Thermodynamics for the Interaction of ϵ -Dinitrophenyl-L-lysine and Bovine Colostral Anti-Dinitrophenyl Immunoglobulin G2

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The effect of varying the temperature over a wide range (4–60°C) on the binding of ϵ -dinitrophenyl-L-lysine to bovine colostrum anti-dinitrophenyl immunoglobulin G2 yielded a non-linear van't Hoff plot. The extent of curvature was indicative of a large positive heat-capacity change, and the thermodynamic parameters, calculated by using a non-linear least-squares computer procedure, revealed an enthalpy–entropy-compensation mechanism for hapten–antibody binding. The enthalpy factor was found to be the primary contributor for the complex-formation at low temperatures, but at increasing temperatures the entropy factor assumed greater importance. At physiological temperature (39°C), the entropy factor was the major contributor to the free energy of reaction.

Earlier thermodynamic investigations (Barisas *et al.*, 1971, 1972; Epstein *et al.*, 1956; Johnston *et al.*, 1974; Singer, 1956), of hapten–antibody interactions revealed the standard enthalpy change (ΔH°) to be quite large, and it was therefore interpreted as the driving force for hapten–antibody binding. The possible involvement of enthalpy–entropy compensation in hapten–antibody binding was suggested some years ago (Epstein *et al.*, 1956) as well as more recently (Halsey & Biltonen, 1975). Thermodynamic analysis of the binding data obtained from equilibrium-dialysis measurements for the interaction of ϵ -Dnp-L-lysine with bovine colostrum anti-Dnp IgG1 and rabbit anti-Dnp IgG (Szewczuk & Mukkur, 1977*a*) and bovine colostrum anti-Dnp Fab' fragment (IgG1) (Szewczuk & Mukkur, 1977*b*) over a wide range of temperature revealed enthalpy–entropy compensation as a mechanism for hapten–antibody binding. Representation of binding data as van't Hoff plots revealed non-linearity with a positive heat-capacity change (ΔC_p°). In addition, plotting the pertinent thermodynamic parameters in the form of an enthalpy–entropy compensation plot revealed a single compensation temperature (T_c°), thus suggesting a single kind of change in protein–solvent conformation (Szewczuk & Mukkur, 1977*b*). Since previous investigations from our laboratory indicated a

conformational difference between bovine colostrum IgG1 and IgG2 (Mukkur & Fang, 1976*a,b*; Tewari & Mukkur, 1975), it was the objective of the present investigation to evaluate the thermodynamics for the interaction of ϵ -Dnp-L-lysine and bovine colostrum anti-Dnp IgG2 and examine if the mechanism of the latter hapten–antibody interaction could also be described by an enthalpy–entropy-compensation process.

Materials and Methods

Immunization

Pregnant cows were immunized by three intramuscular inoculations with a total of 10mg of Dnp-conjugated pig IgG (31 Dnp groups/molecule) in aluminium hydroxide adjuvant, at monthly intervals, starting 4 months *pre partum*. The immune colostrum was collected at 12h *post partum*.

Isolation of anti-Dnp IgG2

The principle underlying the isolation of hapten-specific IgG2 was the same as that described for the isolation of anti-Dnp IgG1 (Mukkur & Fang, 1977). Briefly, the method involved isolation of IgG2 as described by Tewari & Mukkur (1975), followed by isolation of specific anti-Dnp IgG2 by the use of an immuno-adsorbent, which comprised bromoacetyl-cellulose linked to dinitrophenylated human serum albumin (13 Dnp groups/molecule of albumin) (Robbins *et al.*, 1967). The details of the immuno-adsorption method as applied to the bovine immunoglobulin system were described previously (Mukkur & Fang, 1976*a,b*; Mukkur, 1972; Szewczuk &

Abbreviations used: Dnp, dinitrophenyl; Dnp-Pig IgG, dinitrophenylated pig immunoglobulin G; IgG1, immunoglobulin G1; IgG2, immunoglobulin G2.

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Mukkur, 1977a,b). On further re-adsorption with the dinitrophenylated immunoabsorbent, the antibody was found to be at least 95% active, a factor that was taken into consideration in the calculation of the binding data. The antibody preparation was pure as judged by standard criteria such as immunoelectrophoresis, Ouchterlony gel diffusion and analytical ultracentrifugation (Tewari & Mukkur, 1975).

Binding studies

Equilibrium dialysis with micro-chambers was used for all binding experiments. The bovine colostrum anti-Dnp IgG2 was dialysed exhaustively against 0.14M-NaCl/0.01M-sodium phosphate, pH 6.0. ϵ -Dnp-L-[^3H]lysine (New England Nuclear Corp., Boston, MA, U.S.A.; 3.22Ci/mol) was used as the hapten. Buffer was added to one side of the micro-equilibrium-dialysis chambers, and the hapten and antibody were on the other side. The equilibration period was 48h at all temperatures investigated. Although the concentration of antibody used was a constant 240 μM , the range of hapten concentrations used in the binding studies varied from 5mM to 1 μM . The specific radioactivity of the ^3H -labelled stock hapten solution was determined to be 7.44×10^{10} d.p.m./mol.

The concentrations of the hapten after equilibration from both sides of the equilibrium-dialysis chambers were determined by counting radioactivity of 20 μl samples of ^3H -labelled hapten in 5.0ml of Aquasol (New England Nuclear) in a Nuclear-Chicago (mark II) liquid-scintillation counter. The radioactivity (c.p.m.) was converted into d.p.m. and the efficiency was adjusted for ^3H quenching. Corrections for non-specific binding were made, for all temperatures, from plots of the radioactivity bound by normal bovine colostrum IgG2 at various hapten concentrations as described previously (Mukkur & Fang, 1976a).

For all calculations, the molecular weight of the anti-Dnp IgG2 was taken as 146000 (Tewari & Mukkur, 1975). The number of sites (n) available for hapten was determined from the Scatchard (1949) plot. The calculation of total affinity constant, K_t , was made from the plot of $\log Q' [r/(n-r)C]$ against r (see Fig. 1), and referred to as the 'log Q' plot' (Mukkur *et al.*, 1974). The curves passing through the data points were computer-fitted with the non-linear least-squares procedure of Marquardt (1963).

Calculation of thermodynamic parameters

ΔG° , ΔH° and ΔS_T° (uncorrected for the entropy of mixing) were calculated as follows, the derivation of equations having been described previously (Szewczuk & Mukkur, 1977a).

At equilibrium:

$$\Delta G^\circ = -RT \ln K_t \quad (1)$$

where R is the gas constant, T is the absolute temperature and K_t is the total-affinity constant.

$$\Delta G^\circ = \Delta b - \Delta a T \ln T + CT \quad (2)$$

where C is the integration constant.

$$\Delta H^\circ = \Delta b + \Delta a T, \text{ and } a = \Delta C_p^\circ \quad (3)$$

Substitution of eqns. (2) and (3) into the equation for the free-energy change as a function of temperature ($-\Delta S = \Delta G - \Delta H/T$) yields:

$$\Delta S_T^\circ = \Delta a + \Delta a \ln T - C \quad (4)$$

Substitution of eqn. (1) into eqn. (2) yields:

$$\ln K_t = \frac{\Delta a}{R} \ln T - \frac{\Delta b}{R} \times \frac{1}{T} - \frac{C}{R} \quad (5)$$

The graphic representation of the van't Hoff plot consists of a plot of $\ln K_t$ against $1/T$. Also non-linear variation of association constants with temperature could be described by eqn. (5). The three unknown constants in eqn. (5) may be calculated by simultaneous equations or by a least-squares computer procedure as described previously by Mukkur *et al.* (1974).

The unitary free-energy change (ΔF_u) and the unitary entropy change (ΔS_u) were calculated from relationships described previously (Kauzmann, 1959; Karush, 1962).

Results

The binding isotherms represented as the Scatchard and the $\log Q'$ plots for the interaction of ϵ -Dnp-L-lysine with bovine colostrum anti-Dnp IgG2 at different temperatures were used for the calculation of thermodynamic parameters. The number of combining sites per molecule of bovine colostrum anti-Dnp IgG2 was 2 at all temperatures investigated, i.e. 4 (Mukkur & Fang, 1976b), 15, 25, 37, 52 and 60°C (Fig. 1).

The variation in the total-affinity constant, K_t , as a function of temperature for the interaction of ϵ -Dnp-L-lysine and bovine colostrum anti-Dnp IgG2 gave a non-linear van't Hoff plot (Fig. 2). The non-linear least-squares computer-fitted curve could be represented by the following relationship:

$$\ln K_t = (147 \pm 0.60) \ln T + \frac{(4.33 \pm 0.00) \times 10^4}{T} - (942.93 \pm 0.00)$$

The standard heat-capacity change (ΔC_p°) was calculated to be $+1171.93 \pm 2.51 \text{ J } (280.10 \pm 0.60 \text{ cal}) \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. The standard free-energy change (ΔG°) and the unitary free energy change (ΔF_u) were negative for the interaction of ϵ -Dnp-L-lysine with bovine colostrum anti-Dnp IgG2 (Table 1). The standard enthalpy changes increased linearly with increasing temperature. Between 4 and 25°C, ΔH°

Fig. 1. Scatchard and $\log Q'$ plots for the interaction of bovine colostral anti-Dnp IgG2 at various temperatures: (a and a') 4°C; (b and b') 15°C; (c and c') 25°C; (d and d') 37°C; (e and e') 52°C; (f and f') 60°C

$Q' = r/(n-r)C$, where r is equal to total amount of hapten bound (mol)/total amount of antibody (mol) n is the antibody valence, and C is the equilibrium concentration of free hapten (M).

Fig. 2. van't Hoff plot for the interaction of ϵ -Dnp-L-lysine with bovine colostral anti-Dnp IgG2

The bars represent the computer-calculated uncertainties in the values of K_t at various temperatures.

was negative, but increased to a positive value when the temperature was raised above 25°C. The standard entropy changes, ΔS_T^0 , uncorrected for the entropy of mixing, were negative between 4 and 15°C, but

increased to increasingly positive values between 25 and 60°C. The unitary entropy change, ΔS_u , also increased with an increase in temperature like ΔS_T^0 , and was positive between 15 and 60°C (Table 1).

The compensation effects of the thermodynamic parameters for the interaction of ϵ -Dnp-L-lysine and anti-Dnp IgG2 were obtained by plotting various thermodynamic functions [in J (kcal)] against temperature as shown in Fig. 3. A very slight decrease in the standard free energy, ΔG^0 , was observed, and remained apparently constant at approx. 20.9 kJ (5 kcal)/mol. The temperature at which $-T\Delta S_T^0$ exactly equalled ΔH^0 was 25°C. However, if ΔS_u was utilized in the compensation plot, the temperature at which $-T\Delta S_u^0$ equalled ΔH^0 was 22°C.

Representation of ΔH^0 and ΔS_T^0 at various temperatures as an enthalpy-entropy compensation plot yielded a straight line with a single slope of 300 K (Fig. 4).

Table 1. Thermodynamic functions for the interaction of ϵ -Dnp-L-lysine and bovine colostral anti-Dnp IgG2. ΔG° , ΔH° and ΔS_T° were calculated by using equations described in the 'Materials and Methods' section. The proportional errors given for various thermodynamic parameters represent computer-calculated uncertainties.

Temperature (°C)	ΔG°			ΔH°			ΔS_T°			ΔF_u			ΔS_u		
	(kJ/mol)	(kcal/mol)	(J/mol per degree)	(kJ/mol)	(kcal/mol)	(J/mol per degree)	(J/mol per degree)	(cal/mol per degree)	(kJ/mol)	(kcal/mol)	(J/mol per degree)	(cal/mol per degree)	(J/mol per degree)	(cal/mol per degree)	(J/mol per degree)
4	-20.20 ± 4.10	-4.83 ± 0.98	-33.68 ± 0.75	-8.05 ± 0.18	-48.62 ± 18.03	-11.62 ± 4.31	-29.71 ± 4.10	-7.10 ± 0.98	-15.23 ± 18.03	-3.64 ± 4.31	-15.23 ± 18.03	-3.64 ± 4.31	-15.23 ± 18.03	-3.64 ± 4.31	-15.23 ± 18.03
15	-19.95 ± 4.44	-4.77 ± 1.06	-20.79 ± 0.79	-4.97 ± 0.19	-2.97 ± 15.14	-0.71 ± 3.62	-50.27 ± 4.44	-7.09 ± 1.06	30.42 ± 15.15	7.27 ± 3.62	30.42 ± 15.15	7.27 ± 3.62	30.42 ± 15.15	7.27 ± 3.62	30.42 ± 15.15
25	-20.08 ± 4.60	-4.80 ± 1.10	-9.07 ± 0.84	-2.17 ± 0.20	37.07 ± 18.20	8.86 ± 4.35	-30.04 ± 4.60	-7.18 ± 1.10	70.08 ± 18.20	16.75 ± 4.35	70.08 ± 18.20	16.75 ± 4.35	70.08 ± 18.20	16.75 ± 4.35	70.08 ± 18.20
37	-21.00 ± 4.81	-5.02 ± 1.15	4.98 ± 0.84	1.19 ± 0.20	83.30 ± 18.33	19.91 ± 4.38	-31.34 ± 4.81	-7.49 ± 1.15	116.69 ± 18.33	27.89 ± 4.38	116.69 ± 18.33	27.89 ± 4.38	116.69 ± 18.33	27.89 ± 4.38	116.69 ± 18.33
52	-22.59 ± 5.06	-5.40 ± 1.21	22.59 ± 0.75	5.40 ± 0.18	138.70 ± 18.45	33.15 ± 4.41	-33.43 ± 5.06	-7.99 ± 1.21	172.09 ± 18.45	41.13 ± 4.41	172.09 ± 18.45	41.13 ± 4.41	172.09 ± 18.45	41.13 ± 4.41	172.09 ± 18.45
60	-23.68 ± 5.31	-5.66 ± 1.27	32.00 ± 0.92	7.65 ± 0.22	167.19 ± 18.45	39.96 ± 4.43	-34.81 ± 5.31	-8.32 ± 1.27	200.58 ± 18.54	47.94 ± 4.43	200.58 ± 18.54	47.94 ± 4.43	200.58 ± 18.54	47.94 ± 4.43	200.58 ± 18.54

Fig. 3. Compensation effect for the interaction of ϵ -Dnp-L-lysine with bovine colostral anti-Dnp IgG2. The bars represent the computer-calculated uncertainties in the values of the indicated parameters at various temperatures; $\bullet$, ΔG° ; $\blacklozenge$, ΔH° ; $\blacksquare$, $-T\Delta S_T^\circ$.

Fig. 4. Enthalpy-entropy compensation plot for the interaction of bovine colostral anti-Dnp IgG2.

Discussion

The number of combining sites per molecule of anti-Dnp IgG2 was 2 at all temperatures investigated (4–60°C). The binding of ϵ -Dnp-L-lysine by bovine colostral anti-Dnp IgG2 was found to involve an enthalpy factor that was highly tempera-

ture-dependent, as shown by a non-linear van't Hoff plot (Fig. 2). Similar observations were previously made for interaction of ϵ -Dnp-L-lysine with anti-Dnp antibodies of bovine (IgG1 and its subunit, Fab') and rabbit origin (IgG) (Szewczuk & Mukkur, 1977a,b). The extent of the curvature was indicative of a large positive standard heat-capacity change (ΔC_p°), which was assumed to be independent of temperature to permit an assessment of the relative importance of the enthalpy and entropy factors. This large and positive standard heat-capacity change could be interpreted as indicative of an unfolding of an antibody-combining site. However, the influence of subtle perturbations cannot be ruled out. In addition, the enhanced total-affinity constants (K_t) at low and high temperatures were also in accord with a positive heat-capacity change, again suggesting an unfolding type of conformational change in the antibody-combining sites from their native state. Similar observations were previously made for the interaction of ϵ -Dnp-L-lysine with bovine anti-Dnp IgG1 (colostral) and rabbit IgG (Szewczuk & Mukkur, 1977a,b). The concept of low- and high-temperature-unfolded states is not new and has previously been described in terms of a two-state denaturation reaction (Brandt, 1964; Lumry *et al.*, 1966). Similar general observations have previously been made, such as the enhanced binding of Crystal Violet (Blei & Carrol, 1960) and Congo Red (Hajro-witz *et al.*, 1952) for heat-denatured serum albumin as compared with its native state. The unfavourable entropy factor for ϵ -Dnp-L-lysine binding at low temperatures was apparently compensated for by the negative enthalpy factor, resulting in a favourable free-energy change. Thus it appears that the enthalpy factor was a primary contributor to the driving force for the binding process at low temperatures, as suggested previously (Barisas *et al.*, 1971, 1972; Johnston *et al.*, 1974), but at increased temperatures the entropy factor assumed greater importance, as was evident from the plot showing the compensation effect (Fig. 3). At physiological temperature (39°C), the entropy factor was the major contributor to the free energy of reaction. The high enthalpy change [-50.58 to -84.44 kJ (-12.1 to -20.2 kcal)/mol of ligand bound] reported by Johnston *et al.* (1974) could be related to the higher affinity of the immunoglobulin molecule used by them in their investigation. Also, it is not clear if their thermodynamic data were corrected for non-specific ligand binding.

A mechanism for hapten-antibody interaction could therefore be rationalized in terms of an enthalpy-entropy compensation effect, which has previously been used to explain various enzymic catalytic reactions (Hammes, 1964). The predominance of the enthalpy factor at low temperatures could be explained by simultaneously breaking and forming hydrogen bonds (Eisen & Siskind, 1964; Ghose &

Karush, 1974) in the antibody-combining site, thereby resulting in the formation of compensating bonds (Hammes, 1964). Eisen & Siskind (1964) and Ghose & Karush (1974) have suggested that the hydrogen-bonding possibly formed for each bound hapten could account for the observed enthalpy change. If one assumed that the negative value of ΔH_{total} at low temperatures was due to $\Delta H_{\text{binding}}$ of ϵ -Dnp-L-lysine with specific antibody, the trend towards increasing positive values with increasing temperatures would be compensated by $\Delta H_{\text{solvent}}$, thus suggesting a possible source of contribution of solvent-protein interactions.

The entropy effects could also be rationalized in terms of a compensation mechanism. The observed trend in the standard (ΔS_T°) and unitary entropy changes, ΔS_u , for the interaction of ϵ -Dnp-L-lysine with anti-Dnp IgG2 preparation, the values of which are generally quite comparable with those reported in the literature (Karush, 1962), might be considered to be a compensating effect of $\Delta S_{\text{internal}}^\circ$ and $\Delta S_{\text{solvent}}^\circ$, where the former is assumed to be more important at lower temperatures. However, with increasing temperatures, if the structured water in the antibody-combining site could be assumed to break down (Epstein *et al.*, 1956), $\Delta S_{\text{solvent}}^\circ$ would have a positive value. The possible predominance of $\Delta S_{\text{internal}}^\circ$ at low temperatures (although it is an unfavourable effect that is counterbalanced by a contribution of the enthalpy factor to the free-energy change) is supported by increased volume changes in antibody molecule as a result of hapten binding (Ohta *et al.*, 1970), and conformational changes such as those observed by optical rotatory dispersion (Cathou & Haber, 1967), circular dichroism (Bennich & Dorrington, 1972; Cathou *et al.*, 1968) and spectral properties in the u.v. and visible regions (Mukkur & Fang, 1976a,b, 1977; Little & Eisen, 1967; Rubinstein & Little, 1970; Ghose & Karush, 1974). The increase in the standard (ΔS_T°) and unitary (ΔS_u) entropy with increasing temperatures is suggestive of a greater role of hydrophobic bonding in the formation of hapten-antibody complex at those temperatures.

The fact that a single slope with a T_c° of 300 K was observed in the compensation plot for the interaction of ϵ -Dnp-L-lysine with anti-Dnp IgG2 was indicative of a single kind of a change in protein-solvent conformation. The latter should not be construed to mean a single process and probably is a resultant of a number of events such as formation of different types of bonds, disruption of structured water in the antibody-combining site occurring as a result of hapten-antibody complex-formation etc. A constant compensation temperature (T_c°) of 300 K was also reported for the interaction of ϵ -Dnp-L-lysine and bovine colostral anti-Dnp IgG1, rabbit anti-Dnp IgG (Szewczuk & Mukkur, 1977a) and bovine colostral anti-Dnp Fab' fragment (IgG1) (Szewczuk &

Mukkur, 1977b), the significance of which is not clear at the present time. The data in the present paper therefore indicated that the mechanism for the binding of ϵ -Dnp-L-lysine to anti-Dnp IgG2 could also be described as an enthalpy-entropy compensation process.

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