J. Biochem. 123, 827-831 (1998)

Temperature Dependency of Thermodynamic Parameters in Interactions between Hen Egg-White Lysozyme (HEL) and Anti-HEL Antibodies[1]

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Received for publication, October 21, 1997

We examined temperature dependency of thermodynamic parameters in the interactions between hen egg-white lysozyme (HEL) and anti-HEL antibody, D1.3, and two mutant antibodies. The ΔH° values appeared to decrease biphasically in the temperature range from 10 to 45°C with the apparent inflection point around 30°C. The ΔG° calculated from the K_A values showed only small differences because of entropy and enthalpy compensation. It has been argued that large negative values of heat capacity change (ΔCp°), if observed, are mainly derived from hydrophobic interactions. However, the observed ΔCp° values were too high to be ascribed only to hydrophobic interactions. Moreover, addition of methanol did not cause a decrease in the absolute value of ΔCp°.

Key words: differential titration calorimetry, entropy-enthalpy compensation, heat capacity change, hydrophobic interactions.

The antigen-antibody complex is stabilized by van der Waals', ionic, and hydrophobic interactions, as well as the formation of hydrogen bonds (*1*). The most important enthalpic contributions on formation of the complex include hydrogen bonds, van der Waals' forces and salt bridges (*2*). On the other hand, the predominant entropic contributions are known to involve changes in solvent entropy at the solvent-solute interface (*i.e.*, hydrophobic effects in aqueous solution), loss of bond configurational entropy on attainment of compact structure, and the decrease in molecular translational entropy on complex formation, of which only the hydrophobic interactions seem to favor the association between antigen and antibody (*2*). Since the temperature derivatives of both ΔH° and ΔS° are functions of heat capacity change (ΔCp°):

$$(\partial \Delta H^\circ / \partial T)p = \Delta Cp^\circ, \quad (1)$$

$$(\partial \Delta S^\circ / \partial T)p = \Delta Cp^\circ / T, \quad (2)$$

that of the ΔG° should be expressed by the following equation:

$$\frac{\partial \Delta G^\circ}{\partial T} = \frac{\partial (\Delta H^\circ - T\Delta S^\circ)}{\partial T} = -\Delta S^\circ. \quad (3)$$

When ΔCp° shows a large, negative value and the absolute value of ΔS° is much smaller than that of ΔCp°, entropy-enthalpy compensation should always be observed when temperature is changed (*3*). In protein-protein interactions, the contribution of the enthalpic terms is large at higher temperature, and the contribution of the entropic terms becomes larger at lower temperature in general (*2*). This should result in a large, negative ΔCp°. Thus, the entropy-enthalpy compensation in changes of temperature and the presence of a large, negative ΔCp° seem to be inseparable in protein-protein interactions. On the other hand, it has been argued that large, negative values of ΔCp°, if observed, are mainly derived from hydrophobic interactions (*4, 5*). In the present study, we examined the temperature dependency of K_A and ΔH° in the interaction between antibody and protein antigen in order to address the origin of ΔCp°.

For that purpose, we used Fv fragments of a monoclonal antibody, D1.3, specific for hen egg-white lysozyme (HEL), and two mutant antibodies (*6, 7*). These mutant antibodies were selected from among the 13 antibodies prepared in the previous study, of which mutant #2 showed the smallest absolute value of ΔH° at 30°C and mutant #11 showed the largest absolute value of ΔS° at 30°C.

MATERIALS AND METHODS

Preparation of Fv Fragments of D1.3 and Its Two Derivatives—The structure of Fv fragment of D1.3 (designated Wild-0), and the construction and characterization of mutant antibodies #2 and #11 were as described previously (*7*). Differences in amino acids among these three antibodies are summarized in Table I. Large amounts of the three Fv fragments were prepared by the procedure described previously (*7*). The HEL used in this study was a product of SIGMA (3 times crystallized, dialyzed, and lyophilized; no. L8867).

Measurement of the Thermodynamic Parameters of the HEL-Fv Fragment Interaction by Differential Titration Calorimetry (DTC) Analysis—The association constants

[1] This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture in Japan, and the Fujita Health University.

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Abbreviations: HEL, hen egg-white lysozyme; DTC, differential titration calorimetry.

and association enthalpies of the binding of Fv fragments to HEL were determined calorimetrically using an Omega titration calorimeter from MicroCal (*8*). The experimental conditions of the DTC analysis were as described previously (*7, 8*) except that the temperature was changed from 10 to 45°C in 5-degree intervals. The experiments were performed in 50 mM sodium phosphate buffer (pH 6.8) and 200 mM NaCl, and in one experiment, methanol was added at the final concentration of 5%.

TABLE I. **Differences in amino acids in the heavy chains of the Fv fragments analyzed.**

	CDR I				CDR II				CDR III
	27	30	32	46	52	53	54	56	96
Wild-O	F	T	Y	E	W	G	D	N	R
#2	N	T	F	Q	W	G	N	D	W
#11	N	S	F	Q	R	S	N	N	R

The amino acid sequences of these three Fv fragments were described previously (*7*).

RESULTS

Measurements of the Association Enthalpies (ΔH°) of Interactions between HEL and Anti-HEL Antibody in the Temperature Range from 10 to 45°C—A large amount of Fv fragment of D1.3 was synthesized in *Escherichia coli* and purified by HEL-linked affinity column chromatography. The ΔH° of interactions between this Fv fragment and HEL was directly measured by the DTC method (*8*) at 5-degree intervals in the temperature range from 10 to 45°C. This analysis also allowed us to calculate the K_A from the titration profile of ΔH *versus* amounts of added antigen. Therefore, the other two parameters, ΔG° and ΔS°, in-

TABLE II. **Thermodynamic parameters for the association of Fv with HEL.**

clone condition	T	n	K_A	ΔH^0	ΔG^0	$-T\Delta S^0$
	K		10^7/M	kJ/mol	kJ/mol	kJ/mol
Wild-O	283.6	1.08 ± 0.00	86.2 ± 32.0	-75.7 ± 0.4	-48.5 ± 0.8	27.4 ± 1.2
	288.2	1.08 ± 0.00	34.0 ± 4.5	-78.6 ± 0.4	-46.8 ± 0.4	31.4 ± 0.5
	293.0	1.07 ± 0.00	37.2 ± 6.7	-80.7 ± 0.4	-48.1 ± 0.4	32.7 ± 0.7
	298.2	1.05 ± 0.00	17.1 ± 3.0	-85.2 ± 0.4	-46.8 ± 0.4	38.2 ± 0.9
	303.2	1.06 ± 0.00	8.65 ± 1.00	-85.2 ± 0.4	-46.0 ± 0.4	41.2 ± 0.7
	308.2	1.06 ± 0.00	6.42 ± 0.86	-92.4 ± 0.4	-46.0 ± 0.4	46.4 ± 0.8
	313.2	1.05 ± 0.00	2.37 ± 0.14	-102.0 ± 0.4	-44.3 ± 0.0	57.7 ± 0.4
	318.2	1.07 ± 0.00	1.41 ± 0.15	-106.2 ± 0.8	-43.5 ± 0.4	62.7 ± 1.3
#2	283.5	0.995 ± 0.006	1.47 ± 0.20	-39.7 ± 0.4	-38.8 ± 0.3	0.87 ± 0.7
	288.3	0.937 ± 0.006	1.17 ± 0.14	-41.4 ± 0.4	-39.0 ± 0.3	2.8 ± 0.7
	293.3	0.935 ± 0.008	0.787 ± 0.100	-45.1 ± 0.4	-38.7 ± 0.3	6.3 ± 0.8
	298.3	0.995 ± 0.006	0.755 ± 0.073	-46.0 ± 0.4	-39.2 ± 0.3	6.9 ± 0.7
	303.2	0.833 ± 0.008	0.569 ± 0.069	-50.6 ± 0.8	-39.1 ± 0.3	11.2 ± 1.0
	308.3	0.897 ± 0.009	0.429 ± 0.052	-56.0 ± 0.8	-39.1 ± 0.3	16.8 ± 1.1
	313.3	0.929 ± 0.013	0.369 ± 0.057	-61.2 ± 1.3	-39.3 ± 0.4	21.6 ± 1.5
	318.3	0.882 ± 0.010	0.215 ± 0.022	-69.4 ± 1.3	-39.5 ± 0.3	31.0 ± 1.4
#11	283.7	0.960 ± 0.002	2.65 ± 0.19	-66.0 ± 0.4	-40.3 ± 0.2	26.0 ± 0.5
	288.8	0.967 ± 0.004	1.88 ± 0.20	-68.6 ± 0.4	-40.2 ± 0.3	28.4 ± 0.7
	293.4	0.971 ± 0.005	1.26 ± 0.13	-74.8 ± 0.4	-39.8 ± 0.3	35.0 ± 0.8
	298.1	0.940 ± 0.007	0.745 ± 0.087	-78.2 ± 0.8	-39.2 ± 0.3	39.0 ± 1.1
	303.2	0.802 ± 0.006	0.477 ± 0.043	-85.3 ± 1.3	-38.7 ± 0.3	46.4 ± 1.2
	308.3	0.941 ± 0.008	0.303 ± 0.025	-90.7 ± 0.8	-38.7 ± 0.3	52.7 ± 1.3
	313.3	0.918 ± 0.008	0.152 ± 0.009	-101.6 ± 1.3	-38.2 ± 0.2	64.8 ± 1.3
	318.3	0.801 ± 0.018	0.089 ± 0.011	-107.4 ± 3.3	-37.0 ± 0.2	71.1 ± 3.8
Wild-O 5% MeOH	283.4	1.08 ± 0.00	61.8 ± 11.1	-76.1 ± 0.4	-47.7 ± 0.4	28.4 ± 0.8
	288.2	1.08 ± 0.00	29.6 ± 5.3	-81.5 ± 0.4	-46.8 ± 0.4	34.9 ± 0.8
	293.4	1.07 ± 0.00	14.7 ± 1.8	-84.9 ± 0.4	-46.0 ± 0.4	39.0 ± 0.6
	298.1	1.02 ± 0.00	9.80 ± 1.13	-90.3 ± 1.3	-45.6 ± 0.4	44.7 ± 1.7
	303.7	1.05 ± 0.00	6.40 ± 0.42	-94.1 ± 0.4	-45.1 ± 0.0	48.5 ± 0.4
	308.2	1.07 ± 0.00	4.34 ± 0.79	-97.4 ± 0.8	-45.1 ± 0.4	52.7 ± 1.3
	313.2	1.05 ± 0.00	2.49 ± 0.29	-102.0 ± 0.8	-44.3 ± 0.4	57.7 ± 0.8
	318.3	1.07 ± 0.00	1.18 ± 0.10	-112.0 ± 0.8	-43.1 ± 0.4	69.4 ± 1.3

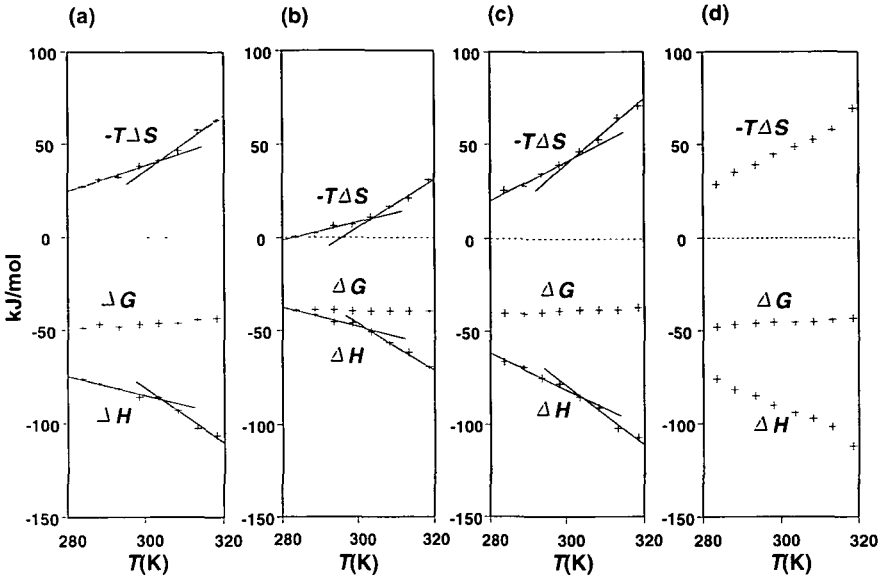

Fig. 1. **Temperature dependency of thermodynamic parameters.** The data of ΔG, ΔH, $-T\Delta S$ at each temperature in Table II are plotted. The samples are (a) Wild-O, (b) #2, (c) #11, and (d) Wild-O in 5% methanol solution. Since the ΔH° values appeared to decrease biphasically in the temperature range from 10 to 45°C with the apparent inflection point around 30°C in (a), (b), and (c), and since $-T\Delta S$ appeared to increase biphasically, we drew straight lines based on the five leftmost points and the four rightmost points, respectively.

volved in antigen-antibody interactions can be calculated. Table II summarizes the data. Three parameters, ΔG°, ΔH°, and $-T\Delta S^\circ$, at various temperatures are plotted in Fig. 1a. The ΔH° appeared to decrease biphasically from -75.7 kJ·mol^{-1} at 10°C to -106.2 kJ·mol^{-1} at 45°C. Since the apparent inflection point was observed around 30°C, ΔCp° was calculated 594 J·K^{-1}·mol^{-1} at lower than 30°C and 1,267 J·K^{-1}·mol^{-1} at higher than 30°C. Bhat *et al.* (*9*) reported that ΔCp° of FvD1.3 complexed with HEL is 1,580 J·K^{-1}·mol^{-1} in the temperature range from 11 to 50°C.

Measurements of the ΔH° of Interactions between HEL and Two Mutant Antibodies—Fv fragments of mutant antibodies #2 and #11 were analyzed by the above method. The data are summarized in Table II. Three parameters, ΔG°, ΔH°, and $-T\Delta S^\circ$, are plotted in Fig. 1, b and c. In both cases, the ΔH° also appeared to decrease biphasically from -39.7 kJ·mol^{-1} at 10°C to -69.4 kJ·mol^{-1} at 45°C for mutant #2, and from -66.0 kJ·mol^{-1} at 10°C to -107.4 kJ·mol^{-1} at 45°C for mutant #11. Since the apparent inflection points were observed around 30°C, ΔCp° of mutant #2 was calculated to be -535 J·K^{-1}·mol^{-1} at lower than 30°C and $-1,258$ J·K^{-1}·mol^{-1} at higher than 30°C, and that of mutant #11 $-1,120$ J·K^{-1}·mol^{-1} at lower than 30°C and $-1,505$ J·K^{-1}·mol^{-1} at higher than 30°C. ΔG° of mutant #2 was moderately constant in this temperature range, showing values between -39.5 and -38.7 kJ·mol^{-1}. ΔG° of mutant #11 changed from -40.3 at 10°C to -37.0 kJ·mol^{-1} at 45°C.

Measurement of the ΔH° of Interaction between HEL and Anti-HEL Antibody in 5% Methanol Solution—Addition of methanol to the solution should decrease the effects of hydrophobic interaction (*10*). The ΔH° of interaction between HEL and Wild-O antibody was measured in 5% methanol solution by the DTC method. The data are summarized in Table II. Three parameters, ΔG°, ΔH°, $-T\Delta S^\circ$, are plotted in Fig. 1d. Throughout the temperature range examined, the K_A values were slightly smaller from those without methanol, and the ΔH° showed slightly lower values than those without methanol. Although the ΔH° also decreased from -76.1 kJ·mol^{-1} at 10°C to -112 kJ·mol^{-1} at 45°C, a distinct biphasic decrease was not recognized. Therefore, the average ΔCp° was calculated to be 1,045 J·K^{-1}·mol^{-1} in this temperature range from 10 to 45°C.

DISCUSSION

In the present study, we examined the temperature dependency of thermodynamic parameters involved in interactions between antigen and antibody. While the differences of ΔH° at 10 and 45°C were large, that is, -30.5, -29.7, and -41.4 kJ·mol^{-1} for Wild-O, and mutants #2 and #11, respectively, those of ΔG° were small, 5.0, 0.29 and 4.6 kJ·mol^{-1}, respectively. These phenomena, that is, entropy-enthalpy compensation, are always observed when ΔCp° has a large, negative value and the absolute value of ΔS° is much smaller than that of ΔCp° (*3*). Mutant #2 showed a typical example. ΔS° changed from -3.09 J·K^{-1}·mol^{-1} at 10°C to -97.4 J·K^{-1}·mol^{-1} at 45°C, while ΔCp° was -535 J·K^{-1}·mol^{-1} at lower than 30°C and $-1,258$ J·K^{-1}·mol^{-1} at higher than 30°C. This resulted in the constancy of ΔG°. Differences in amino acid residues at certain sites may induce different interactions at the contact surfaces. This would result in changes in bonds such as hydrogen bonds and van der Waals' contacts. The large differences of ΔH° among antibodies might originate from such changes at the contact surface. As long as the proper association between antigen and antibody is achieved, however, the entropy-enthalpy compensation in changes of amino acid residues still worked, as shown in the previous study (*7*). Because the three antibodies showed different ΔH° values but similar ΔCp° values to each other, we suggested that the origin of ΔCp could derive from the factors which determine the absolute values of ΔH°.

The DTC analysis gave us two experimental data: ΔH°, which is obtained directly from the heat generated, and K_a, which is calculated from the titration profile. The ΔH° values at each temperature can be calculated from the van't Hoff equation using the K_A at various temperatures:

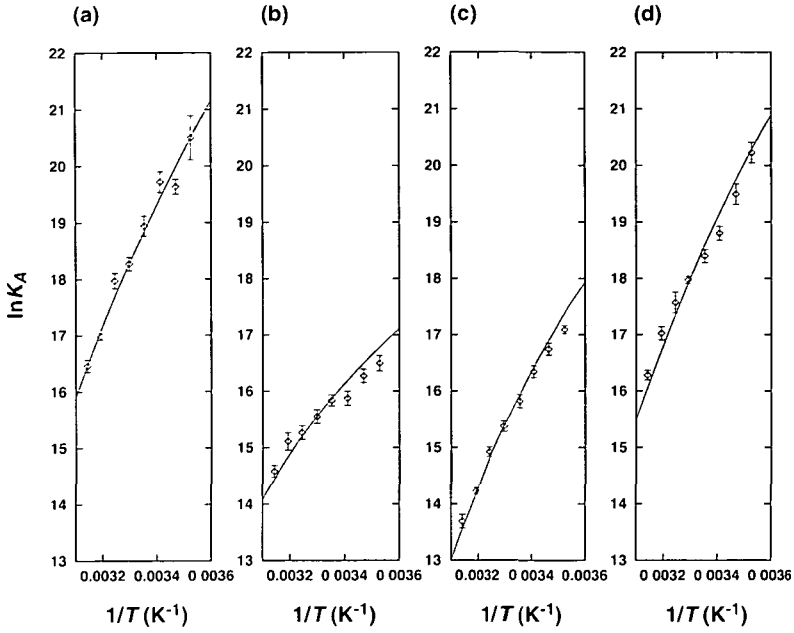

Fig. 2. **van't Hoff plot.** The values of lnK_A *versus* $1/T$ are plotted according to the data in Table II. The lines indicate the values calculated from the van't Hoff equation (Eq. 4) using the values of ΔH. The samples are (a) Wild-O, (b) #2, (c) #11, and (d) Wild-O in 5% methanol solution.

$$\frac{d\ln K_A}{d(1/T)} = -\frac{\Delta H^\circ}{R} \quad (4)$$

As shown in Fig. 2, the K_A based on the van't Hoff equation and the ΔH° at various temperatures showed practically the same values as those calorimetrically obtained in all the four cases. Although these results were as expected, the actual measurements showed large discrepancies between these two values in some cases. Sigurskjold and Bundle (*11*) analyzed temperature dependency of binding constants of oligosaccharide to the antibody. Although they also showed a biphasic curve with two apparent intrinsic enthalpy estimations, ΔH° measured by the DTC method and the ΔH° calculated from van't Hoff analysis showed large discrepancies. The occurrence of different ΔCp° values at low and high temperature was also reported by Herron *et al.* (*12*) for the binding of fluorescein with anti-fluorescyl antibody. Although the reason for the occurrence of different ΔCp values was not clear, we discussed the contribution of hydrophobic interactions to the association using the estimated values.

The present study indicated the occurrence of large, negative values of ΔCp° in interactions between HEL and anti-HEL antibodies. It has been argued that large, negative values of ΔCp°, if observed, are mainly derived from hydrophobic interactions (*4, 5*). This interpretation is based on the observations showing good correlations between the magnitude of ΔCp° and the area of exposed non-polar surfaces, ΔA_{np}, in water. The following values have been indicated (*13*): for the transfer of hydrocarbon in water at infinite dilution to the pure liquid state, $\Delta Cp^\circ/\Delta A_{np} = -1.38 \pm 0.38$ J/mol/deg/Å^2; and for protein folding, $\Delta Cp^\circ/\Delta A_{np} = -1.05 \pm 0.13$ J/mol/deg Å^2. The sum of the areas of the solvent-buried surfaces of HEL and D1.3 after complex formation was calculated to be 1,290 Å^2 (*14*). ΔCp° values of three kinds of antibody examined in the present study are -594 to $-1{,}120$ J·K^{-1}·mol^{-1} at lower than 30°C, and $-1{,}267$ to $-1{,}538$ J·K^{-1}·mol^{-1} at higher than 30°C. These values correspond to -0.460 to -0.868 J/mol/deg/Å^2, and -0.982 to -1.192 J/mol/deg/Å^2, respectively. In the case of HEL-anti-HEL interactions, most of the amino acids that are located at the contact surface and involved in hydrogen bonding and van der Waals' contact are polar and hydrophilic in nature (*14*). Therefore, the actual area of non-polar surfaces buried after the complex formation should be much smaller than 1,290 Å. It has been shown that at least three residues, W52 (Trp at the 52nd residue), G53, and D54, are involved in the direct contact with HEL among the seven residues in which mutations were introduced in the present study (*14*). While the hydrophobic interactions between the antibody and HEL should have had different effects on the binding of the three antibodies, ΔCp° values appeared to be similarly high, although different. Moreover, the values calculated for HEL and anti-HEL antibodies still seemed to be equivalently high to the above values for the transfer of hydrocarbon and the protein folding. Therefore, it seems unreasonable to ascribe the origin of ΔCp° observed in the present study only to hydrophobic interactions. Measurements of the thermodynamic parameters in 5% methanol solution indicated smaller K_A values than those without methanol. These data are consistent with the notion that addition of methanol should decrease the effects of hydrophobic interactions. On the other hand, ΔCp° of interactions between HEL and Wild-O in 5% methanol solution showed a constant value, $-1{,}045$ J·K^{-1}·mol^{-1}, in the temperature range from 10 to 45°C. Although this value is higher than that at higher than 30°C, $-1{,}267$ J·K^{-1}·mol^{-1}, it is lower than that at lower than 30°C, -594 J·K^{-1}·mol^{-1}. Thus, we suggested that the contribution of hydrophobic interactions to the large, negative ΔCp would not be large in the interaction between HEL and anti-HEL antibodies. Essentially the same conclusion as ours has been reported by Bhat *et al.* (*9*). Hibbits *et al.* (*15*) reported the calorimetric analysis of the association between HEL and HyHEL-5 antibody and concluded that the ΔCp value experimentally observed ($-1{,}421$ J·K^{-1}·mol^{-1}) agrees closely with the ΔCp estimated from polar and nonpolar surface areas

buried upon association. In our previous paper (*16*), we estimated the thermodynamic parameters involved in pre- and post-processes of formation of the activated complex and found a strong compensation between changes in entropy and changes in enthalpy caused by substitutions of amino acids. Thus, the K_A values are mainly determined in the post-process, in which direct non-covalent bonds are formed at the contact surface. This observation supports the idea that the contribution of the hydrophobic interactions to the association is not large.

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