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Abstract

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ROLE OF HYDROPHOBIC INTERACTION IN HAPTEN-ANTIBODY BINDING

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Abstract. The precipitation reaction of bovine serum albumin coupled with p-azophenylleucine with homologous antibody was inhibited by several structurally related haptens. The isobutyl group substituent on α-carbon atom of the leucine residue contributed more than -5.8 Kcal/mol to the free energy of binding. This value was consistent with the free energy change expected from the transfer of n-butane from an aqueous environment to liquid n-butane. The observed contribution was explained, in terms of the hydrophobic interaction of the isobutyl group with the antigen binding site of the antibody molecule. These results were also compared with other hapten-antibody systems.

The binding specificity of hapten with antihapten antibody has been examined by many investigators. These investigators have related the haptenic structure to the thermodynamic parameters of hapten-antibody interaction obtained from the precipitation inhibition test (1, 2) and equilibrium dialysis (3), which have been the two major procedures used in these investigations.

In this paper we report on some results of a precipitation inhibition study, and discuss the individual contributions of some chemical groups in hapten molecules to the hapten-antibody interaction. The haptenic group selected for the study was p-azobenzoylleucine, and the precipitation reaction with homologous antibody was inhibited by several structurally related haptens. The inhibition experiments indicate that the isobutyl group, substituent on the α-carbon atom of the leucine residue, contributed more than -5.8 Kcal/mol to the free energy of binding. The results provide evidence that hydrophobic interaction plays an important role in the binding of hapten and antihapten antibody.

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MATERIALS AND METHODS

Preparation of antigen. D, L-Leucine was reacted with p-nitrobenzoylchloride and then, the nitro group was reduced by the method of Landsteiner and Van der Scheer (4). The purity of p-nitrobenzoylleucine (PNBL) and p-aminobenzoylleucine (PABL) was confirmed by measuring the melting point: PNBL, 224.5-225.5°C and PABL, 180-182°C.

PABL (0.12 mmoles) was diazotized by equimolar sodium nitrite at 0-5°C for 60 min. The excessive sodium nitrite was removed with ammonium sulfamate until the solution fail to produce a blue response on starch-iodide paper. Diazotized PABL was coupled to 0.2 g of bovine serum albumin (BSA) in 20 ml of 0.01 M borate buffer, pH 9.0, by adding diazotized PABL in small portions over a period of 2 hours, maintaining the pH at 9.0 by addition of 1 N NaOH. The solution was then kept at 0-4°C overnight. After adjusting the pH to 7.0 with HCl, the solution was dialyzed at 0-4°C against 1,000 ml of 0.1 M NaCl changed daily with new solution for seven days.

Preparation of antiserum. The dialyzed solution of antigen (BSA-conjugated p-azobenzoylleucine; abbreviated BSAL) which contained 5 mg of protein per ml solution was homogenized with an equal volume of Freund complete adjuvant. A total dose of 2 ml of this mixture was injected in divided dosages subcutaneously to the palmar, plantar, and abdominal skin of a young rabbit. Two weeks later, the same procedure was repeated. At the end of the sixth week after the first injection, about 30 ml of antiserum was prepared from the rabbit.

Antibodies produced against antigenic determinants other than the introduced haptenic group were precipitated by addition of BSA at the optimum amount which produced maximum precipitation with the antiserum. The optimum amount was determined as described in the section on precipitation reaction. This reaction was completed in 60 min at 37°C. The precipitate, which contained complement as well as antigen-antibody complex, was then removed by centrifugation at 15,000×g for 20 min. The supernatant was used as the specific antiserum to the homologous hapten.

The specificity of this antiserum was confirmed by the double diffusion technique of Ouchterlony: the precipitine line was observed between BSAL but not between BSA and antiserum.

Assay of precipitation reaction. The antigen solution was serially diluted with 0.15 M saline solution buffered with 0.008 M phosphate (PBS), pH 7.0. After addition of a given amount of antiserum to the antigen solution, the mixture was incubated for 60 min at 37°C, then allowed to stand for two days at 4°C. The precipitates were centrifuged, washed once with 1.0 ml of PBS and the protein amount in the precipitate was measured by the method of Lowry *et al.* (5).

Inhibition of precipitation by haptens. The amount of BSAL required for optimum precipitation was determined, and the precipitation reaction at this antigen-antibody ratio was inhibited with various haptens by the same procedure described above. The reaction mixture contained: hapten 1.0 ml (PBS

in place of hapten for control), BSAL 1.0 ml (48 μg, the optimum amount) and antiserum 0.5 ml (20 times diluted). The addition was in this sequence. Abbreviations and formulae for the haptens used in this experiment are summarized in Table 1. The nonspecific binding of haptens to serum protein is almost negligible because of the low concentration of antiserum (6).

TABLE 1. INHIBITION HAPTENS USED IN THE PRECIPITATION REACTION BETWEEN BSAL AND HOMOLOGOUS ANTIBODY

Hapten	Abbreviation	Formula[b]
p-Aminobenzoylleucine	PABL[a]	H_2N-C_6H_4-CONH-CHR-COOH
p-Aminohippuric acid	PAH[a]	H_2N-C_6H_4-CONH-CH_2-COOH
p-Nitrobenzoylleucine	PNBL[a]	O_2N-C_6H_4-CONH-CHR-COOH
p-Nitrobenzamide	PNBA	O_2N-C_6H_4-$CONH_2$
N-Acetyl-L-leucine	NAL[a]	H_3C-CONH-CHR-COOH
D, L-Leucine	Leu	H_2N-CHR-COOH
p-Aminobenzoic acid	PABA[a]	H_2N-C_6H_4-COOH

[a] Dissolved in 1 N NaOH, pH adjusted to 7.0 with HCl, diluted to desired concentrations with PBS and stocked at 4°C.
[b] -R = -$CH_2CH(CH_3)_2$

RESULTS AND DISCUSSION

The effect of haptens on the precipitation reaction of BSAL with homologous antibody was tested (Fig. 1). The results were interpreted by applying the theory of heterogeneous antibody binding sites described by Pauling *et al.* (1). They assumed a Gaussian distribution for the free energy of binding between a

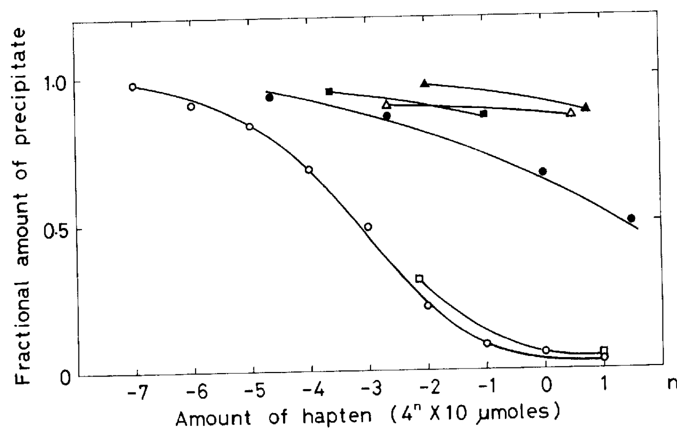

Fig. 1. Precipitation inhibition by PABL and other structurally related haptens. The points are from experimental data and the curves are from theoretical calculations (1). ○, PABL; □, PNBL; ●, NAL; △, PAH; ▲, Leu; ■, PNBA.

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haptenic group and heterogenous antibody. If the precipitation reaction between multivalent hapten and antibody was interfered by a monovalent inhibition hapten, two parameters (K_O' and σ) can be calculated from the equation derived by Pauling *et al.*. K_O' is the average inhibition constant of a hapten, expressed as a relative value to that for the homologous hapten (usually, K_O' for the homologous hapten is taken as unity). K_O' can be considered as a relative binding constant of the hapten to antibody. The heterogeneity index, σ, is the standard deviation in the error function, and is zero for a homogeneous group of binding sites and increases with antibody heterogeneity. The experiment with the homologous hapten, PABL, was carried out at nine different concentrations to determine the degree of agreement between the experimental data and the theoretical curve. As evident from the figure, the experimental points are in fair agreement with the theoretical curve. On other haptens, however, only a few points were tested.

The values of K_O' and σ for haptens employed were calculated and are shown in Table 2. Since values smaller than 10^{-4} for K_O' and greater than 7.0

TABLE 2. CALCULATION OF THE AVERAGE HAPTEN INHIBITION CONSTANT (K_o'), HETEROGENEITY INDEX (σ) AND RELATIVE FREE ENERGY OF BINDING (ΔG_{rel})

Hapten	K_o'	σ	ΔG_{rel} (Kcal/mol)
PABL	1.0	3.0	0
PAH	$<10^{-4}$	8.0	>5.8
PNBL	0.63	3.0	0.29
PNBA	$<10^{-4}$	8.0	>5.8
NAL	1.5×10^{-3}	7.0	4.1
Leu	$<10^{-4}$	8.0	>5.8

For abbreviations of haptens, see Fig. 1 legend.
Values for K_o' and σ were calculated from the theoretical equation of Pauling *et al.* (1).
Values for ΔG_{rel} were expressed at 37°C.

for σ are not reliable, the apparent difference among these results for PAH, PNBA and Leu (Fig. 1) has no significance. The relative free energy change of binding of a hapten to antibody (ΔG_{rel}) is also shown in Table 2. ΔG_{rel} was calculated from the following equation,

$$\Delta G_{rel} = -RT \ln K_O',$$

where R is the gas constant and T is the absolute temperature. The difference in ΔG_{rel} between different haptens represents the differential free energy of binding. Comparisons of structurally related haptens differing in only one substitution group, therefore, permit the estimation of the differential contribution of the groups.

The contribution of the isobutyl group to the free energy of hapten binding with antihapten antibody is larger than 5.8 Kcal/mol (Table 2). The significance of nonpolar groups in the association of hapten with antibody has been pointed out by Nisonoff *et al.* (2) and Karush (3). The contribution of α-phenyl group in the hapten, phenyl-(benzoylamino)-acetic acid, was estimated to be 4.1 Kcal/mol by Karush (3). Karush suggested that this value agrees closely with that of the change in free energy for the transfer of 1 mol of benzene from an aqueous environment to liquid benzene (-4.1 Kcal/mol, see 7). He inferred from this fact that the phenyl group induces a quasi-crystalline structure of water molecules in its immediate vicinity when the hapten is free but is in intermolecular contact with the nonpolar side chains of the protein when the hapten is bound with the antibody. The same argument is valid in our case. The change in free energy for the transfer of 1 mol of n-butane from an aqueous environment to liquid n-butane is -5.8 Kcal/mol (8). This value may be a little less negative for isobutane because of the branching effect of the carbon chain. Therefore, a contribution larger than 5.8 Kcal/mol of isobutyl group strongly suggests that the nonpolarity of the isobutyl group is the main factor for the contribution observed, as the phenyl group is in the phenyl-(benzoylamino)-acetic acid system. It must be mentioned, however, that direct comparison of these two systems may not be very proper because of the difference in the precipitation inhibition test and the equilibrium dialysis method.

If the London dispersion force between the isobutyl group and its nearest neighbors in the antibody binding site is taken into account, the large contribution of the isobutyl group is fully explained. To evaluate the dispersion force, however, it would be necessary to measure the temperature dependence of the contribution, by the equilibrium dialysis technique.

There is another possibility that the large ΔG_{rel} observed for PAH is due to the differential conformation around the peptide bond in PAH and PABL. IR spectra of PAH and PNBL eliminated this possibility since strong absorption bands were observed around 1520–1530 cm^{-1} (amide II) in both spectra, indicating *trans* orm is the preferential conformation in both haptens.

It is interesting that in some hapten-antibody systems, some nonpolar groups seem to confer only limited contribution. In the p-azophenyltrimethylammonium ion system (6, 9), p-(*tert*-butyl)-azophenyl group (steric analogue of p-azophenyltrimethylammonium ion) contributes only 1.5 Kcal/mol more than the p-azophenyl group.

There is only a small difference (0.29 Kcal/mol) between the contribution of the amino and the nitro group of PABL and PNBL molecules, respectively (Table 2). The position of these substituents corresponds to the diazo group in the immunization antigen (BSAL). The contribution order of these groups is

reversed in the p-azophenyl arsonate system (6, 10).

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