Immobilized Catalytic Antibodies in Aqueous and Organic Solvents[1]

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Abstract: We describe the *first report of immobilized catalytic antibodies* and their behavior in aqueous as well as organic solvents. The results indicate that under aqueous conditions lipase-like catalytic antibodies bound to an inorganic support retain the same activity and stereospecificity they exhibit in free solution. Furthermore, this immobilization imparts added stability in organic solvents. In addition, one abzyme retained a modest activity in 40% aqueous dimethyl sulfoxide. However, attempts to catalyze the reverse reaction (transesterification) under anhydrous conditions were unsuccessful. The use of abzymes as "synthetic reagents" or in "immobilized-abzyme reactors" is suggested.

In the past few years, numerous publications have appeared on catalytic antibodies.[2] Studies in our laboratories have shown antibodies to be modest catalysts for acyl-transfer reactions.[3] Recently, we described the use of a racemic phosphonate **1** to obtain abzymes with either an (*R*)-**2** or (*S*)-**3** substrate specificity (Figure 1).[4] The structures of these compounds are similar to those which naturally occurring lipases can utilize as substrates.[5]

It has been reported that catalytic antibodies function in reverse micelles.[6] Furthermore, it has been established that enzymes (namely lipases and proteases) can act as catalysis in water-miscible or water-immiscible solvents.[7,8] Since enzymes may often exhibit altered activity in organic solvents,[7a] we decided to test the feasibility of using our lipase-like antibodies under these unnatural conditions. To determine abzyme stability in organic solvents,[9] we placed lyophilized antibody 2H6 (specific for the (*R*)-**2** ester) and 21H3 (specific for the (*S*)-**3** ester) into hydrophobic solvents such as hexane, ether, chloroform, and benzene and water-miscible solvents such as acetonitrile and dioxane. After 1 h, the solvent was removed and the antibodies were redissolved in buffer and tested for catalytic activity. In all cases, the rates of the antibody-catalyzed reactions were reduced to less than 5% of their original levels. Although enzymes often show remarkable activity in nonaqueous media,[10] drastic reductions in catalytic efficiency can occur. This is often attributed to phenomena such as aggregation[11] or irreversible conformational change.[12] Several papers describing enzyme immobilization that retard these types of inactivation[13] led us to examine catalytic antibodies bonded to inorganic supports. Herein, we present the *first report of immobilized catalytic antibodies* and their behavior in aqueous as well as organic solvents.

Table I. Kinetic Parameters for the Hydrolysis of (*R*)-**2** by 2H6 and 2H6-1 and (*S*)-**3** by 21H3 and 21H3-1[a]

antibody	K_m (10^{-3} M)	k_{cat} (min^{-1})	k_{cat}/k_{uncat}[b]	K_i ($\times 10^{-6}$)
2H6	3.99	4.6	80 000	2.0
2H6-1	2.20	4.0	72 000	1.2
21H3	0.39	0.09	1 600	0.19
21H3-1	0.20	0.06	1 100	0.05

[a] Conditions: Assay conditions (pH, buffer, buffer concentrations, ionic strength, substrates, and cosolvents) were the same as reported in ref 4. Antibody concentration (nonimmobilized) was determined from a bicinchonic acid (BCA), while immobilized antibody was measured by amino acid analysis. [b] The first-order rate constant for the hydrolysis in the absence of antibody was determined to be 5.56×10^{-5} min^{-1}.

Numerous methodologies exist for protein immobilization to a wide variety of solid supports.[14] Of utmost importance in any immobilization procedure is the preservation of biological activity. Two common methods are typically employed for immobilizing immunoglobulins. One relies on oxidation of the carbohydrate moiety attached to the Fc region of the antibody that produces aldehyde groups.[15] These can be covalently bonded to amino groups (reductive amination) or hydrazide groups (hydrazone formation) on the support.[16] The second method relies on utilizing a heterobifunctional cross-linker.[17] Typical methodology here requires initial coupling of the linker to the support followed by immunoglobulin coupling through lysine residues, usually forming a covalent amide bond. Because either method should be feasible, we have examined both techniques utilizing abzymes 2H6 and 21H3. A site-directed coupling through the carbohydrate func-

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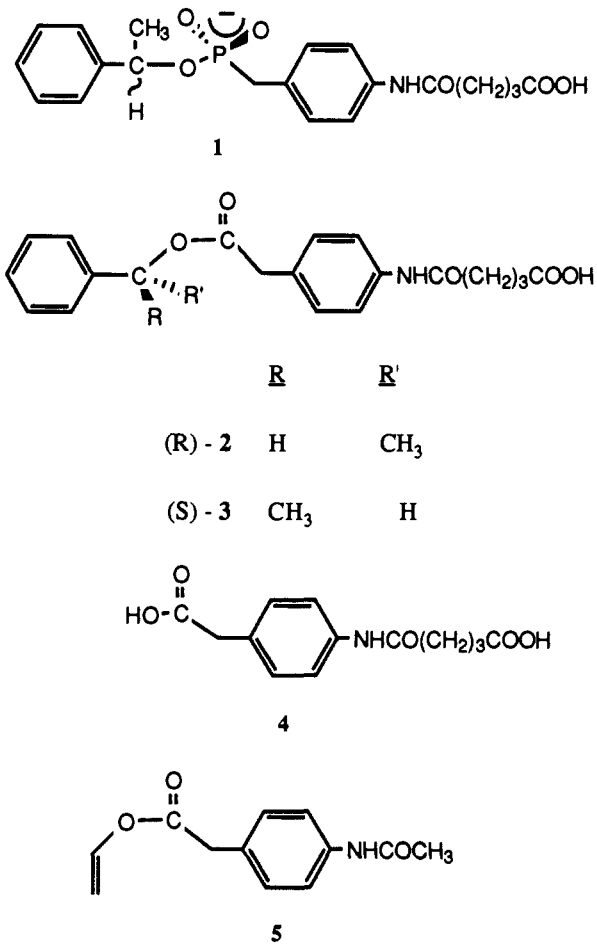

Figure 1. Structures of antigen (**1**), substrates (**2**, **3**), product inhibitor of reaction (**4**), and substrate for the transesterification reaction (**5**).

tionality has proven to be quite disastrous, while heterobifunctional cross-linking has been reproducible.

The catalytic monoclonal antibodies (total of 11) were covalently immobilized on porous glass beads via a slightly modified three-step procedure of Ligler.[17] In this procedure (Figure 2) the glass beads (Sigma Chemical Co.) are initially cleaned with a 5% nitric acid solution followed by a 1:1 mixture of concentrated hydrochloric acid/methanol and then concentrated sulfuric acid. After each acid wash, the beads are rinsed with distilled water. The clean silica surface can now be silanized (inert atmosphere) by use of a 2% toluene solution of MTS ((3-mercaptopropyl)-trimethoxysilane). After 3 h, the excess MTS is removed and the silanized beads are treated with the heterobifunctional cross-linker (*N*-[(γ-maleimidobutyryl)oxy]succinimide ester). The excess reagent is removed, and the beads are dried at room temperature. The activated beads are now resuspended in PBS (phosphate-buffered saline, pH 7.4) with the immunoglobulin for 2 h. The beads are finally rinsed with PBS and can be stored at 4 °C in a suspended form (PBS solution) or filtered to dryness. The porous glass support used in this study was selected because it does not shrink or expand in the presence of nonaqueous solvents. Typical, reproducible loadings were in the range of 0.8 mg of protein/g of beads. It is noteworthy that the binding pocket need not be blocked with hapten during the coupling procedure for catalytic activity to be maintained. Particle size (120–200 and 200–400 mesh) and pore diameter (240–2000 Å) has been shown to affect the activity of an immobilized enzyme.[18] The optimum pore diameters were found to be 1000 Å at 200–400 mesh and 2000 Å at 120–200 mesh. At the optimum pore sizes, the antibodies coupled to 120–200-mesh beads were approximately 1.5 times more active than those coupled to the 200–400-mesh beads when at substrate concentrations near K_m.

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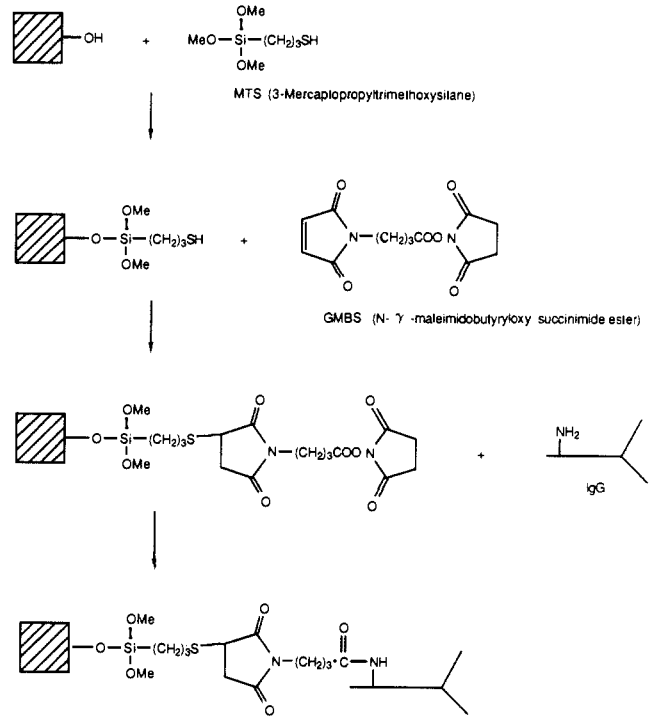

Figure 2. Procedure for coupling immunoglobulins to the inorganic support.

While all 11 catalytic antibodies were successfully immobilized, kinetic studies were performed on only two of the antibodies (2H6 and 21H3, 120–200 mesh, 2000 Å), chosen because of their rates of reaction and enantioselectivity, which we previously described.[4] Immobilized abzyme 2H6 (2H6-I) was completely enantiospecific for ester (*R*)-**2** (hydrolysis of less than 2% of the opposite stereoisomer would have been detected). Its initial rate of hydrolysis measured as a function of substrate (*R*)-**2** concentrations was described by the Michaelis–Menten equation (Table I). The kinetic constants obtained were quite comparable with our previously reported constants when the abzyme was free in solution,[4] including inhibition results (Table I). This suggests that substrate diffusion is not rate limiting probably due to the linker length and modest k_{cat}.[19] When stored with thymerosal (phosphate buffer, 50 mM, pH 7.4), the 2H6-I preparation showed no loss of catalytic activity after 3 months. In addition, 2H6-I can be reused after a reaction (at least three times, with no observable loss of activity) via simple buffer washings on a filter funnel. This process is also useful for removing **4** (K_i = 50 μM, product inhibition) by use of a 15% dioxane/50 mM, pH 5, citric acid solution. This technique should prove to be quite beneficial, since strong product inhibition is commonly seen in catalytic antibody reactions.[4,20] Most important, one may now start thinking of using abzymes in continuous-flow immobilized reactor systems[21] since reaction product inhibitors may be conveniently removed.

In contrast to the *R* stereospecificity of 2H6-I, immobilized abzyme 21H3 (21H3-I) was completely specific for the hydrolysis of the antipodal (*S*)-α-methyl benzyl ester **3**. It likewise displayed saturation kinetics comparable to that previously reported for free 21H3 immunoglobulin in solution (Table I). The 21H3-I preparation was also reusable. In this case, product inhibition was not detected (K_i for **4** > 0.4 mM); hence, the citric acid/dioxane wash was not needed.

The immobilized abzymes were tested for their stability in organic solvents under the same conditions described above that led to inactivation of the uncoupled catalytic antibodies. To our

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delight, both preparations remained functional. With this knowledge, we looked at catalytic activity in 40% solutions of dipolar aprotic solvents (dioxane, DMF, DMSO, CH_3CN). These solvent mixtures cause precipitation of free antibody, rendering them inactive. Solvents of this type would be useful in cases where substrate solubility is a problem. While there was modest catalytic activity with 21H3-1 in aqueous DMSO (40% original activity; complete stereoselectivity), these conditions otherwise proved to give only minor rate accelerations (<10%) with 2H6-1. These results were somewhat disappointing in lieu of findings that immobilized chymotrypsin retains complete activity in water-miscible solvents up to 95%.[22] However, the fact that one abzyme did retain a *significant* amount of activity suggests other immobilized abzymes may be fully catalytic in the presence of certain concentrations of water-miscible organic solvents.

Since the immobilized abzymes are stable in an organic environment, we next attempted to catalyze the reverse reaction (transesterification) under anhydrous conditions. Both immobilized abzymes were tested with (1) *sec*-phenethyl alcohol (neat) and enol ester **5** (4 mM), (2) dioxane or DMSO, *sec*-phenethyl alcohol (4 mM), and enol ester **5** (4 mM), or (3) a biphasic mixture (buffer/butyl ether), *sec*-phenethyl alcohol (4 mM), and 4-acetamidophenylacetic acid (2 mM). However, no rate acceleration over background was observed regardless of conditions tried. Thus, while substrate specificity and reactivity are remarkably similar between the abzymes described and typical lipases under aqueous conditions, their behavior may differ significantly in water-miscible or water-immiscible organic solvents.[7,8,23] Future directions will have to address these differences, possibly through the design of new haptens.

In conclusion, we have demonstrated that catalytic antibodies immobilized on an inorganic support retain the same activity and stereoselectivity they exhibit in free solution. In addition, this immobilization imparts added stability in organic solvents and can afford catalytic activity in an aqueous dipolar aprotic medium. These findings suggest the possibility of utilizing abzymes as "synthetic reagents" or in "immobilized-abzyme reactors" and will be the subject of future reports.

Experimental Section

Materials and Methods. All the controlled-pore glass beads used in this study were purchased from Sigma. The silanizing reagent, (3-mercaptopropyl)trimethoxysilane (MTS), was obtained from Petrarch Systems, Inc., of Huls America (Bristol, PA) and dissolved in anhydrous toluene from Aldrich. The heterobifunctional cross-linker, *N*-[(γ-maleimidobutryl)oxy]succinimide ester (GMBS) was from Calbiochem (La Jolla, CA). All IgG antibodies used were previously described[4] and stored frozen in phosphate-buffered saline (PBS, 10 mM phosphate buffer, 160 mM NaCl, pH 7.4) until ready for immobilization. Assay conditions including pH, buffer concentrations, ionic strengths, substrates, and reaction products were identical with those described by Janda et. al.[4] Kinetic measurements and treatments of the data obtained for both free and immobilized abzyme were determined in a similar fashion as described elsewhere.[4]

General Procedure for Catalytic Antibody Immobilization on Glass Beads. The glass beads selected for eventual antibody immobilization were first cleaned by a series of washes carried out on a hot plate in an open beaker equipped with a stir bar. The beads (4–5 g) were heated (90–100 °C) and stirred for 1 h in each of the following solutions: 5% nitric acid (100 mL), 1:1 concentrated hydrochloric acid/methanol (200 mL), concentrated sulfuric acid (150 mL), and finally in doubly distilled water (150 mL). Following each cleaning step, the beads were collected, rinsed extensively with doubly distilled water, and then air dried for at least 20 min on a medium sintered-glass filter. After the final water rinse the cleaned beads were placed in an open beaker on top of an oven (30 °C) and allowed to air dry overnight.

The beads were then stirred under a nitrogen atmosphere in a 2% (v/v) solution on MTS in dry toluene (100 mL) for 3 h. The silanized beads were collected in a glass filter, rinsed with dry toluene, and again allowed to air dry in an oven-warmed beaker overnight.

A solution of the heterobifunctional cross-linking reagent, GMBS, was prepared by first dissolving in a minimum amount of dimethylformamide (DMF) and then diluting to a final concentration of 2 mM with absolute ethanol. The silanized beads were stirred in the GMBS solution (100 mL) for 2.5 h, then rinsed thoroughly with PBS, and allowed to air dry on a glass filter.

Once dry, the beads coated with silane and cross-linker were weighed and then resuspended in 15–20 mL of PBS in a 50-mL polypropylene centrifuge tube with a screw cap. The antibody to be immobilized, also in PBS (8.7 mg/mL for 2H6, 8.2 mg/mL for 21H3), was added in such an amount to expose 4 mg of antibody to each 1 g of beads. Immobilization was achieved by rocking the centrifuge tube on a peptide shaker for 2 h. Excess (nonimmobilized) antibody was removed by rinsing with PBS on a glass filter. The immobilized abzyme was then resuspended in PBS and stored in 4 °C until assayed for catalysis. The actual amount of antibody bound to the beads by this procedure was determined by subjecting weighed samples of dried beads to acid hydrolysis, followed by amino acid analysis with high-performance liquid chromatography (HPLC).

Organic Solvent Stability Studies. To determine the stability of the immobilized abzymes 2H6-1 and 21H3-1 in organic solvents, we first removed approximately 1 g of each from its PBS storing solution by air drying (separately) on a glass filter funnel. The dried beads were then divided into 1-mL eppendorf tubes and suspended in various organic solvents on a shaker. After 1 h the beads were removed from the organic solvent, rinsed with pH 9.0 ATE buffer (0.1 M aces (2-[(carbamoylmethyl)amino]ethanesulfonic acid), 0.052 M Tris, and 0.052 M ethanolamine), and air dried on a glass filter. The immobilized abzymes were then resuspended in pH 9.0 ATE and tested for their catalytic activity in comparison to immobilized abzyme that had not been exposed to organic solvent.

Immobilization Stability Studies. The immobilized catalytic antibody 2H6-1 was stored at 4 °C in a solution containing 2% thymerosal, phosphate buffer (50 mM, pH 7.4), and 100 mM NaCl. Periodically, the immobilized antibody was withdrawn as a slurry, placed on a filter funnel, and washed several times with phosphate buffer (50 mM, pH 7.4). After the final washing, the beads were air dried and the desired amount was weighed and transferred to the assay solution. Kinetic measurements were made as described above.[4]

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