Thermodynamics of Hapten Binding to MOPC 315 and MOPC 460 Mouse Myeloma Proteins†

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ABSTRACT: This paper reports the thermodynamic parameters for the binding of various haptens to mouse plasmacytoma (MOPC) proteins 315 and 460 at 4 and 25°. Haptens examined are N^{ϵ}-2,4-dinitrophenyl-L-lysine (Dnp-lysine), 2-methyl-1,4-naphthoquinone (menadione), and 2,4-dinitro-1-naphthol (dinitronaphthol) for both proteins, together with Dnp-glycine, Dnp-aminocaproic acid, and N^{ϵ}-2,4,6-trinitrophenyl-L-lysine (Tnp-lysine) for protein 315 only. Dissociation constants from independent fluorescence quenching and equilibrium dialysis experiments agree well and binding free energies $\Delta G_b°$ are calculated. The protein concentration dependence of the binding of Dnp-lysine to protein 315 at 4° is examined. Binding enthalpies, ΔH_b, obtained by direct flow calorimetry are found to agree with van't Hoff values obtained from equilibrium measurements. At 25° ΔH_b for systems involving polynitroaromatic ligands ranges from −12.1 to −20.2 kcal (mol of ligand bound)$^{-1}$ and provides the driving force for the ligand–protein association. ΔH_b for systems involving menadione are conspicuously low. The change in heat capacity on binding, ΔC_p, is calculated from the temperature variation of the calorimetric ΔH_b and ranges from −110 to −300 cal deg^{-1} (mol of ligand bound)$^{-1}$. The various thermodynamic quantities are discussed in terms of structural features of the proteins and haptens. Calorimetric measurement of ΔH_b for association in water of tryptophan with Dnp-aminocaproic acid and Tnp-aminocaproic acid yields −3.8 to −7.4 kcal (mol of hapten)$^{-1}$ and −2.6 to −7.5 kcal (mol of hapten)$^{-1}$, respectively, depending upon choices of dissociation constants for the complexes. Charge-transfer association of ligands with protein tryptophan is discussed as a possible contribution to the spectral and thermodynamic parameters of binding.

That enthalpic factors provide the driving force for hapten binding by anti-2,4-dinitrophenyl (Dnp)[1] antibodies was originally deduced on the basis of van't Hoff calculations (Eisen and Siskind, 1964). Subsequently, direct calorimetric examination of hapten binding by anti-Dnp and anti-Tnp antibodies (Barisas *et al.*, 1971, 1972) showed very large exothermic heats of binding, larger than could be demonstrated by van't Hoff calculations. Moreover, no simple mechanistic explanation of the source of such large thermal effects has emerged. Since the proteins produced by MOPC 315 and MOPC 460 exhibit substantial affinity for a broad spectrum of polynitrophenyl and related haptens, these materials have been accorded wide study as homogeneous models for antibody systems. We therefore thought it desirable to characterize, as thoroughly as practicable, the thermodynamics of binding of a number of haptens by proteins 315 and 460. Our goals in this study were four. First, we wished to see whether large negative hapten binding enthalpies were characteristic of these polynitrophenyl-binding immunoglobulins. This being the case we wanted, second, to establish thermodynamic parameters for such high-enthalpy binding in the reproducible, homogeneous systems which these proteins afford. Since each conventionally raised antibody sample is unique, thermodynamic experiments such as we had previously done could not be reproduced by other investigators. Third, we hoped to correlate the structures of the hapten molecules with the observed binding thermodynamics and perhaps to identify at the molecular level the sources of the large thermal effects. Finally, there has been some disagreement among investigators concerning the affinities of these proteins for various haptens. We hoped, by careful examination of several of the more important systems simultaneously and under parallel conditions, to put on record a set of dissociation constants possessing, at least, internal consistency.

We should note that the calorimetric measurements required hundreds-of-milligram amounts of these proteins. Samples of such size require a huge investment of time and facilities for preparation and could provide materials for a number of interesting studies. If the nondestructive nature of flow calorimetry did not make possible recovery of the starting material quantitatively and virtually unchanged, such experiments would never have been undertaken.

Material and Methods

Plasmacytomas and Protein Purification. Plasma cell tumors MOPC-315 and 460 were generously provided by Dr. Michael Potter (National Cancer Institute, Bethesda, Md.) and were maintained by serial transplantation as subcutaneous solid tumors in Balb/c mice (Cumberland Farms, Tenn.). Proteins 315 and 460 were isolated from the sera of these mice as mildly reduced and alkylated 7S molecules wherein 5–6 cysteine residues were carboxymethylated. Purification of the

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[1] Abbreviations used are: Dnp, 2,4-dinitrophenyl; Tnp, 2,4,6-trinitrophenyl; PBS, phosphate-buffered saline (0.15 M sodium chloride–0.01 M sodium phosphate, pH 7.4). Nomenclature of immunoglobulins follows the recommendations of the World Health Organization (*Bull. W.H.O. 30*, 447, 1964). Myeloma proteins are designated by numbers and the tumors that produce them by preceding letters; *e.g.*, tumor MOPC-315 produces protein 315.

proteins was accomplished by their specific adsorption onto a column of N^{ϵ}-Dnp-L-lysyl-Sepharose 4B followed by elution with Dnp-glycine (Goetzl and Metzger, 1970a).

For protein 315 the molecular weight and $\epsilon_{278}^{1\%}$ were taken as 150,000 and 1.35 ml/mg, respectively (Underdown *et al.*, 1971), while, for protein 460, the same values were taken to be 150,000 and 1.55 ml/mg, respectively (Jaffe *et al.*, 1969).

Haptens. N^{ϵ}-Dnp-L-lysine (Dnp-lysine), Dnp-glycine, and 2-methyl-1,4-naphthoquinone (menadione) were purchased from the Sigma Chemical Co., St. Louis, Mo., while 2,4-dinitro-1-naphthol (dinitronaphthol) was obtained from Eastman Organic Chemicals, Rochester, N. Y. These commercially available compounds were recrystallized prior to use. Dnp-aminocaproic acid, Tnp-aminocaproic acid, and N^{ϵ}-Tnp-L-lysine (Tnp-lysine) were gifts of Professor S. J. Singer. [^{3}H]Dnp-lysine was prepared using [3,5,6-^{3}H]-1-fluoro-2,4-dinitrobenzene (10,400 Ci/mol) by the technique of Underdown *et al.* (1971).

Hapten concentrations were determined spectrophotometrically. The molar extinction coefficients (M^{-1} cm^{-1}) used at neutral pH were: Dnp-lysine, ϵ_{360} = 17,400 (Carsten and Eisen, 1953); Tnp-lysine, ϵ_{348} = 15,400 (Little and Eisen, 1966); Dnp-aminocaproic acid, ϵ_{365} = 17,800 (Carsten and Eisen, 1963); Dnp-glycine, ϵ_{360} = 15,890 (Ramachandran and Sastry, 1962); dinitronaphthol, ϵ_{395} = 14,630 (Underdown *et al.*, 1971); menadione, ϵ_{252} = 20,000 (Underdown *et al.*, 1971); Tnp-aminocaproate, ϵ_{348} = 15,700 (Little and Eisen, 1966).

Equilibrium Dialysis. Measurements were performed at 4 and 25° in PBS. Dissociation constants for protein binding of [^{3}H]Dnp-lysine were obtained by standard dialysis procedures in 100-μl Lucite cells (Eisen *et al.*, 1968). Protein binding of the unlabeled haptens was estimated by competitive inhibition of [^{3}H]Dnp-lysine binding (Karush, 1956; Jaffe *et al.*, 1971). Measurements were generally made at fixed protein concentrations of 0.064 mg/ml (protein 315) and 2.5 mg/ml (protein 460). In the case of the binding of Dnp-lysine to protein 315 the effect on equilibria of varying protein concentration was also investigated over the range 0.040–12.0 mg/ml.

Fluorescence Quenching. Protein–hapten dissociation constants in PBS were determined at 4 and 25° by fluorescence titration in an Aminco-Bowman spectrofluorimeter (Eisen and Siskind, 1964; Barisas *et al.*, 1971). Attenuation of exciting (280 nm) and emitted (345 nm) radiation by hapten was corrected for by titrating with the same hapten solution a solution of tryptophan having an absorbance at 280 nm equal to that of the protein solution under study. The maximum extent of fluorescence quenching, Q_{max}, was determined by titrating protein and tryptophan solutions with a highly concentrated solution of hapten. When the binding constant for a particular protein–hapten system was less than 5×10^5 M^{-1}, equilibrium dialyses were run in parallel with the titrations in order to ensure an accurate extrapolation of quenching to saturation of all protein sites (Eisen and McGuigan, 1971). Both proteins were examined at a concentration of 0.050 mg/ml.

Calorimetric measurements were performed using a flow calorimeter (Sturtevant and Lyons, 1969; Velick *et al.*, 1971; Barisas *et al.*, 1971) based on the Beckman Model 190B microcalorimeter. In the instrument used for this study, the platinum tubing comprising the flow system was 1×500 mm compared with 0.5×1000 mm in earlier models. This change reduces viscous heating by a factor of approximately 32 over that observed in these previous instruments. On the other hand, this larger tubing somewhat reduces both the efficiency of the mixer and the rate of thermal equilibration of flowing fluids with the heat sink. Consequently, flow rates must be adjusted to give a suitably increased residence time in the calorimeter for the solution after mixing.

Experiments were performed in PBS at 4 and 25° and all appropriate corrections for viscous heating and heats of dilution were applied. In most experiments protein and hapten solutions were flowed into the calorimeter at rates of 0.055 and 0.158 ml per min, respectively. The protein concentration in the drive syringes was generally about 10 mg/ml which corresponds to a combining site concentration after mixing of approximately 33 μM. The precise protein concentration in a given solution was determined from the absorbancy of the solution at 278 nm, together with the molecular weight and extinction coefficient of the protein. The actual concentration of combining sites was then set equal to the protein concentration, multiplied by the number of sites per molecule capable of reversibly binding hapten as determined by fluorescence quenching measurements. Because of relatively weak binding in many of the protein–hapten systems, heats of binding, ΔH_b, per mole of combining sites were measured over a range of hapten concentrations [Hp], the hapten always being present in large excess, and the heat extrapolated to infinite hapten concentration by a double-reciprocal plot of $1/\Delta H_b$ *vs.* 1/[Hp]. The binding enthalpy at saturation is then the reciprocal of the y intercept.

Tryptophan–hapten equilibria were characterized at 25° by both differential spectrophotometry and by direct calorimetry. The haptens examined were Dnp-aminocaproate and Tnp-aminocaproate. The spectrophotometric method for obtaining dissociation constants in these systems has been previously described (Little and Eisen, 1967). The heats of interactions were determined by mixing in the calorimeter various concentrated tryptophan solutions (0.02–0.05 M, 0.220 ml/min) with a single solution of hapten (0.002 M, 0.044 ml/min). After appropriate corrections, the data were analyzed in a manner analogous to those from the spectrophotometry to obtain dissociation constants and heats of formation for the tryptophan–hapten complexes.

Results

Purification. Both purified proteins were substantially free of irreversibly bound hapten. From absorbance measurements made on concentrated (~10 mg/ml) solutions at the absorption maximum of Dnp-glycine (360 nm) we conclude that 5 and 4% of the combining sites of proteins 315 and 460, respectively, contained hapten remaining from the purification procedures.

Dissociation Constant Determinations. Good agreement was obtained between the protein–hapten dissociation constants determined by equilibrium dialysis and those obtained by fluorescence quenching titration. Table I compares the values of negative logarithms of the dissociation constants, pK_d, obtained by the two methods. Agreement of our dissociation constants with the literature values cited in the table is, on the whole, quite satisfactory. We would expect affinities of Dnp-aminocaproate to resemble those cited for Dnp-lysine while Tnp-lysine should resemble Tnp-aminocaproate which is bound by protein 315 at 4° with $pK_d > 7$ (Eisen *et al.*, 1970). The only significant disagreement is for protein 315 binding Dnp-glycine at 25°, Pecht and coworkers (1972a) reporting a pK_d a full unit higher than we observe. Finally we should note that our data preserve for each protein and temperature the sequences of binding affinities for three haptens reported earlier (Jaffe *et al.*, 1971); for protein 315, Dnp-lysine >

TABLE I: Dissociation Constant Determinations for Proteins 315 and 460 with Various Haptens.

System	Measurements at 4°					Measurements at 25°				
	pK_d[a] (Equil Dialysis)	pK_d[b] (Fluorescence)	a[c]	Q_{max}[d] (%)	pK_d (Lit.)	pK_d (Equil Dialysis)	pK_d (Fluorescence)	a[c]	Q_{max}[d] (%)	pK[d] (Lit.)
Protein 315 + Dnp-lysine	6.85	6.92	0.94	75	7.20[e]	5.93	6.00	0.91	70	6.30[j]
+Dnp-glycine	4.79	4.92	0.93	71		3.89	4.04	0.94	68	5.17[j]
+Dnp-aminocaproate	6.51	6.77	0.91	69		5.78	5.85	0.91	66	6.3[k]
+Tnp-lysine	7.48	7.68	0.96	76		6.32	6.42	0.93	73	
+Dinitronaphthol	5.34	5.54	0.95	69	5.05[f]	4.82	4.77	0.93	64	
+Menadione	5.62	5.70	0.92	66	5.80[f]	4.89	4.96	0.92	62	
Protein 460 + Dnp-lysine	5.32	5.59	0.91	50	5.48[g]	4.55	4.77	0.92	48	5.00[l]
+Dinitronaphthol	6.48	6.16	0.91	51	6.42[h]	5.60	5.36	0.91	48	
+Menadione	5.01	5.12	0.93	36	4.21[i]	4.85	4.77	0.91	32	

[a] Systems involving Dnp-lysine examined by direct equilibrium dialysis; others by competitive inhibition of Dnp-lysine binding. [b] These dissociation constants determined by fluorescence quenching titrations. [c] Sips index of heterogeneity from fluorescence measurements. [d] Fraction of protein fluorescence quenched upon saturation by hapten. [e] Eisen *et al.* (1968). [f] Eisen *et al.* (1970). [g] Jaffe *et al.* (1969). [h] Underdown *et al.* (1971). [i] Jaffe *et al.* (1971). [j] Pecht *et al.* (1972a). [k] Pecht *et al.* (1972b). [l] Rosenstein *et al.* (1972).

menadione > dinitronaphthol, while for protein 460, dinitronaphthol > Dnp-lysine > menadione. The values from the two methods were averaged to yield a mean pK_d from which the binding free energy, $\Delta G_b°$, was calculated as $-2.303 \cdot RT(pK_d)$. Also the van't Hoff binding enthalpy was estimated from the values for the mean pK_d at 4 and 25° as ΔH_b (van't Hoff) = $2.303R[pK_d(25°) - pK_d(4°)]/[1/(298.2) - 1/(277.2)]$. The free energy and van't Hoff enthalpy of binding appear in Table II and are discussed in connection with the calorimetric results in the next section. In the case of the fluorescence measurements, the extent of site saturation by hapten was measured over a range of free hapten concentrations and the data were treated in accord with the model of Sips (1948). Included in Table I are the extents of fluorescence quenching, Q_{max}, upon saturation by hapten and the Sips indices, a, of heterogeneity in hapten binding. As expected all these indices are 0.91 or over, indicating highly homogeneous protein preparations. Moreover the values for Q_{max} exhibit good internal agreement and are in good accord with published values. For example, Q_{max} at 4° for the systems protein 315 + Dnp-lysine, protein 460 + Dnp-lysine, and protein 460 + dinitronaphthol have been cited as 69, 46, and 46%, respectively (Eisen *et al.*, 1968; Jaffe *et al.*, 1971). Our data indicate that Q_{max} is relatively insensitive to temperature and even to the precise nature of the hapten chromophore, being rather a characteristic of the individual protein. This has long been assumed in the case of conventionally raised antibody populations (Eisen and Siskind, 1964). The slightly lower Q_{max} values for menadione doubtless reflect the rather low molar extinction coefficient of menadione (2500) at the 355-nm fluorescence emission maximum of protein tryptophan (Morton, 1965).

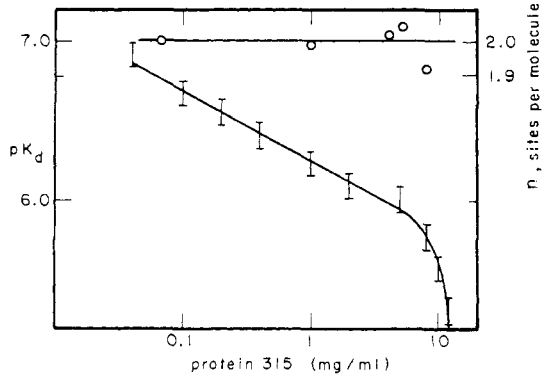

FIGURE 1: Dependence of the dissociation constant, K_d, and valence for Dnp-lysine binding by protein 315 on protein concentration at 4°. $pK_d = -\log K_d$. The data were obtained by equilibrium dialysis. Vertical bars indicate association constants (left scale) and their uncertainties, and open circles indicate protein valence (right scale).

The fluorescence titrations also permit evaluation of the number of sites per molecule which reversibly bind hapten. To obtain Sips plots of optimum linearity, it was necessary to assume that, for both proteins, this quantity was 1.8 (2 theoretical). The fraction of sites occupied by hapten remaining from the purification process (~5%) clearly represents a lower limit for the fraction of sites unavailable for hapten binding, since combining sites not binding residual hapten may nonetheless be denatured. Thus the figure of 1.8 sites/molecule (90% site purity) for the protein preparations seems quite reasonable.

To see if equilibrium measurements on the protein 315–Dnp-lysine system were sensitive to variations in protein concentration and to insure that we would be justified in combining free energy values obtained by fluorescence titration and equilibrium dialysis (0.064 mg/ml) with enthalpic measurements obtained calorimetrically (2.5 mg/ml), we investigated the dependence of the dissociation constant for the protein 315–Dnp-lysine complex on protein concentration. The results are indicated in Figure 1. There is evident a marked concentration dependence of the dissociation constant, presumably resulting from extensive protein–protein interactions which merit further study. It appears to be safe for present purposes to ignore these interactions for the following reasons. In terms of $\Delta G_b°$, the difference between the values at the concentrations used in equilibrium and thermal measurements with protein 315 is only 0.4 kcal/mol. Both types of measurement with protein 460 were made at approximately the same concentration. In the thermal experiments the protein solution underwent a fourfold dilution in the calorimeter, from 10 to 2.5 mg

TABLE II: Thermodynamic Parameters for Hapten Binding to Proteins 315 and 460.

System	Measurements at 4°			Measurements at 25°			ΔC_p[d] (calorim) (cal/(deg mol))	ΔH_b[e] (van't Hoff (kcal/mol)
	$\Delta G_b°$[a,b] (kcal/mol)	ΔH_b[c] (kcal/mol)	$\Delta S°$ (cal/(deg mol))	$\Delta G_b°$[a,b] (kcal/mol)	ΔH_b[c] (kcal/mol)	$\Delta S°$ (cal/(deg mol))		
Protein 315 + Dnp-lysine	−8.7	−12.8	−14.6	−8.1	−16.6	−28.3	−180	−16.6
+Dnp-glycine	−6.2	−13.8	−27.5	−5.4	−19.9	−48.6	−291	−16.1
+Dnp-aminocaproate	−8.4	−12.1	−13.3	−7.9	−14.4	−21.3	−111	−14.9
+Tnp-lysine	−9.6	−12.3	−9.8	−8.7	−18.1	−31.6	−276	−21.8
+Dinitronaphthol	−6.9	−13.9	−25.2	−6.5	−20.2	−45.9	−301	−13.8
+Menadione	−7.2	−7.9	−2.6	−6.7	−12.1	−18.1	−201	−13.3
Protein 460 + Dnp-lysine	−6.9	−10.1	−11.5	−6.4	−15.8	−31.6	−270	−14.2
+Dinitronaphthol	−8.0	−14.8	−24.4	−7.5	−18.7	−37.6	−186	−15.1
+Menadione	−6.4	−4.1	+8.3	−6.7	−9.3	−9.3	−249	−4.5

[a] Throughout this table "mol" refers to 1 mol of hapten bound to protein combining sites. [b] Calculated from dissociation constant measurements as $RT \ln K_d$. [c] From calorimetric data corrected for fraction of protein sites incapable of reversibly binding hapten. [d] Calculated from calorimetric binding enthalpies as $[\Delta H_b(T_2) - \Delta H_b(T_1)]/[T_2 - T_1]$. [e] Calculated from dissociation constants as $R[\ln K_d(T_2) - \ln K_d(T_1)]/[(1/T_2) - (1/T_1)]$.

per ml, with no significant enthalpy change, indicating that if the variation of binding with concentration is to be attributed to protein association, such association is not accompanied by a large enthalpy change.

Calorimetric Measurements. The extrapolation of binding enthalpies to infinite hapten concentration and thus to saturation of all combining sites is illustrated in Figure 2. The hapten concentration, [Hp], is that of the *free* species and the enthalpy of binding, ΔH_b, is the negative of the heat evolved per mole of combining sites reacted with excess hapten. Similar extrapolations were made for all systems examined. The extrapolated binding enthalpies were combined with the binding free energies calculated from the dissociation constants of Table I to yield the thermodynamic parameters which comprise Table II. Unitary free energies (Kauzmann, 1959) are obtained from the values listed in the table by adding −2.2 and −2.4 kcal per mol at 4 and 25°, respectively, and unitary entropies by adding +8 cal/(deg mol). Also included in Table II are values for the change, ΔC_p, in heat capacity on binding calculated from the temperature dependence of the calorimetrically measured hapten binding enthalpies with the assumption that ΔC_p is independent of temperature.

There is little in the literature against which our enthalpy data can be compared. A value of −13.6 kcal/mol of hapten bound has been reported previously for the van't Hoff binding enthalpy of Dnp-lysine to protein 315 at 20° (Michaelides and Eisen, 1973). This accords reasonably well with the value of −15.6 kcal/mol interpolated from our calorimetric measurements. Generally, we must resort to comparing our own van't Hoff and calorimetric enthalpies. Application of the van't Hoff relation to two equilibrium measurements made at different temperatures in a system where $\Delta C_p \neq 0$, and where, therefore, the binding enthalpy is temperature dependent, yields the binding enthalpy corresponding to a temperature approximately midway between the temperatures of observation. Calculation shows this temperature to be $[(T_2T_1)/(T_2 - T_1)] \ln (T_2/T_1)$ where T_2 and T_1 are the temperatures of observation. For measurements at 25° and 4° then, the van't Hoff binding enthalpy corresponds to 14.4°; and we can interpolate our calorimetric measurements to this temperature. The agreement between the 14.4° calorimetric and van't Hoff binding enthalpies, ΔH_b^{cal} and $\Delta H_b^{v.H.}$, respectively, may be expressed in the two following quantities

$$d_1 = 1/n\Sigma(\Delta H_b^{cal} - \Delta H_b^{v.H.})$$

$$d_2 = [1/n\Sigma(\Delta H_b^{cal} - \Delta H_b^{v.H.})^2]^{1/2}$$

where n is the number of systems studied. For the nine systems we examined, $d_1 = 0.79$ kcal/mol. d_1 indicates the systematic disagreement between the two types of determination and shows that the van't Hoff binding enthalpies are, on the average, that much larger negative than those determined by calorimetry. We consider this error to be well within acceptable limits. d_2 is the root mean square disagreement between ΔH_b^{cal} and $\Delta H_b^{v.H.}$ and, as such, is a measure of the random errors present in the determinations. For the systems studied $d_2 = 3.0$ kcal/mol. This quantity, though not unreasonable considering that one of the measurements involved is a van't Hoff determination, would fall to 2.1 kcal/mol (and d_1 to 0.06 kcal/mol) if the protein 315–Tnp-lysine system were excluded. It is in this system alone that agreement between calorimetric and van't Hoff enthalpies is poor. Based on our experience

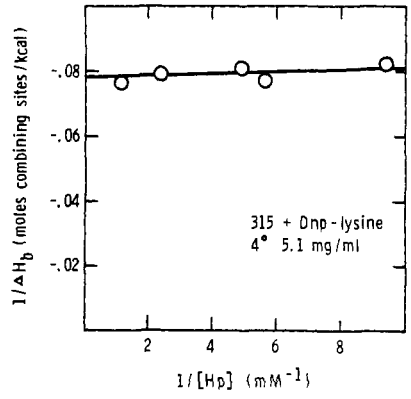

FIGURE 2: Extrapolation to infinite hapten concentration of ΔH_b for Dnp-lysine binding by protein 315: temperature, 4°; protein concentration, 5.1 mg/ml.

TABLE III: Equilibrium Constants and Thermodynamic Parameters for Tryptophan–Hapten Association at 25°.

L-Trp +	K_d[a] (M)	K_d[b] (M)	K_d[c] (M)	$\Delta G_b°$[d] (kcal/mol)	ΔH_b[e] (kcal/mol)	ΔS_b (cal/(deg mol))
Dnp-aminocaproate	0.33	0.31	0.17	−0.70	−3.8	−10.5
Tnp-aminocaproate	0.10	0.29	0.17	−0.74	−4.4	−12.2

[a] Spectrophotometric value (Little and Eisen, 1967). [b] Spectrophotometric value (this study). [c] Calorimetric value. [d] Calculated from dissociation constant (*b*) as $RT \ln K_d$. [e] Calorimetric value.

with the techniques, we estimate the standard error in the van't Hoff enthalpies to be 2 kcal/mol while that in the direct calorimetry should be approximately 1 kcal/mol. These error estimates would imply $d_2 = 2.2$ kcal/mol. Finally, if we assign to $\Delta G_b°$ the very reasonable standard error of about 1 kcal/mol, then the standard error in ΔS will be about 4.7 cal/(deg mol) at 25°.

Tryptophan–Hapten Interaction. It has been previously suggested (Little and Eisen, 1967), on the basis of spectral evidence, that charge-transfer interaction of one or more protein tryptophan residues with the Dnp or Tnp nucleus is involved in Dnp- or Tnp-hapten binding by conventionally raised antibody populations directed against these determinants. Proteins 315 and 460 exhibit difference spectra on Dnp-hapten binding which suggest similar charge-transfer interaction. The changes in hapten absorption spectra which accompany polynitrophenylhapten binding to either conventionally raised antibody or proteins 315 or 460 are reproduced when these haptens are mixed with concentrated solutions of tryptophan. Dissociation constants and molar extinction coefficients of tryptophan complexes with Dnp-aminocaproate and Tnp-aminocaproate were reported in the previously mentioned study (Little and Eisen, 1967). We wished to see if the tryptophan–hapten complexes exhibited, as do antibody complexes with these haptens, substantial negative enthalpies of formation. We measured K_d for the systems and the agreement with previous results was not good. We concluded that, because the maximum solubility of tryptophan in water at 25° is approximately 0.05 M and because the dissociation constants for the complexes are 0.1–0.3 M, such spectrophotometric methods simply cannot give precise enough values of K_d to permit a van't Hoff determination of ΔH_b. We turned to calorimetric techniques and obtained the results in Table III. $\Delta G_b°$ was calculated from our spectrophotometric results, and ΔH_b was derived from calorimetric measurements. The major uncertainty in these results derives from the uncertainties in the dissociation constants. Although these errors do not introduce large uncertainties into $\Delta G_b°$, the extent of saturation of available hapten with tryptophan is small, so that the values calculated for ΔH_b at saturation are almost linearly dependent upon those assumed for K_d. If our calorimetric data are analyzed using the three available sets of dissociation constants, ΔH_b for Dnp- and Tnp-aminocaproate would range from −3.8 to −7.4 kcal (mol of hapten)$^{-1}$, respectively, depending upon the choice of dissociation constants.

Discussion

Underdown *et al.* (1971) had noted a protein concentration effect on the valence of protein 315 for binding Dnp-lysine. Because our equilibrium and calorimetric measurements necessarily had to be performed at substantially different protein concentrations, we wished to examine further this concentration dependence to insure that our equilibrium and calorimetric data could justifiably be compared. Our experiments show the dissociation constant, rather than the valence, for Dnp-lysine binding by protein 315 to be markedly concentration dependent. The dissociation constant increases by about a factor of ten as the protein concentration is raised from 0.04 to 10 mg per ml while the valence remains constant at 1.92–2.04 mol of hapten/mol of protein (Figure 1). We do not know why our results should be in disagreement with those of Underdown *et al.* (1971), especially since both our measurements and theirs were performed in the same laboratory. We can only suggest that techniques for handling protein 315 have been improved since the previous study, leading to greater stability during dialysis. As discussed earlier in this paper, we do not believe this concentration dependence of the dissociation constant has great relevance to our thermodynamic results. Even if we had obtained the same results as Underdown *et al.* (1971), our calculations would remain unaffected since $\Delta G°$, calculated from dissociation constants, would then be concentration independent while our calorimetric measurements of ΔH (which do depend on the protein valence) would have been performed at protein concentrations where the valence was observed to be 1.9 or greater. It should be noted that various workers may have used quite different protein concentrations in their equilibrium dialysis studies with these proteins, and it is possible that some of the discrepancies reported for protein 315 affinities for various haptens arise from not taking this concentration dependence into account.

It has been noted that protein 315 is more nearly representative of anti-Tnp than anti-Dnp antibodies (Eisen *et al.*, 1970). On the basis of our measured dissociation constants, we concur since affinities of protein 315 are higher for Tnp-lysine than for any other hapten. On the other hand, Q_{max} for protein 315 averages, for polynitroaromatic ligands, over 70% and this is rather more like anti-Dnp than anti-Tnp antibodies. We feel that the most one can say is that protein 315 (and 460, for that matter) belongs to that functional class of immunoglobulins which also contains anti-Dnp and anti-Tnp antibodies.

The binding of polynitroaromatic ligands by proteins 315 and 460 appears thermodynamically fully parallel to Dnp- and Tnp-hapten binding by conventionally raised anti-Dnp and anti-Tnp antibodies. Large negative binding enthalpies and changes in heat capacity on binding characterize both classes of system. Other types of antibody–hapten and antibody–antigen systems for which thermodynamic data are available do not exhibit such large binding enthalpies. For example, ΔH_b for the bovine serum albumin–anti-bovine serum albumin system at 0° is 0 ± 2 kcal/mol (Singer, 1967) while for the *p*-azophenylarsonate system at 23°, ΔH_b is −0.8 ± 2.6 kcal/mol (Epstein *et al.*, 1956). Our data thus support the conclusion advanced by Eisen *et al.* (1970) that these myeloma proteins

have high structural homology to anti-Dnp and anti-Tnp antibodies. If we accept these similarities between myeloma proteins and conventionally raised antibodies, then some comment is in order concerning the discrepancies reported in previous papers (Barisas *et al.*, 1971, 1972) between the van't Hoff and calorimetric binding enthalpies for hapten binding by conventionally raised antibody populations. In this study no such systematic discrepancy was observed and we conclude that the difficulty in previous work lay in the measurement by fluorescence quenching of the most probable dissociation constant, K_d^0, for the heterogenous populations. If the heterogeneity of binding free energies in these samples did not precisely obey the Sips distribution, or if there was a correlation between Q_{max} and K_d for various molecules in the samples, then our determinations of K_d^0 would be systematically in error, since analysis of the data involved extrapolating plots of log $[\theta/(1-\theta)]$ *vs.* log [Hp] from the region where the data were taken ($\theta \sim 0.8$) to $\theta = 0.5$ where log [Hp] = log K_d^0. We feel, therefore, that the discrepancy between calorimetric and van't Hoff enthalpies for hapten binding by anti-hapten antibodies is not of importance.

The outstanding thermodynamic feature of the systems examined is the hapten binding enthalpies. These quantities are all large and negative; and, in all but one case, it is the enthalpic term which provides the driving force for the hapten binding to the protein. The exceptional case involves menadione (+ protein 460 at 4°), and its binding enthalpy to either protein at either temperature has the lowest magnitude among the haptens studied. Clearly menadione is incapable of generating large heats of binding, in contrast with the sterically very similar dinitronaphthol. One might suggest that this reflects the less electron-deficient nature of the menadione π system when compared with polynitroaromatic ligands. The heats of binding exhibited by dinitronaphthol, while the largest for any ligand, are nonetheless approximately comparable to those of the Dnp- and Tnp-haptens. This indicates further that hapten steric factors are relatively unimportant in determining the binding enthalpies, but rather that it is the presence or absence of a polynitroaromatic nucleus which is critical. Another feature of interest in the thermodynamic data is that Dnp-glycine, while having very favorable heats of binding to protein 315 when compared to Dnp-aminocaproate, is much inferior to that ligand in terms of binding affinity. The difference in specificity resides in the entropic term and this might reflect an unfavorable entropy arising from burying the negative charge of the Dnp-glycine in the hydrophobic environment of the combining site. It appears that the side chains of Dnp-lysine and Dnp-aminocaproic acid are long enough to permit their charge(s) access to the solvent while the haptenic group is bound to the protein.

An uncertainty of 1 kcal/mol in ΔH_b at each temperature corresponds to an uncertainty of 70 cal/(deg mol) in ΔC_p. Although precise interpretation of the values for ΔC_p given Table II is not possible, it is interesting to note that all the values except that for protein 315 + Dnp-aminocaproate are within ±70 cal/(deg mol) of the mean value −240 cal/(deg mol). Furthermore the values observed here accord well with those reported in previous studies of polynitrophenylhapten binding by conventionally raised anti-Dnp and anti-Tnp antibodies (Barisas *et al.*, 1971, 1972). It is tempting to suggest that a large negative ΔC_p is a fundamental feature of the binding of electron-deficient aromatic ligands by immunoglobulins having high affinities for such compounds.

Charge-transfer association of polynitrophenyl ligands with tryptophan groups apparently cannot contribute much to the free energies of antibody–hapten association if the thermodynamics of the interaction in the combining site resembles that in bulk water. If, however, the combining site is relatively hydrophobic, then the thermodynamics of transfer of the nonpolar Dnp or Tnp group into the site must be taken into account. This is an entropically favorable process involving only a small enthalpy change (Breslauer, 1972); moreover, the fact that the tryptophan residues are confined to a combining site of restricted dimensions increases their effective concentration and should thus lead to an increase in the entropy term favoring the tryptophan–hapten interaction.

In the case of protein 315, the amino acid sequences of both the light chain (Schulenburg *et al.*, 1971) and heavy chain (Francis *et al.*, 1973) are known. Affinity-labeling studies have shown that two tryptophans are very possibly in contact with the bound hapten. In the light chain, Tyr-34 is affinity labeled by *m*-nitrobenzenediazonium fluoroborate (Goetzl and Metzger, 1970b) and by *N*-bromoacetyl-*N'*-Dnp-ethylenediamine (Haimovich *et al.*, 1972) and this residue is in close proximity to Trp-37. In the heavy chain, Lys-54 is specifically labeled by N^{α}-bromoacetyl-N^{ϵ}-Dnp-L-lysine (Haimovich *et al.*, 1972) and Trp-49 is also nearby. If both residues could interact with the hapten, perhaps as much as −10 kcal/mol of enthalpy change could be ascribed to charge transfer. Taken together with favorable entropic contributions to the binding free energy, arising from hapten transfer into the nonpolar site and from proximity effects on effective tryptophan concentration, this model gives some crude indication as to how the thermodynamics of hapten binding to these proteins is to be rationalized.

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