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Structural and Thermodynamic Basis of Affinity in Anti-Dinitrophenyl Antibody†

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ABSTRACT: The thermodynamic quantities of the anti-dinitrophenyl antibody–hapten interaction are reported for rabbit, goat, and guinea pig antibodies. Rabbit and goat antibodies had similar exothermic enthalpy changes for their reaction with 2,4-dinitrophenyl-L-lysine (−13.9 and −14.8 kcal/mol, respectively). The enthalpy change with guinea pig antibody was much less exothermic (−8.7 kcal/mol), and this value was the same for two guinea pig antibody preparations that differed in affinity by almost two orders of magnitude. A heterogeneous goat anti-dinitrophenyl antibody preparation was fractionated on a molecular sieve column in the presence of a bivalent ligand, a procedure that has been reported to separate antibodies according to differences in the depth of interaction with the ligand. The relationship of these differences in apparent site *depth* to changes in interactions with the hapten tail was examined by comparing the affinities of various fractions for two haptens. The results show that the presumed *deeper* sites have stronger interactions with the hapten tail. These studies suggest that the heterogeneity of anti-dinitrophenyl antibodies with respect to affinity results from differences in entropy driven lysyl side-chain interactions which arise from a heterogeneity in antigen binding site *depth*.

The physical-chemical principles governing antibody–antigen interaction will be revealed only when the continually emerging antibody structure information can be quantitatively correlated with sound thermodynamic data for interaction with various ligands. Such correlation will provide a "thermodynamic mapping" of the antigen binding site in terms of the energetics of specific ligand–antigen binding site interactions. In addition such studies will lead to an understanding of the thermodynamic basis of variation in affinity within a heterogeneous antibody population. Essential to any such efforts, however, is the acquisition of reliable and meaningful thermodynamic quantities.

Since most antibody preparations constitute a heterogeneous population of molecules, derived thermodynamic quantities generally represent a complex average value and their relationship to a true thermodynamic value is ambiguous. This is particularly true for enthalpy changes estimated by a van't Hoff analysis based on average association constants. Barisas et al. (1972) have shown, for example, that the van't Hoff ΔH for the binding of Dnp[1]- and Tnp-lysine to their respective antibodies underestimated the true enthalpy change, determined calorimetrically, by as much as 10 kcal/mol. It is therefore apparent that meaningful enthalpy changes for hapten–antibody reactions can best be obtained by direct calorimetric measurements.

In this communication the results of thermodynamic studies on the binding of Dnp-lysine and dinitroaniline to anti-Dnp antibodies from rabbit, goat, and guinea pig are reported. These results support previous conclusions that

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[1] Abbreviations used are: Dnp, dinitrophenyl; Dnp-lysine, *N*-2,4-dinitrophenyl-L-lysine; Tnp, trinitrophenyl; Dnp-D-GL, copolymer of D-glutamic acid and D-lysine with dinitrophenyl groups on ε-amino; bis-Dnp-lysine, *N,N'*-didinitrophenyl-L-lysine.

the heterogeneity in affinity is primarily due to differences in the entropy change for the interaction (Halsey and Biltonen, 1975a).

Using a method first described by Warner and Schumaker (1970), goat anti-Dnp antibody was fractionated into two populations: those that can form dimeric or polymeric complexes with a bivalent hapten and those that can only form monomeric complexes with the ligand. Carson and Metzgar (1974) and Wilder et al. (1975) showed that this fractionation was most likely based on differences in antigen binding site *depth*[2] such that the monomer-forming antibody molecules had combining sites too *deep* to be cross-linked by the bivalent ligand. In the studies reported below the affinities of these two populations of antibodies for Dnp-lysine and dinitroaniline were measured. The results provide independent support for the conclusion that the column procedure separates molecules according to differences in the depth of the interaction.

Methods

Antibody Preparations. Rabbit anti-Dnp (preparation 1) was prepared from sera obtained from rabbits immunized 4 weeks previously with 5 mg of Dnp-bovine γ-globulin in complete Freund's adjuvant (Halsey and Biltonen, 1975a). At 10 weeks these same rabbits were given an intravenous challenge (5 mg with no adjuvant) and bled 7 days later. The anti-Dnp antibody from this secondary response (preparation 2) was isolated in a similar manner. Sera was obtained from a goat that had been challenged five times with Dnp-bovine γ-globulin. The primary immunization was intramuscularly with 20 mg of Dnp-bovine γ-globulin in complete Freund's adjuvant and the booster immunizations were with 40 mg in incomplete Freund's adjuvant. Guinea pig antibody was isolated from sera obtained from the family 13 strain of inbred pigs that had been immunized with 0.4–0.6 mg of Dnp-keyhold limpet hemocyanin in complete Freund's adjuvant. Tnp-serum albumin was used to precipitate the antibody and dinitrophenol was used to dissolve the washed precipitate. For all these preparations the antibody was separated from antigen and hapten by the split column (DEAE-cellulose-Dowex 1) method described by Eisen et al. (1967). The guinea pig I preparation was further fractionated on a DEAE-52 column to purify the γ_2 isotype. The pigs used to produce the guinea pig III antibody had been pretreated with Dnp-D-GL. This procedure leads to a preferential depression of the capacity to produce high affinity anti-Dnp antibody (Davie et al., 1972). This antibody preparation contained both the γ_1 and γ_2 isotypes.

Separation of antibody according to apparent differences in combining site depth was effected by mixing 4.5 mg of goat anti-Dnp antibody with equimolar bis-Dnp-lysine. Following a 30-min incubation, this mixture was applied to a Sephadex G-200 column (2.1 cm × 42.5 cm) which had been equilibrated with phosphate-buffered saline (0.15 *M* NaCl–0.01 *M* phosphate (pH 7.3)). The sample was eluted at a rate of 5 ml/hr and 1-ml fractions were collected. Fractions were pooled as indicated, dialyzed several days vs. 0.05 *M* Dnp, passed through a Dowex 1 column, and then concentrated by vacuum dialysis. The percentage of hapten blocked sites for pools I, II, and III was estimated by analysis of the spectra to be 30, 31, and 35%, respectively. No dimer or polymeric antibody was observed when the goat antibody was chromatographed on the G-200 column without bivalent ligand.

The affinity of the different antibody preparations for Dnp-lysine and dinitroaniline was determined by a Sips analysis of fluorescence quenching experiments at 25°C (Eisen and Siskind, 1964). This method is often not extremely accurate with "hyperimmune" sera due to greater asymmetry in the affinity distribution and heterogeneity in Q_{max}. However, the resulting uncertainty in the free energy change is rather small. As a check on the fluorescence quenching method, the affinities of both the goat and rabbit antibody preparations for Dnp-lysine were also examined by equilibrium dialysis.

The calorimetric measurements of the antibody–hapten reaction were made with an LKB batch microcalorimeter (see Halsey and Biltonen, 1975a). Generally, antibody concentrations of 0.5–1 mg/ml and hapten concentrations of $\sim 5 \times 10^{-4}$ *M* were mixed and heats of about 150 μcal measured. The heats were corrected for the fraction of unreacted sites, $f_u = 1/[1 + K^a(H)^a]$ where [H] represents the final free hapten concentration and a the Sips heterogeneity coefficient. These corrections in the reported ΔH values were less than 2% for all antibodies except the goat antibody where an 8% correction was required.

Results and Discussion

The thermodynamic quantities associated with the binding of Dnp-lysine and dinitroaniline to anti-Dnp antibody preparations from rabbit, goat, and guinea pig are summarized in Table I. A range in affinity for Dnp-lysine of almost two orders of magnitude is observed but the range in the affinity for dinitroaniline is less than a factor of 2. These observations suggest that the differences in affinity of the antibodies for Dnp-lysine may be found primarily in the interaction of the lysyl side chain with the antigen-combining region.

A priori, it is reasonable to assume that the free energy for binding Dnp-lysine is the sum of the free energies of interaction of the dinitrophenyl and lysyl moieties and that the dinitrophenyl contribution is equivalent to the free energy change for dinitroaniline binding. To the extent that this assumption is correct, the Dnp group interaction appears to provide most of the favorable free energy change for binding Dnp-lysine. A similar conclusion was made by Eisen and Siskind (1964) for rabbit antibodies. The affinities of the rabbit and guinea pig antibodies for dinitroaniline were all in the range of $3–5 \times 10^6$ l./mol; and for preparations 1, 2, and 5 this interaction provided about 90% of the total free energy change. Although preparation 4 had a similar affinity for dinitroaniline, it had a much higher affinity for Dnp-lysine. In this case the apparent contribution of the lysyl group was about 25% or 2.9 kcal/mol.

Comparison of the Calorimetric ΔH's for Antibody from Different Species. The calorimetric enthalpy changes measured for the binding of Dnp-lysine to the two rabbit preparations and the goat preparation (Table I) were about −14 kcal/mol (i.e., −13.7 to −14.8 kcal/mol). The rabbit antibody examined calorimetrically by Barisas et al. (1972) had a similar enthalpy change (−14.1 kcal/mol). It appears that the high affinities that are generally observed for anti-Dnp antibodies are due to conserved structural features that provide large exothermic enthalpy changes upon interaction with Dnp-lysine. For the rabbit and goat antibodies, the en-

[2] Depth is operationally defined here in terms of these fractionations with bivalent ligands and no structural description is implied. Since the antigen combining region appears to be a cleft lined with the hypervariable residues, the fractionation may reflect differences in the length of this antigen binding crevice.

Table I: Hapten Binding Thermodynamics of Different Anti-DNP Antibody Preparations.[a]

Antibody preparation[b]	Hapten	K^c (l./mol)	ΔG (kcal/mol)	ΔH^d (kcal/mol)	ΔS (cal/ (mol deg)
1. Rabbit anti-Dnp	Dnp-lysine	2.2×10^7	−10.0	−13.9 ± 0.4 (8)	−13.1
	Dinitroaniline	3.2×10^6	−8.8		
2. Rabbit anti-Dnp	Dnp-lysine	3.9×10^7	−10.3	−13.7 ± 0.7 (3)	−11.4
	Dinitroaniline	5×10^6	−9.13		
3. Goat anti-Dnp	Dnp-lysine	4×10^7	−10.4	−14.8 ± 0.1 (2)	−14.9
4. Guinea pig anti-Dnp-I	Dnp-lysine	7×10^8	−12.1	−8.7 ±1.0 (4)	+11.4
	Dinitroaniline	5.5×10^6	−9.2		
5. Guinea pig anti-Dnp-III	Dnp-lysine	8.5×10^6	−9.45	−8.8 ± 1.0 (2)	+2.2
	Dinitroaniline	3.4×10^6	−8.9		

[a] The standard state for these thermodynamic quantities is 1 mol/l. [b] The percentage of sites blocked with hapten after purification and the Sips coefficients for Dnp-lysine for these five preparations are: 1 (17%, 1.0), 2 (44%, 0.45), 3 (12%, 0.3), 4 (6%, 0.37), and 5 (2%, 0.63). [c] An average of two to three fluorescence quenching titrations. The equilibrium constants obtained by equilibrium dialysis for preparations 1 and 3 were 2.67×10^7 and 2×10^7 l./mol, respectively (for Dnp-lysine). [d] The indicated error refers to the standard error of the mean or the range in the case of only two measurements. The number in parentheses indicates the number of calorimetric experiments.

tropy changes are negative, suggesting that the hydrophobic interaction of the lysyl side chain provides a relatively minor contribution to the overall driving force insofar as one would expect large positive entropy contributions for hydrophobic interactions. As discussed below, *differences* in interactions with the hapten tail may be the origin of heterogeneity in affinity. The calorimetric ΔH's for the two guinea pig antibody reactions with Dnp-lysine were both about −9 kcal/mol. For these antibodies positive entropy changes are associated with the binding reaction. This finding suggests that hydrophobic interactions involving the lysyl side chain are more important in guinea pig antibody than in rabbit and goat antibody.

Entropic Basis of Heterogeneity. In a previous paper (Halsey and Biltonen, 1975a), the separation of a rabbit anti-Dnp antibody pool into fractions with different binding affinity was reported. When these five fractions, which varied in affinity over a 20-fold range, were examined calorimetrically, they appeared to be enthalpically similar, indicating that the heterogeneity in affinity was primarily the result of differences in the entropy of interaction. In Table I the enthalpy data for preparations 4 and 5 suggest a similar homogeneity for guinea pig anti-Dnp antibody. Here the two antibody fractions, differing in affinity by a factor of 82 and in free energy by 2.65 kcal/mol, have no significant difference in binding enthalpy. Since the enthalpy change is virtually identical (i.e., −8.7 kcal/mol for both), the difference in affinity for Dnp-lysine seen in preparations 4 and 5 (Table I) is due to a difference in the entropy of interaction. A logical basis for such differences is in the degree of hydrophobic interactions involving the lysyl side chain.

The ratio of affinities (Dnp-lysine/dinitroaniline) for conventionally immunized guinea pigs was 127 and for the Dnp-D-GL pretreated pigs the ratio was 3.5. To the extent that this ratio is proportional to antibody interaction with the lysyl tail part of the hapten, the pigs that were pretreated with Dnp-D-GL produce a lower affinity antibody molecule that appears to interact minimally with the tail part of the hapten molecule. Since the affinities of these two preparations for dinitroaniline are essentially the same, the higher affinity observed for Dnp-lysine in the antibodies from conventionally immunized pigs is therefore due to additional interactions with the lysyl side chain.

Carson and Metzgar (1974) have shown that in a population of rabbit anti-Dnp antibodies there exists a heterogeneity in apparent antigen combining site *depth*. Further, they found that the antibody fraction with *deeper* sites also had the higher affinity for Dnp-lysine. A priori, one would predict that increasing the effective combining site depth in anti-Dnp antibody would improve the entropy of the interaction with Dnp-lysine by reducing the contact of the lysyl group with water. The large negative heat capacity changes observed for Dnp-lysine binding (Barisas et al., 1972) and for removing Dnp-lysine from water (Halsey and Biltonen, 1975b) indicate that such hydrophobic interactions could be important. It is possible that these two guinea pig antibody populations differ significantly in antigen combining site *depth* and that the antibody from the Dnp-D-GL treated guinea pigs has shallower sites than conventional antibody.

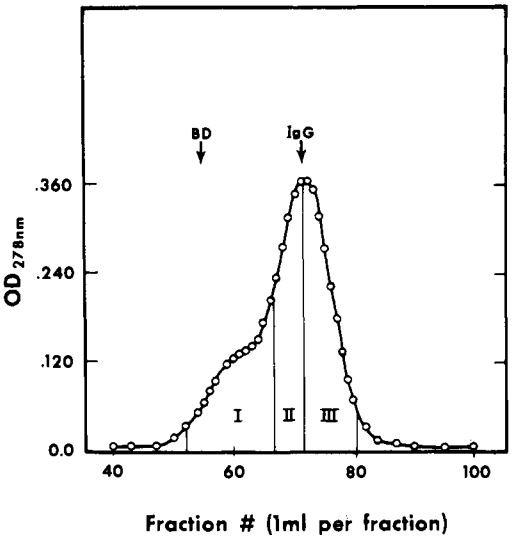

FIGURE 1: G-200 filtration of goat anti-Dnp antibody and bis-Dnp-lysine. BD indicates where blue dextran eluted and IgG indicates where goat anti-Dnp antibody without ligand eluted. Pool I should contain dimers and polymers and pools II and III should contain antibody monomers.

Heterogeneity, Site Depth, and Hapten Tail Interactions. Since sufficient quantities of goat anti-Dnp antibody were available, the relationship between differences in affinity, antigen combining site *depth*, and interactions with the hapten tail was investigated. Warner and Schumaker (1970) have demonstrated that anti-Dnp antibody can be fractionated with bivalent haptens into populations that can

Table II: Interactions with the Hapten Tail of Goat Antibodies from G-200 Pools.

	Ratio of Equilibrium Constants[a] $\frac{K_{(\text{Dnp-lysine})}}{K_{(\text{dinitroaniline})}}$	$\Delta(\Delta G)^b$ (kcal/mol)
I	$\frac{3.9 \times 10^7 (\pm 0.1)}{1.64 \times 10^7 (\pm 0.3)} = 2.38$	0.5
II	$\frac{1.4 \times 10^8 (\pm 0.7)}{2.1 \times 10^7 (\pm 0.1)} = 6.6$	1.1
III	$\frac{1.9 \times 10^8 (\pm 0.6)}{2.9 \times 10^7 (\pm 0.7)} = 6.6$	1.1

[a] Each affinity reported is average of two to four fluorescence quenching titrations. Errors refer to standard error of the mean. [b] $\Delta(\Delta G) = -RT \ln K_{(\text{Dnp-lysine})} + RT \ln K_{(\text{dinitroaniline})}$.

form dimer antibody complexes and those that can only form monomers. This heterogeneity was concluded to be due to differences in combining site *depth* (Carson and Metzgar, 1974) such that the monomer-forming antibodies were viewed as having sites too deep to allow intermolecular cross-linking.

In Figure 1 the G-200 elution profile of a goat antibody and bis-Dnp-lysine mixture is shown. With this bivalent ligand about 75% of the antibody eluted as an IgG monomer and 25% was dimeric (or polymeric). The fractions were pooled as indicated in the figure, and after purification the affinity of each of the pools for Dnp-lysine and dinitroaniline was measured. The results are shown in Table II. For all these pools the affinity for dinitroaniline was found to be similar but the monomer-forming antibody (pools II and III) had much higher affinity for Dnp-lysine than did pool I. The ratios of affinities for the two ligands in Table II demonstrate clearly that depth differences implied from the elution position on the G-200 column *are* correlated with improved or deeper interaction with Dnp-lysine.

As an initial estimate, the $\Delta(\Delta G)$ shown in Table II can be taken as a measure of the apparent free energy of the interaction with the hapten tail. Pools II and III which are monomer forming and thus presumably have *deeper* sites are seen to have much greater interaction free energy with the lysyl moiety than pool I, the dimer-forming antibodies. These apparent differences are not sufficiently accurate to justify a detailed discussion of the energy differences. Such a quantitative treatment will be possible when equilibrium dialysis and calorimetric measurements can be made on antibody fractions in which the percentage of hapten-blocked sites is lower than in these preparations. It bears noting that the correlation between the apparent *depth* and hapten tail interaction is not an artifact due to the relatively large percentage of hapten-blocked sites. Since the sites with highest affinity for Dnp-lysine are preferentially blocked, the observed correlation would be more pronounced if all sites were available.

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