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Antibody-Antigen Binding in Organic Solvents

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We describe, for the first time, the action of antibodies in anhydrous organic solvents. It has been demonstrated that the binding of a hapten, 4-aminobiphenyl, to the immobilized monoclonal antibody 2E11 is strong and specific not only in water but also in a variety of non-aqueous media. Further, the strength of interaction between antibody and hapten has been related to the hydrophobicity of the solvent: the more hydrophobic the solvent, the weaker the protein-ligand interaction.

In recent years, it has been established unequivocally that enzymes can act as catalysts in anhydrous organic solvents and that in this unnatural milieu enzymes exhibit a number of remarkable new properties (1-3). To test the generality of biochemical reactions in non-aqueous media, we have examined another type of biological interaction, that between an antibody and antigen, in various organic solvents. We report herein that not only does an antibody bind its antigen in organic solvents, but this interaction is strong and of a highly specific nature.

EXPERIMENTAL PROCEDURES

Tritiated [2,2'-^{3}H]-4-aminobiphenyl was obtained from Midwest Research Institute and had a specific activity of 30 mCi/mol. The organic solvents used in this study were of the highest purity commercially available and were dried by gentle shaking with 3-Å molecular sieves (Linde) overnight.

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Monoclonal antibody 2E11 was raised against the hapten 4-aminobiphenyl as follows. Five female BALB/By CJ mice (Jackson Laboratories) were immunized by intraperitoneal injection with 100 μg each of a 4-aminobiphenyl-bovine gamma globulin conjugate emulsified with an equal volume of complete Freund's adjuvant (Gibco). The antigen was prepared by reaction of diazotized 4-aminobiphenyl with the protein. At 20 and 50 days, the mice were boosted with 100μg of antigen in incomplete Freund's adjuvant. After 10 days, serum samples were collected by tail bleeding and assayed for antibody titer by ELISA. Three days prior to fusion, each mouse was given a final immunization of the same antigen in 100μl of phosphate buffered saline (PBS)(10 mM phosphate buffer, pH 7.2, 0.15 M NaCl) injected into the tail vein. The myeloma cell line used for cell fusion, SP-2, was maintained in suspension in Dulbecco's modified Eagle's medium supplemented with 20% fetal-calf serum, glutamine, penicillin, streptomycin, and non-essential amino acids (GIBCO). Cell fusion was performed as described earlier (4).

To produce the antibody, cells were grown as ascites in pristane-primed CAF_1 mice. The monoclonal antibody was purified by precipitation from mouse ascites fluid with ammonium sulfate (40% saturation). The precipitate was redissolved in PBS to give a final concentration of approximately 10 mg/ml, and extensively dialyzed against PBS. The antibody was then purified to homogeneity on a Bio-Rad Affi-Gel Protein A MAPS II column. The antibody concentration was measured by amino acid analysis, the Bio-Rad protein assay system, and absorbance at 280 nm.

The binding constant of purified antibody for 4-aminobiphenyl in water was measured by radioimmunoassay. Antibody dilutions (1:1 to 1:500) in a total volume of 100 μl (PBS/10% normal horse serum (Sigma)) were added to 100 μl of a solution of 4-aminobiphenyl containing approximately 1000 cpm. After a 2 hr incubation at 7 °C (at which time equilibrium has been attained), 200 μl of saturated ammonium sulfate was added to the assay mixture and stirred at room temperature for 15 min. The precipitate was removed by a 10 min centrifugation (10,000 g), and 200 μl of the supernatant was counted in a scintillation counter.

Antibody was immobilized on porous glass beads (120-200 mesh, 1984 Å mean pore diameter, Sigma) using an adaptation of the general method of Weetall (5). Two g of glass beads was cleaned with 50 ml of nitric acid (5%) at 100 °C for 45 min and then extensively washed with distilled water. The beads were subsequently added to 25 ml of 3-aminopropyltriethoxysilane (10%, pH 3.5) and incubated at 75 °C for 3 hr with stirring. The aminated beads were then extensively washed with distilled water, dried overnight in an oven at 100 °C, treated with 25 ml of 2.5% glutaraldehyde (in 50 mM phosphate buffer, pH 7.0) for 2 hr at room temperature with stirring, and washed with distilled water. The activated beads were resuspended in 5 ml of 50 mM phosphate buffer (pH 7.0) containing approximately 5 mg each of antibody and non-radioactive 4-aminobiphenyl. After incubation overnight at room temperature, the beads were washed first with distilled water and then with 50 mM phosphate buffer, pH 7.0, finally resuspending them in 25 ml of the aforementioned buffer. Fifty mg of sodium borohydride was added to the

suspension, and after incubation at 4 °C overnight the beads were rinsed with distilled water and stored in buffer. In order to measure accurately the amount of antibody bound to the beads, a sample was subjected to acid hydrolysis, followed by amino acid analysis using HPLC (6).

The binding constant of the immobilized antibody for 4-aminobiphenyl in anhydrous organic solvents was determined as follows. The immobilized antibody was dried by filtration through a sintered glass funnel, followed by air drying for 15 min. Glass beads (100 mg) to which antibody had been attached were suspended in 200 µl of solvent, and 20 µl of tritiated 4-aminobiphenyl (containing approximately 1500 cpm) in dimethyl sulfoxide was added (the final concentration of antibody binding sites was approximately 5 µM). The suspension was incubated overnight at 7 °C with vigorous agitation (it was demonstrated in separate experiments that equilibrium was established after approximately 5 hr). The beads were then pelleted by centrifugation, and up to 150 µl of the supernatant was counted in a scintillation counter. The dissociation constant was determined at a number of different concentrations of antibody and ligand in order to ensure accuracy. As a control, the same process was carried out in the absence of glass beads, in the presence of glass beads which did not contain antibody, in the presence of glucose isomerase (a model enzyme) immobilized on porous glass beads (7), and in the presence of bovine serum albumin immobilized on porous glass beads. The specificity of binding was analyzed by performing the binding assays described above in the presence of a 333-fold molar excess (6 µM 4-aminobiphenyl to 2 mM competitor) of various competitors such as 2-aminobiphenyl, biphenyl, aniline and 4-phenylphenol.

RESULTS AND DISCUSSION

A monoclonal antibody (2E11) was successfully raised against the antigen 4-aminobiphenyl. The dissociation constant of the complex formed by 2E11 and 4-aminobiphenyl in aqueous solution was found to be 2.1 µM. The presence of a 333-fold molar excess of putative competitors with structures resembling 4-aminobiphenyl had negligible effect on the binding (Table I). Thus, the interaction between 2E11 and 4-aminobiphenyl is very specific.

In order to present the antigen to the antibody in organic solvents, we first immobilized the antibody, thus avoiding the problem of handling very small quantities of lyophilized antibody (antibodies, like other proteins (8), are insoluble in organic solvents). The porous glass support used in this study was selected because it does not shrink or expand in the presence of non-aqueous solvents. The attachment process described above resulted in a binding of a total of about 1 mg of antibody per 1 g of porous glass beads. By comparing the binding capacity of the immobilized antibody with that of its free predecessor and assuming that both have

Table I. Effect of competitors on binding of 4-aminobiphenyl to immobilized monoclonal antibody 2E11

Competitor[a]	Inhibition of binding in water[b], %	Inhibition of binding in acetonitrile[c], %
2-aminobiphenyl	18	0
4-aminobiphenyl	100	100
biphenyl	38	22
aniline	24	0
4-phenylphenol	30	0

Footnotes to Table I: [a]Competitor molecules were present in the reaction mixture at 2 mM. [b]4-Aminobiphenyl was at the necessary concentration to give approximately 50% binding in the absence of competitors. [c]The total binding in acetonitrile was 35% in the absence of competitors.

identical affinities for the antigen, it was determined that approximately 60% of the immobilized antibody was competent in binding the antigen in water.

The binding constant for 4-aminobiphenyl to 2E11 in acetonitrile was determined to be 23 μM. (This binding was completely reversible: the regenerated antibody, washed extensively with acetonitrile, had the same affinity for 4-aminobiphenyl.) To verify that this binding in the non-aqueous solvent was the result of a specific interaction, like that in aqueous solution, we performed a number of controls. First, the immobilized antibody was boiled in aqueous suspension for 30 min. The resultant antibody could no longer bind 4-aminobiphenyl in either water or acetonitrile. This observation demonstrates that the intact, non-denatured antibody is necessary for binding in both media. Second, an immobilized model enzyme, glucose isomerase, was used instead of 2E11 in the binding assay. Since no detectable binding was observed, it can be concluded that 4-aminobiphenyl is not binding non-specifically to the protein and/or glass bead surface.

Table I describes the effect of four competitors on the binding of 4-aminobiphenyl to 2E11 in water and acetonitrile. In aqueous solution, 2-aminobiphenyl, biphenyl, aniline, and 4-phenylphenol compete slightly for the 4-aminobiphenyl binding site, as evidenced by the fact that when they are present in a 333-fold molar excess, there is a significant drop in the binding of 4-aminobiphenyl. However, in acetonitrile only biphenyl (which is the strongest competitor in water) shows any significant

Table II. The binding of 4-aminobiphenyl to immobilized monoclonal antibody 2E11 in organic solvents of different hydrophobicities.

Solvent	Dissociation constant[a] (μM)	Log P[b]
water	2.1 ± 0.2	-
dioxane	16 ± 2	-1.10
acetonitrile	23 ± 2	-0.33
1-propanol	46 ± 5	+0.28
1-butanol	51 ± 7	+0.80
1-pentanol	790 ± 360	+1.30

Footnotes to Table II: [a]The dissociation constant for the binding of 4-aminobiphenyl to the immobilized monoclonal antibody was determined as described in the experimental section. Values are reported ± standard error (error increases with increasing binding constant since the % of ligand bound decreases). The total binding of 4-aminobiphenyl to 2E11 was in the range of 30 - 40% in most cases. [b]P is the octanol/water partition coefficient and log P is a measure of the hydrophobicity of the solvent (10): the greater the partition coefficient, the more hydrophobic the solvent.

inhibition of the binding of 4-aminobiphenyl to the antibody. Furthermore, a 333-fold molar excess of 2-aminobiphenyl (2 mM) over 4-aminobiphenyl (6 μM) in dioxane, acetonitrile, 1-propanol, 1-butanol, and 1-pentanol did not affect the binding of 4-aminobiphenyl (data not shown). Since one would expect that the non-specific binding properties of 2- and 4-aminobiphenyl to be similar (see below), it can be concluded again that the binding of 4-aminobiphenyl to 2E11 in organic solvents is highly specific.

To verify that a non-specific hydrophobic binding site of a protein would indeed bind both 2- and 4-aminobiphenyl, we used immobilized bovine serum albumin (BSA) in the place of antibody in a competition experiment. The binding constant of 4-aminobiphenyl for BSA immobilized onto glass beads was determined to be 150 μM in acetonitrile. In the presence of a 333-fold molar excess of 2-aminobiphenyl, there was no appreciable binding of 4-aminobiphenyl. Thus, although the latter did bind to BSA, this binding was non-specific, since it was inhibited by the presence of 2-aminobiphenyl.

It has been shown previously that enzyme specificity (9) and reactivity (10-14) are related to the hydrophobicity of the organic solvent used as the reaction medium. Table II shows that as the hydrophobicity of the solvent decreases, the antibody-antigen affinity increases. Thus this interaction is favored in hydrophilic solvents.

The use of antibodies as biological detectors is well established (14). We have described here that an antibody can function specifically in anhydrous organic solvents. In addition to its mechanistic interest, this finding, if general, may significantly broaden the applications of antibodies as biosensors by extending their use to the determination of lipophilic, water-insoluble compounds.

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