to obtain a higher binding affinity. The attainment of a better surface complementarity may be produced by an increase in the number of Tyr side chains in the antibody combining site.

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