The Thermodynamics of Hapten and Antigen Binding By Rabbit Anti-Dinitrophenyl Antibody†

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ABSTRACT: Rabbit anti-dinitrophenyl antibody from a serum pool was obtained as five fractions of purified specific antibody by a limiting antigen precipitation method. Each fraction had a different binding affinity for ϵ-*N*-2,4-dinitrophenyl-L-lysine. The free energy changes for hapten binding to the five antibody fractions varied from −8.35 to −10.0 kcal/mol. An average ΔH of −13.9 kcal/mol was measured for the fractions with a batch calorimeter. The results indicate no significant correlation between enthalpy changes and free energy changes. However, a statistically significant correlation between the free energy changes and the entropy changes was found. The enthalpy of the anti-dinitrophenyl antibody interaction with multivalent dinitrophenyl human serum albumin was determined. These are the first enthalpy measurements of an antibody-antigen reaction in which the intrinsic binding enthalpy between the antibody and the determinant group is known. The ΔH for the antigen binding reaction was −10.1 kcal/mol which is 3.8 kcal/mol less exothermic than the ΔH for the hapten binding reaction. The interactions that could lead to such a difference in enthalpy are discussed.

A combination of thermodynamic and structural information can be expected to provide a complete understanding of the nature of the antibody-antigen interaction. The necessary quantitation to measure the thermodynamics of these interactions has recently become possible due to advancements in microcalorimetric instrumentation. The first applications of this new instrumentation with immunochemical systems were the experiments of Barisas *et al.* (1971) who examined the binding of Dnp-lysine[1] to anti-Dnp antibody. The van't Hoff calculations of Velick *et al.* (1960) had indicated that the antibody-hapten reaction was enthalpically driven. Although this fact was confirmed by Barisas *et al.* (1971), the calorimetric measurements demonstrated that the reaction was considerably more exothermic than the van't Hoff estimates had indicated. Thus, with anti-Tnp antibody, Barisas *et al.* (1972) calculated a van't Hoff ΔH of −10 kcal/mol and then measured a ΔH of −21 kcal/mol with the calorimeter. The large difference in the two values demonstrates the importance of measuring the ΔH directly if unambiguous thermodynamic information is desired.

Barisas *et al.* (1971) measured the ΔH of binding for their serum pool at several different concentrations of Dnp-lysine. They reasoned that the ΔH obtained at limiting concentrations of Dnp-lysine reflected the binding energy of the high affinity antibody, whereas the ΔH determined at saturating hapten concentrations represented an average binding energy for the entire population of antibodies. The ΔH at saturation was dependent on the accuracy in estimating N, the number of available combining sites per molecule, but the ΔH at low hapten concentration was not. The two estimates obtained, −22.8 and −15 kcal/mol for the low and high hapten concentrations, respectively, constituted an unusually large range in enthalpies. In a subsequent paper (Barisas *et al.*, 1972) using different preparations the enthalpy heterogeneity question was examined again. Although no *conclusion* on this point was made about the anti-Dnp antibody, there appeared to be no significant heterogeneity in enthalpy for the anti-Tnp antibody. Since there remained some uncertainty because of possible problems in the estimation of N, particularly for the anti-Dnp antibody, it was felt that this question of enthalpy heterogeneity should be *directly* examined.

Fractions of different binding affinity were prepared from rabbit serum by the fractional precipitation method (Eisen and Siskind, 1964). The ΔH of the antibody-Dnp-lysine binding reaction was then measured with the batch calorimeter. The results obtained suggest that antibody to Dnp-lysine is homogeneous with respect to enthalpy over a 1.3 kcal/mol range of binding free energy.

Much of what is known about the antibody-antigen interaction has been inferred from studies on the antibody-hapten reaction. One would like to compare the interaction of antibody and the free hapten to the interaction of antibody and the hapten coupled to a protein carrier. In general, the antibody-antigen reaction is difficult to examine with optical techniques because the system is precipitated, except possibly at very high antigen excess. The batch calorimeter has the advantage that an optically clear solution is not required as is the case with many standard biochemical techniques. A previous calorimetric enthalpy measurement of an antibody-antigen reaction was made by Steiner and Kitzinger (1956), who examined the interaction of bovine serum albumin with a crude preparation of antibovine serum albumin antibody. They reported a ΔH of -3 ± 2 kcal/mol for this reaction. This system was quite complex due to antibodies reacting with many different determinant groups on the albumin molecule. To examine the thermodynamics of a more clearly defined antibody-antigen system, the interaction of purified anti-Dnp antibody with Dnp human serum albumin has been studied. Since the thermodynamic properties of the intrinsic binding reaction with Dnp-lysine was known, the energetic aspects of the antibody-Dnp-antigen interaction could be evaluated.

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[1] Abbreviations used are: Dnp-lysine, ϵ-*N*-2,4-dinitrophenyl-L-lysine; Dnp, dinitrophenyl; PBS, phosphate-buffered saline (0.15 M NaCl-0.01 M KH_2PO_4 (pH 7.3-7.4); Tnp, trinitrophenyl.

Experimental Procedure

Immunization. Four rabbits were immunized with Dnp bovine IgG in PBS that was emulsified with Freund's Complete Adjuvant (Miles Laboratories). Each rabbit was given 5 mg (2 ml of 2.5 mg/ml) of antigen subcutaneously into the footpads and on the back of the neck. The rabbits were bled after 4 weeks and the serum was pooled. The titer of this pool was approximately 1.6 mg of antibody/ml based on the precipitation of Dnp bovine serum albumin.

Preparation of Antibody. Antibody to the dinitrophenyllysyl group was purified from the 110-ml serum pool by the fractional precipitation method of Eisen and Siskind (1964). Five fractions of different affinity were obtained by adding the antigen, Dnp bovine serum albumin, in limiting amounts (approximately 15–20% of the amount required for maximum precipitation). The mixture was incubated 1 hr at 37° and then overnight at 4°. The precipitate was spun down and the supernate saved for isolation of the next fraction. The precipitate was washed five or six times with cold PBS and readily dissolved in 10 ml of 0.1 M Dnp-glycine. The antibody–antigen–hapten mixture was then fractionated on a split column of DEAE-cellulose and Dowex-1 (20 ml of DEAE-cellulose over 15 ml of Dowex) as described by Eisen *et al.* (1967). The protein containing fractions were pooled and concentrated by precipitation with ammonium sulfate (0.3 g/ml) and then dialyzed against PBS. The euglobulins were precipitated by dialysis against 0.001 M phosphate buffer (pH 7.4) and removed by Millipore filtration (0.8 μ pore size) and the antibody was dialyzed exhaustively against PBS.

Analysis of Antibody. The absorption spectrum from 400 to 240 nm of each antibody fraction was recorded with a Cary Model 14 spectrophotometer. The antibody absorption at 278 nm was obtained by subtracting the hapten contribution at this wavelength (Eisen *et al.*, 1967). The extinction coefficient of rabbit IgG was assumed to be $E_{278\,nm}(1\%)$ 15.5. The concentration of antibody bound Dnp-glycine was estimated from the optical density at 360 nm by assuming that the extinction coefficient was the same as the unbound, $E_{360\,nm}(M)$ 15,890 (Ramachandran and Sastry, 1962). The fraction of blocked sites was 17% or less for these fractions. The molecular weight of rabbit IgG was taken to be 150,000.

Preparation of Antigen. The Dnp–protein conjugates were prepared by mixing 2,4-dinitrobenzenesulfonate with the protein at a mass ratio of 2:1. After a 24-hr incubation at 20°, the conjugated proteins were exhaustively dialyzed against water and then put through a Dowex-1 column. The conjugates were dialyzed *vs.* PBS and stored in the frozen state. Protein concentrations and the number of covalently linked Dnp groups were determined by spectral analysis in 0.1 N NaOH. The bovine IgG, bovine serum albumin, and human serum albumin had 51, 15, and 30 Dnp groups, respectively.

Fluorescence Quenching Titrations. An Aminco-Bowman spectrophotofluorometer was used to measure the affinity of the antibody fractions for Dnp-lysine at 25°; 2-ml samples of the antibody solution (0.03 mg/ml) were titrated with 0.1 ml of Dnp-lysine ($\sim 10^{-5}$ M) in PBS. The hapten was added in 0.003–0.01-ml increments. The excitation and emission wavelengths were 280 and 341 nm, respectively. The samples were shielded from the high-intensity beam except during recording of the fluorescence. Appropriate volume and hapten attenuation corrections were made. The hapten attenuation correction was based on the titrations of free tryptophan or normal bovine IgG with Dnp-lysine. The hapten bound was assumed to be $(Q_{obsd}/Q_{max})(N)$(antibody concentration) where Q_{max} is the maximum quenching when all available combining sites are occupied by hapten. In all experiments Q_{max} was taken to be 0.73. The reported equilibrium constants are averages of at least two fluorometric quenching titrations and were calculated from Sips plots of the data (Karush, 1962). The estimated error of the equilibrium constants is about ±30%.

Equilibrium Dialysis. The binding affinity of the antibody fraction with highest affinity was determined by equilibrium dialysis using lucite microdialysis cells divided into two equal compartments by a single thickness of boiled, *N*-ethylmaleimide treated dialysis tubing. A 0.05-ml sample of the hapten, tritiated Ac-Dnp-lysine, was placed on one side of the chamber and 0.05 ml of the antibody solution, containing 0.14 mg/ml of the rabbit anti-Dnp antibody and 1 mg/ml of normal bovine IgG, was placed on the other side. The dialysis cells were incubated for 21 hr at 24.2° and then 0.025 ml was removed from each side and counted in 5 ml of scintillation fluid. The bound hapten concentration was obtained by subtracting the counts on the side without antibody from the counts on the side with antibody. Control experiments with normal bovine IgG indicated that membrane adsorption and nonspecific binding were not significant.

Calorimetry. Enthalpy measurements were made with an LKB Batch microcalorimeter in an air bath maintained at 24.5°. A 2-ml sample of antibody (~1 mg/ml) in PBS was put into the smaller part of the gold reaction chamber and 4 ml of Dnp-lysine ($\sim 5 \times 10^{-5}$ M) in PBS was put into the larger section. A glass syringe, weighed before and after injection, was used to determine the sample volume (solution density was assumed to be 1.005 g/ml). The two solutions to be mixed were adjusted to the same pH (within 0.003 pH unit) just prior to charging the calorimeter. The criterion of thermal equilibration was the absence of a significant base line drift over a 60-min interval with the Kiethley amplifier on the 1-μV scale. After the required equilibration period of about 24 hr, the two solutions were mixed by rotating the calorimetric unit with the amplifier on the 3-μV scale. After return to base line (about 12 min), the unit was rotated two additional times to determine the frictional heats and then an electrical calibration experiment was performed. The heat change in these reactions was calculated by measuring the area of the voltage *vs.* time curves on the recorder chart paper. The total heat measured in these experiments was usually about 0.27 mcal (areas ~30 cm^2). With these small heat quantities, problems with slight base-line shifts can be significant. Experiments with base-line shifts of 0.1 μV or more had to be discarded. Electrical calibrations in PBS using comparable heats gave a precision of 1.6% (standard error of the mean of six experiments). Separate experiments were performed to measure the heats of dilution and mixing of Dnp-lysine with PBS (total ~0.03 mcal). No heat of dilution correction was necessary for the dilution of the antibody solutions. The heat of dilution of bovine IgG with PBS at these concentrations was less than 20 μcal. In the experiments in antigen excess, where relatively high concentrations of antigen (~4 mg/ml) were used, a correction for the dilution and mixing of the antigen was required (~0.150 mcal).

Reagents. The Dnp-lysine and Dnp-glycine were obtained from Cyclo Chemical Company of Los Angeles. The bovine serum albumin was a Sigma fraction V preparation

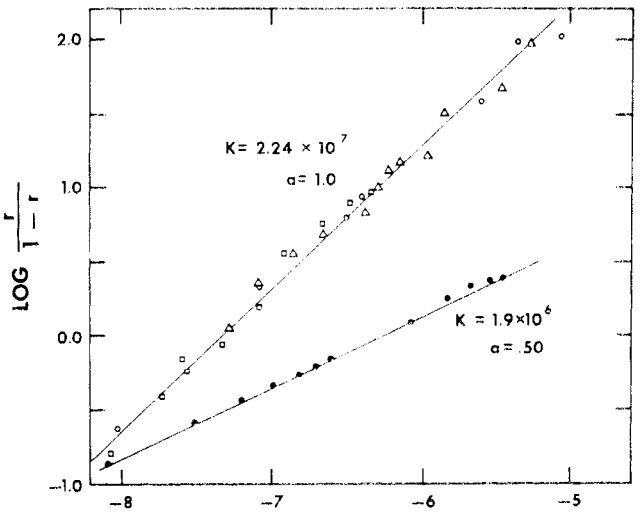

FIGURE 1: Sips plots of fluorescence quenching data for fraction I and fraction IV (r is ratio of bound hapten to antibody sites). The upper curve is data from fraction I. Each symbol (△, ○, □) represents data from a different experiment. The lower curve is the data from one experiment with fraction IV (●). K is the association constant in l./mol and a is the Sips heterogeneity coefficient.

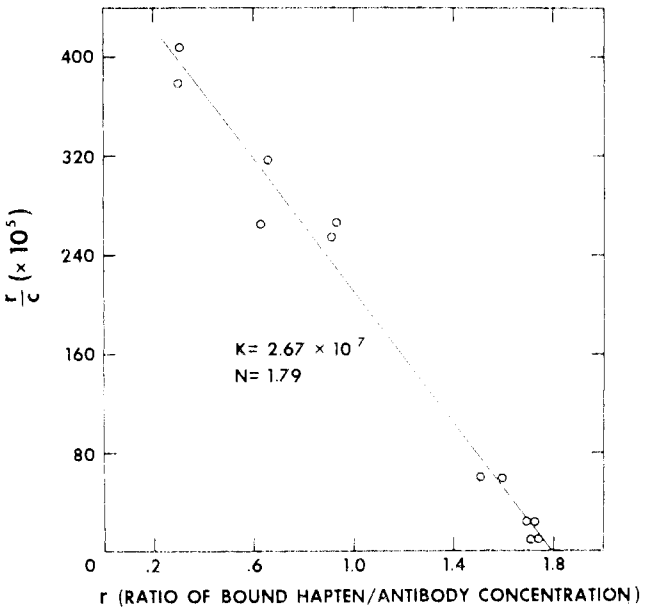

FIGURE 2: Scatchard plot of equilibrium dialysis data using fraction I. (C is the free hapten concentration). The linear least-squares fit of the data is shown. The intercept of this line is 1.79. K is the association constant in l./mol and N is the number of available sites per antibody molecule.

(Lot 33C-2940). The bovine IgG, A grade (Lot 300537), and the human serum albumin fraction V powder (Lot 300402) were purchased from Calbiochem. Tritiated A-Dnp-lysine was prepared by Dr. Elizabeth S. Dugan. The 2,4-dinitrobenzenesulfonic acid, sodium salt, was obtained from Eastman.

Results

Five antibody fractions with specificity for the Dnp-lysyl group were isolated from the serum pool by precipitation with Dnp coupled to a heterologous carrier (*i.e.*, bovine serum albumin). The total overall yield after purification was about 20%. The affinity of these fractions was evaluated by the fluorescence quenching method. The resulting Sips plots for two of these (I and IV) are shown in Figure 1. The data include three separate titrations performed on different days with fraction I. The curve is linear over a three log concentration range. In the lower curve is shown the plot from one titration with fraction IV. The equilibrium constant was evaluated as the reciprocal of the log free hapten concentration where log $r/(1-r)$ was equal to zero. The results of these fluorescence quenching titrations and the spectral determinations of N for the five fractions are summarized in Table I.

Table I: Analysis of Fractions Isolated by Fractional Precipitation of Rabbit Anti-Dnp Serum.

Fraction	K (l./mol)[a]	Sips Heterogeneity Coefficient (a)	N(no. of Free Sites per Antibody Molecule)[b]
I	2.24×10^7	1.0	1.66
II	8.2×10^6	0.51	1.84
III	2.1×10^6	0.42	1.75
IV	1.5×10^7	0.53	1.98
V	1.32×10^6	0.55	1.71

[a] An average of at least two fluorescence quenching experiments at 25°. [b] Obtained from analysis of spectrum.

Table II: Thermodynamic Analysis of the Fractions Isolated from Rabbit Anti-Dnp Serum.

Fraction	ΔG (kcal/mol)	$-\Delta H$ (kcal/mol of Sites) Obsd[c]	Corrected[a]
I	−10.0	13.40	13.40
		12.90	13.00
II	−9.43	11.80	13.30
		14.50	15.60
III	−8.62	11.40	13.80
		12.20	14.80
IV	−8.42	12.50	14.50
V	−8.35	11.10	12.60
	Av[b]	12.50 (±0.39)	13.90 (±0.35)

[a] Enthalpy values corrected for unliganded sites by assuming a Sips distribution of affinities; fraction of sites without ligand = $1/(1 + K^a c^a)$ where c is the free Dnp-lysine concentration, a is the Sips heterogeneity coefficient, and K is the association constant. [b] Error indicated is the standard error of the mean. [c] $\Delta H = (Q_{exp} - Q_{hapten})/((\text{moles of antibody})N)$ where Q_{exp} and Q_{hapten} refer to the measured heats in calories for the mixing of hapten with and without antibody.

The number of available antibody sites, N, was determined by an equilibrium dialysis experiment performed with the rabbit anti-Dnp antibody fraction I and tritiated Ac-Dnp-lysine. This experiment is represented in Figure 2. A value of 1.79 sites/molecule from the equilibrium dialysis experiment was within 6% of the value from the spectral analysis. The equilibrium constant obtained from the Scat-

Table III: Comparison of the High and Low Affinity Fractions of Rabbit Anti-Dnp Antibody.

	ΔG (kcal/mol)	ΔH (kcal/mol)[a]
High affinity (fractions I and II)	−9.72	−13.85 (±0.59)
Low affinity (fractions III, IV, and V)	−8.46	−13.93 (±0.48)
Difference	−1.25	0.08

[a] The errors indicated refer to the standard error of the mean.

Table IV: Enthalpy of the Antibody–Antigen Reaction.

Expt	Molar Ratio of Dnp_{30}-HSA/antibody	ΔH (per mol of Sites) (kcal/mol)
1	19	−10.6
2	24	−9.44
3	0.5 (equivalence)	−10.2

[a] Antibody was rabbit anti-Dnp fraction II, $K = 8.2 \times 10^6$ l./mol and $\Delta H = -13.9$ kcal/mol for Dnp-lysine; antigen (3.9 mg/ml in experiments 1 and 2 and 0.092 mg/ml in experiment 3) was human serum albumin (HSA) with average of 30 Dnp groups/molecule.

chard plot in Figure 2 ($K = 2.67 \times 10^7$ with Ac-Dnp-lysine) was also close to the fluorescence quenching result ($K = 2.24 \times 10^7$ with Dnp-lysine). Further, both the linearity of the Scatchard plot of the equilibrium dialysis data and the Sips coefficient of 1 from the fluorescence titration indicate that this fraction is homogeneous with respect to affinity.

The free energies of binding calculated from the equilibrium constants are presented in Table II. In addition, the eight different enthalpy measurements for the five fractions are shown. Fractions IV and V were not available in sufficient amounts to permit duplicate calorimetric determinations. Since fractions II–V contained a distribution of antibodies (Sips coefficients all approximately 0.5), the lower affinity sites in the distribution were not saturated at the hapten concentrations used and therefore the observed ΔH values were corrected as noted in Table II. An average ΔH of −13.9 kcal/mol was obtained. This value is in good agreement with the ΔH of −14.1 kcal/mol reported by Barisas *et al.* (1972).

In Table III the higher affinity fractions (I and II) are compared with the lower affinity fractions (III, IV, and V) in terms of the free energy changes and the enthalpy change. Although a difference of −1.25 kcal/mol in the free energy of binding was observed, there appears to be no *significant* difference in the binding enthalpy.

The ΔH of the reaction of rabbit antibody specific for the Dnp-lysyl group with Dnp human serum albumin was examined in the batch calorimeter and the results are shown in Table IV. Experiments 1 and 2, performed with the antigen in excess, provided an average ΔH of −10.0 kcal/mol. No detectable precipitation in experiment 1 and a slight amount of precipitation in experiment 2 was observed when the solutions were examined 30 min after mixing. In experiment 3 the enthalpy of the antigen–antibody reaction was examined near equivalence. In this case the solution was turbid when inspected at the conclusion of the experiment. The resulting ΔH of −10.2 kcal/mol is not significantly different from the enthalpy change measured in antigen excess. The errors in these ΔH measurements are estimated to be ±1 kcal/mol. In these experiments no correction for unliganded sites was necessary because the affinity enhancement arising from the multivalent nature of the interaction (see Karush, 1970) assured saturation of the system.

Discussion

The experiments of Eisen and Siskind (1964) provided direct evidence that rabbit anti-Dnp antibody was heterogeneous with respect to affinity. In these experiments fractions of different affinity were prepared by precipitating the antibody pool with limiting amounts of antigen. Their findings and the results presented in Table I demonstrate that when a limiting amount of antigen is added to a heterogeneous population of antibodies, the antibodies with highest affinity are preferentially precipitated. The spread in affinity in the five fractions reported here, differing by a factor of 17, was not as large as found for some of the rabbits of Eisen and Siskind (1964). In their experiments ten fractions were isolated from individual rabbits since only very small amounts of antibody were required to measure the equilibrium constant by fluorescence quenching.

In the enthalpy calculations the number of available antibody combining sites, N, was obtained from the spectral analysis of the purified antibody. The moles of Dnp-glycine bound to the antibody, and hence the moles of sites unavailable, was estimated from the OD at 360 nm. For this calculation it was assumed that the Dnp-glycine extinction coefficient in free solution is the same as when bound. The dinitrobenzene extinction at 360 nm may decrease when the ligand is bound to the antibody (Little and Eisen, 1967) but any such effect should be small, systematic, and have no significant effect on our conclusions.

An error in the enthalpy determination might also occur if antibody not specific for Dnp were isolated along with the anti-Dnp specific antibody. This might occur, for example, if the serum pool contained antibodies to aggregated rabbit IgG. Such contamination would be most significant in the first fraction precipitated from the serum pool. Therefore the number of available binding sites per mole of antibody for this fraction was examined by equilibrium dialysis. The estimate of N obtained was actually slightly larger than the spectral estimate, indicating that antiglobulins were not contaminating our preparation.

The average ΔH of binding measured, −13.9 kcal/mol, indicates that the Dnp-lysine binding reaction of rabbit antibody is enthalpically driven. The reaction actually has an unfavorable unitary entropy change (−5.1 to −10.6 cal/(mol deg)). A similar result was obtained by Barisas *et al.* (1972) for both anti-Tnp and anti-Dnp antibody.

This finding is somewhat surprising insofar as hydrophobic interactions are thought to be involved and therefore one would predict a positive entropy contribution. The negative heat capacity change observed for the binding reaction (Barisas *et al.*, 1972) and the negative heat capacity change for transfering Dnp-lysine from water to ethanol (Halsey and Biltonen, 1975) do, in fact, suggest that hydrophobic interactions are important in spite of the overall negative ΔS of binding. If this is indeed the case then a large nega-

tive entropic contribution is associated with other aspects of the hapten–antibody interaction. A small negative ΔS (~ -8 cal/(mol deg)) would be expected for rigidification of the lysyl chain upon binding. In addition any "tightening" of the protein structure would also make a negative contribution to ΔS. More detailed thermodynamic studies are required to clarify this situation.

Homogeneity in Enthalpy. In the experiments reported here the same method could be used to measure the ΔH of binding of both high and low affinity antibody because the fractions of different affinity were *isolated*. These results for the five fractions presented in Table II indicate that there was no *large* spread in enthalpy for rabbit antibodies of different affinity. Thus, these findings with anti-Dnp antibody are in accord with the conclusions of Sturtevant and his collaborators (Barisas *et al.*, 1972) for anti-Tnp antibody.

There was no significant correlation between the free energy changes and enthalpy changes. The coefficient of correlation between the two quantities was calculated to be -0.14 which is not statistically significant. On the other hand, the correlation between ΔS and ΔG was found to be significant. The slope of the least-squares regression line relating ΔG and ΔS was 4.1 with a coefficient of correlation of 0.64 which is statistically significant ($P < 0.001$). This is also apparent in Table III where the fractions were divided into higher and lower affinity populations. Although a difference in the ΔG of binding of 1.25 kcal/mol is observed, the ΔH values for these two pools appear to be identical. Therefore it follows that any differences in the free energy of binding primarily reflect changes in only the entropy term. For example, the higher affinity antibody molecules may have "deeper" sites and would thus be able to better "remove" some of the lysyl CH_2 groups from contact with water (see Carson and Metzger, 1974). This hydrophobic interaction could provide sufficient entropy gain to account for the increase in affinity. The generality of the homogeneity in enthalpy reported here for anti-Dnp antibody is of course unknown since only a factor of 17 in affinity separates fractions I and V. It is possible that if *very* large differences in affinity (*i.e.*, 10^6–10^9) could be examined one may find that both the entropy and enthalpy terms contribute to the affinity differences.

Antibody–Antigen Binding. Arend and Mannick (1974) have shown that with rabbit anti-human serum albumin and human serum albumin the complexes formed at high antigen excess are primarily $antigen_2$ $antibody_2$ and $antigen_1$ $antibody_1$. In the equivalence zone a much larger structure is presumably formed, *i.e.*, a lattice structure in which the ratio of antibody to antigen is close to 3. The apparent ΔH of the interaction is -10.0 kcal/mol in antigen excess and -10.2 kcal/mol at equivalence. It is noteworthy that no significant difference in the ΔH for the reaction is observed under these different conditions. Thus, there are no apparent enthalpy contributions present in the lattice structure at equivalence that are not present in antigen excess, where smaller aggregates are assumed to predominate. No additional loosening of the binding interaction appears to occur when the larger cross-linked structure was formed.

The ΔH of the antibody–antigen interaction is approximately 4 kcal/mol less exothermic than the antibody–hapten interaction. We can speculate on possible reasons for this difference. It is possible that with the Dnp coupled to the human serum albumin molecule the lysyl side chain cannot make good van der Waals contacts with the residues in the antibody combining site. This is not likely to be the complete explanation for the 4 kcal/mol difference. The difference in ΔH might also reflect a reduction or loosening in the contacts with the dinitrophenyl group required to permit the two proteins to come together.

Although the enthalpy of binding is less negative when the ligand is coupled to human serum albumin, the antibody–antigen reaction is still enthalpically favored. The multivalency of both the antibody and the antigen provides for a significant enhancement in "functional affinity" (see Karush, 1970). Such entropic contributions could improve the free energy change of the antibody–antigen interaction relative to the antibody–hapten reaction. As developed theoretically by Crothers and Metzger (1972) and demonstrated experimentally by Hornick and Karush (1972), these contributions would lead to affinity enhancements of several orders of magnitude. The present results tend to confirm these expectations. Assuming that antigen–antibody affinity is greater than hapten–antibody affinity, the fact that ΔH is less negative for the former reaction requires that the enhanced affinity is the result of a more favorable entropy change.

Acknowledgments

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