Thermodynamics of Antibody–Antigen Reactions. 1. The Binding of Simple Haptens to Two Classes of Antibodies Fractionated According to Affinity†

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ABSTRACT: The thermodynamic parameters which characterize the binding of dinitrophenylglycine and dinitrophenylmethoxypoly(ethylene glycol) to selected affinity classes of equine IgG and IgG(T) antibodies were determined by fluorescence quenching and flow calorimetry. The binding enthalpies and entropies were in all cases large and negative, falling in the ranges −14 to −17 kcal/mol and −18 to −25 eu, respectively. The differences in the enthalpies and entropies of binding for different affinity classes and for different haptens are discussed with reference to differences in the structures of the haptens studied and as indications of differences in binding site structure. In addition, the apparent existence of fluorescent side chains which can transfer energy to either hapten binding site in IgG(T) antibodies but not in IgG antibodies is interpreted as indicative of a smaller average interbinding site distance in IgG(T) than in IgG antibodies.

The experiments described in this report were undertaken in order to obtain a complete thermodynamic characterization of the binding of certain Dnp[1]-haptens to homogeneous populations of equine antibodies of the IgG and IgG(T) immunoglobulin classes. Barisas et al. (1971, 1972) have found, from binding isotherms deduced from fluorometric titrations and direct determinations of binding enthalpies by flow calorimetry, that the binding of Dnp-haptens to heterogeneous rabbit antibodies is driven by large negative enthalpies of binding and is accompanied by large reductions in entropy. Johnston et al. (1974) reported that similar changes in thermodynamic parameters also characterized the binding of Dnp-haptens to homogeneous mouse myeloma proteins. More recently Halsey and Biltonen (1975) and Halsey et al. (1975) have confirmed this characterization of the binding of Dnp-haptens in studies with rabbit antibodies which were fractionated according to affinity and also with goat and guinea pig antibodies. This paper extends the description of the binding of Dnp-haptens by antibody to two purified immunoglobulin classes of horses. Because the antibodies used in this study were also fractionated to near homogeneity in affinity for hapten, some tentative conclusions can be drawn from the differences in the values of the enthalpy and entropy changes found for the binding of different Dnp-haptens to different affinity classes of antibodies. These experiments were done in order to obtain well-characterized preparations of antibodies which could be used in studies (Archer and Krakauer, 1977) in which random-coil divalent Dnp-antigens were used as probes of average antibody hinge conformations.

Materials and Methods

Antibody. Antibodies with specificity for the Dnp determinant were isolated and fractionated according to affinity for the hapten and immunoglobulin class as previously described (Archer et al., 1973). Fractions with the highest (fraction 1) and lowest (fractions 5 and 6) affinities were used in the studies described below. The ratios of absorbances at 360 and 278 nm were in the range 0.008 to 0.013, indicating that the antibodies were substantially free of residual bound hapten. Concentrations of the antibodies were determined spectrophotometrically using molar absorbancies at 278 nm of $(1.941 \pm 0.010) \times 10^5$ L mol^{-1} cm^{-1} for IgG and $(1.880 \pm 0.009) \times 10^5$ L mol^{-1} cm^{-1} for IgG(T). These absorbancies were determined by light-scattering measurements described in the following paper (Archer and Krakauer, 1977).

Haptens. The monovalent haptens Dnp-glycine and Dnp-MP were used. Dnp-glycine was used as purchased and concentrations were calculated from a molar absorbancy of 15 900 L mol^{-1} cm^{-1} at 360 nm (Little and Donahue, 1968). Dnp-MP was prepared as described (Cooke et al., 1974). Its molar absorbancy is maximal at 298 nm where it is 10 900 ± 50 L mol^{-1} cm^{-1}.

Measurement of Hapten Binding. The binding of haptens to antibodies was studied by fluorescence quenching titrations interpreted in a way which allowed for the possibility of unequal quenching by the binding of the first and second hapten (Archer et al., 1973). In this method, the total quenching, Q, is related to the average fractional site occupancy, θ, by the expression

$$Q/\theta = 2q_1 - (q_1 - q_2)\theta \quad (1)$$

where q_1 and q_2 are the reductions in the quantum yield of the antibody consequent to the binding of the first and second haptens respectively. Average site association constants, K, and heterogeneity indices, a, were estimated assuming a Sips distribution of affinities (Sips, 1948). Measurements were made at ambient temperatures (22 to 25 °C) in air-conditioned rooms. The binding of Dnp-glycine was studied in 0.15 M NaCl–0.01 M sodium phosphate buffer, pH 7.8. Measurements were made in a Turner Model 430, a Perkin-Elmer Model MPF-2, or a modified Brice Phoenix Series 2000 light-scattering photometer (Archer, 1975).

Calorimetry. A Beckman Model 190C flow reaction calorimeter (Sturtevant and Lyons, 1969) was operated in a heat

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[1] Abbreviations used: Dnp, 2,4-dinitrophenyl; MP, methoxypoly(ethylene glycol) 750.

TABLE I: Association Constants and Heterogeneity Indices for the Binding of Haptens to Antibodies.

Antibody	Hapten	Log K^a	a^a
Fraction 1 IgG(T)	Dnp-glycine	7.48 ± 0.09	1.07 ± 0.16
Fraction 1 IgG(T)	Dnp-MP	6.64 ± 0.02	1.02 ± 0.05
Fraction 5 IgG(T)	Dnp-glycine	6.20 ± 0.02	0.81 ± 0.03
Fraction 5 IgG(T)	Dnp-MP	6.31 ± 0.02	0.73 ± 0.03
Fraction 6 IgG(T)	Dnp-glycine	6.32 ± 0.02	1.00 ± 0.03
Fractions 1-3 IgG	Dnp-MP	6.33 ± 0.03	0.81 ± 0.03

[a] Listed uncertainties are standard errors.

TABLE II: Reductions in Antibody Fluorescence Consequent to Hapten Binding.

Antibody	Hapten	$q_1{}^a$	$q_2{}^a$	$q_1 + q_2$
Fraction 1 IgG(T)	Dnp-glycine	0.48	0.13	0.61
Fraction 1 IgG(T)	Dnp-MP	0.50	0.12	0.62
Fraction 5 IgG(T)	Dnp-glycine	0.55	0.14	0.69
Fraction 5 IgG(T)	Dnp-MP	0.53	0.08	0.61
Fraction 6 IgG(T)	Dnp-glycine	0.51	0.11	0.62
Fractions 1-3 IgG	Dnp-MP	0.32	0.32	0.64

[a] Differences between these values and those reported previously (Archer et al., 1973) are due to the use of revised antibody extinction coefficients.

burst mode (Krakauer, 1972) to measure the enthalpy of hapten binding to antibody. Solutions of antibody at concentrations of 0.5 to 9×10^{-5} mol L^{-1} were pumped together with an appropriate concentration of hapten for 1 min. Calibration was based on the standard reaction of neutralization of tris(hydroxymethyl)aminomethane by dilute HCl (Öjelund and Wadsö, 1968). The solvent in all calorimetry experiments was 0.15 M NaCl-0.01 M sodium phosphate, pH 7.8. Solutions of antibodies were prepared for calorimetry by chromatography on Bio-Gel P-6 to effect equilibration with solvent components. Heats of dilution of the antibodies and of the haptens were found to be negligible. Corrections for viscous heating effects were applied.

Results

The average equilibrium constants and Sips heterogeneity parameters that characterize the binding of the two haptens to the various fractions of the antibodies are listed in Table I. In general, no large differences in binding affinity were detected among the antibodies or haptens studied, and only limited heterogeneity is indicated by the values of Sips indices which are close to 1.0. The equilibrium constant for the binding of Dnp-glycine to fraction 1 antibody, the highest affinity fraction, was about ten times greater than that for the binding of the same hapten to the lowest affinity fraction, fraction 6. The average equilibrium constant for the binding of Dnp-MP, however, was about the same for all fractions of antibodies, and similar to that for the binding of Dnp-glycine to low affinity fractions. Only a small fraction of the Dnp-specific antibody produced by the immunized horse was in the IgG class. As expected, the IgG antibody isolated by our purification procedure had an affinity for Dnp-haptens which was similar to that of IgG antibodies. The equality of the average equilibrium constants for the binding of Dnp-MP to fraction 5 IgG(T) and a pool of fractions 1-3 IgG is important to note as these fractions were used for the light-scattering studies of the interactions of these antibodies with divalent dinitrophenylated poly(ethylene glycol) (Archer and Krakauer, 1977).

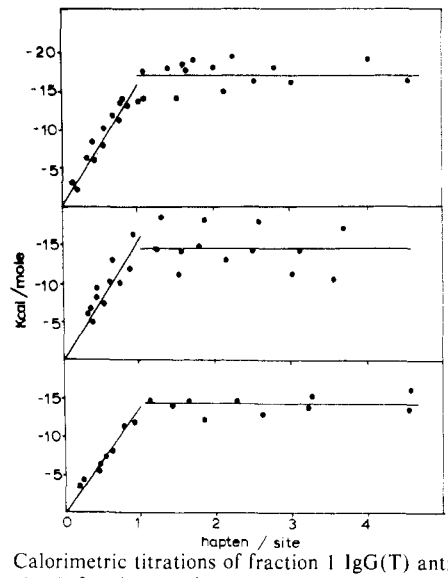

FIGURE 1: Calorimetric titrations of fraction 1 IgG(T) antibody with Dnp-glycine (top), fraction 1 IgG(T) antibody with Dnp-MP (middle), and fraction 6 IgG(T) antibody with Dnp-glycine (bottom). Each point represents the average of three to six measurements. The indicated lines in the region of antibody site excess are the results of least-squares analysis, and those above the equivalence point represent average values.

In Table II are listed the values of the quenching parameters q_1 and q_2. The sum $q_1 + q_2$ represents the reduction in fluorescence of an antibody molecule which results when both binding sites are occupied by hapten. This quantity, listed in the right-most column of Table II, is similar for both IgG and IgG(T) antibodies reacting with both Dnp-glycine and Dnp-MP haptens. There is a marked contrast, however, between the difference between q_1 and q_2 for IgG(T) antibody and their equality in IgG antibody.

The data obtained from the calorimetric determinations of the enthalpies of binding of Dnp-glycine and Dnp-MP to IgG(T) antibodies are displayed in Figure 1 and summarized in Table III. The enthalpies of binding under conditions of excess sites are the slopes of the lines through the origin. The data obtained at hapten excess were also fitted to straight lines and extrapolated to the equivalence point. This procedure was indicated in the case of Dnp-MP by the detection of a slight interaction between nonspecific immunoglobulin and dinitrophenylated poly(ethylene glycol) (Archer and Krakauer, 1977). This procedure was also followed in the case of Dnp-glycine for consistency, although no detectable heat was produced upon mixing of IgG(T) from a horse that had not been immunized against the Dnp group with this hapten. None of the slopes of the lines fitted to the data at hapten excess is of substantial magnitude and significantly different from zero. Similarly, none of the differences between enthalpies obtained at site and hapten excess is statistically significant.

The enthalpy changes, and the entropy changes that may be calculated from them and the free energy changes, are large and negative, in agreement with the results of Barisas et al. (1971, 1972), and of Halsey and Biltonen (1975) for the binding of Dnp-haptens to heterogeneous rabbit antibodies, of Johnston et al. (1974) for Dnp-binding myeloma proteins, and of Halsey et al. (1975) for rabbit, goat, and guinea pig immunoglobulins.

Discussion

The data for the binding of Dnp-glycine to fractions 1 and

TABLE III: Summary of the Thermodynamics of the Binding of Haptens to Antibodies.[a]

Antibody	Hapten	ΔG (kcal/mol)	$\Delta H°$ (kcal/mol) At Site Excess[b]	At Hapten Excess[c]	Grouped Average[d]
Fraction 1 IgG(T)	Dnp-glycine	−10.20 ± 0.12	−16.40 ± 0.43	−17.13 ± 0.65	−16.83
Fraction 1 IgG(T)	Dnp-MP	−9.07 ± 0.04	−16.61 ± 0.87	−15.62 ± 1.41	−16.07
Fraction 5 IgG(T)	Dnp-glycine	−8.53 ± 0.02			
Fraction 5 IgG(T)	Dnp-MP	−8.61 ± 0.03			
Fraction 6 IgG(T)	Dnp-glycine	−8.63 ± 0.03	−14.43 ± 0.14	−14.00 ± 0.32	−14.16
Fractions 1-3 Fractions 1-3 IgG	Dnp-MP	−8.64 ± 0.04			

[a] Listed uncertainties are standard errors. [b] These are the slopes of the lines through the origins in Figure 1 fitted to data obtained at antibody site excess. [c] These are the intercepts at hapten-site equivalence of lines fitted to the data at hapten excess. [d] These are averages of the data in the preceding two columns weighted by the number of points in each group.

6 of IgG(T) give a clear indication that differences in binding affinity of the same hapten to different antibodies are due to differences in the enthalpy binding which reflect differences in the specific interactions of the hapten with the binding sites. This is also supported by the observation that the changes induced in the absorption spectrum of a hapten upon binding to antibodies depend on the affinity of the antibody to which it is bound (Archer et al., 1973). Halsey and Biltonen (1975), in contrast, found that populations of rabbit antibodies differing in average affinity for Dnp-glycine by 1.3 kcal/mol had indistinguishable binding enthalpies. In neither study is the range of the thermodynamic parameters sufficient to definitively resolve the conflict, especially in view of the limited precision of the calorimetric measurements.

This limited precision also conceals the basis for the difference of 1.1 kcal/mol in the affinity of one antibody, fraction 1, for the haptens Dnp-glycine and Dnp-MP. The data obtained with antibody sites in excess, which inspire somewhat greater confidence because of their somewhat better precision, suggest that the reduced affinity for Dnp-MP might be due to a greater loss of entropy. This might conceivably arise from the constraints imposed on the rather long polyethylene oxide tail consequent to the attachment of the Dnp group to the antibody site (Archer and Krakauer, 1977). A similar lower affinity due to a greater loss in entropy was observed by Johnston et al. (1974) when Dnp-glycine rather than Dnp-aminocaproate bound to a mouse myeloma protein. On the other hand, the data at hapten excess, though poorer, argue for the opposite conclusion, while the grouped averages are ambiguous. Thus, although, in this particular case, the lower affinity is more likely than not the result of a greater loss in entropy, a definitive statement to that effect is not justified.

The final comment to be made from the thermodynamic data is that both immunoglobulin classes, IgG and IgG(T), bind Dnp-MP with similar affinities and thus, as has been shown by others (Rockey, 1967), the different biologic behavior of the two antibodies cannot be the result of different intrinsic binding affinities.

In addition to binding free energies and estimates of binding site homogeneity, the fluorescence quenching measurements also yield information which may be indicative of differences in the arrangement in space of the Fab arms of the two antibodies. The marked difference between the reductions in quantum yield (q_1, q_2) of the fluorescence of IgG(T) antibody which are produced by the first and second haptens binding stands in striking contrast to the equality of these reductions for IgG antibody. This qualitative difference together with the similarity of the total reduction in quantum yield ($q_1 + q_2$) suffered by both antibodies when saturated indicates that there is a significant fraction of the tryptophan side chains that can transfer energy to a hapten acceptor bound to either antibody site in IgG(T) but not in IgG antibodies. While such a situation could be realized in more than one way, the simplest explanation that could account for this transfer is that the antibody binding sites are closer together in IgG(T) than in IgG antibodies. The possibility of such transfer, if the Fab arms are close enough, is assured by an estimate of the distance at which there is a 50% probability of transfer (3.7 nm) for tryptophan-Dnp donor-acceptor pairs (Green, 1964). Additional independent evidence that strongly supports the existence of such a difference in the conformations of these two classes of antibodies has been obtained in the studies of their interaction with divalent high molecular weight antigens (Archer and Krakauer, 1977).

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