Pergamon

*Tetrahedron* Vol. 51, No. 28, pp. 7673-7680, 1995
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0040-4020/95 $9.50+0.00

0040-4020(95)00407-9

# A Lipid-coated Catalytic Antibody in Water-miscible Organic Solvents[1]

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**Abstract**: A lipid-coated catalytic antibody was prepared by mixing aqueous solutions of antibody and synthetic glycolipids. The lipid-coated catalytic antibody is soluble in organic solvents and showed a remarkable reactivity for hydrolysis of lipophilic esters in a buffer solution containing 20-80% DMSO (dimethyl sulfoxide). DMSO was added to solubilize the lipophilic esters and a native antibody was denatured in the same condition. Michaelis-Menten kinetics showed that the reduced reactivity of a native antibody in DMSO-buffer solutions is due to the largely deduced $k_{cat}$ value but not the small change of $K_m$ value.

## Introduction

In the past several years, numerous publications have appeared on catalytic antibodies. Various reactions such as hydrolyses of esters and amides, transesterifications, Diels-Alder reactions, decarboxylations, and sigma tropic rearrangements have been reported to be catalyzed by catalytic antibodies.[2] Catalytic antibodies are induced to compounds (haptens) that mimic the transition states in these chemical reactions. Although substrates in these reactions are relatively lipophilic and have some difficulties in solubility in water, the reactions have been carried out always in aqueous buffer solutions because catalytic antibodies are thought to be soluble and stable only in the aqueous phase. In an effort to make catalytic antibodies more attractive catalysts from a synthetic standpoint, catalytic activity of antibodies in organic media should be studied. Several attempts to use native catalytic antibodies without any modifications have been reported in a reversed micellar system,[3] in a physically immobilized system,[4] and in an aqueous-organic biphasic system.[5] However, significant features of catalytic antibodies in organic media or immobilized systems have not been established.

In recent years, enzymes have been interested to use as a catalyst in organic solvents.[6-10] We have reported preparation of a lipid-coated enzyme whose surface is covered by synthetic glycolipid monolayers, that is soluble and acts as a catalyst in most of organic media.[11-15] For example, the lipid-coated lipase can catalyze enantioselective esterification from racemic alcohols and aliphatic acids in the dry isooctane.[1,14]

The catalytic activity of the lipid-coated lipase was 20-100 times higher than those of other systems such as lipase in w/o emulsion,[16-18] direct dispersion of powdered lipase,[19-24] and poly(ethylene glycol)-grafted lipase in organic solvents.[25-27] This lipid-coating technique can be applied widely to other enzymes such as chymotrypsin, phospholipase[15] and glucose oxidase.[12]

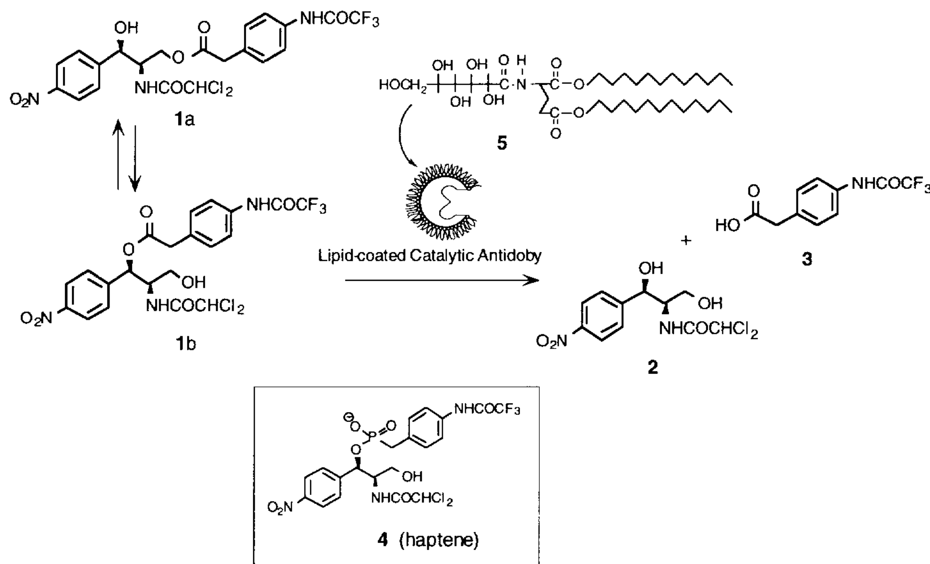


**Figure 1.** Schematic illustration of hydrolysis of ester **1** by catalytic antibody

In this paper, we apply the lipid-coating technique to a catalytic antibody and study a catalytic hydrolysis of lipophilic esters in water-miscible organic media. The catalytic hydrolysis of monoester of chloramphenicol was chosen as an example, because the substrate is lipophilic and the catalytic reaction has been studied in aqueous solution (see Figure 1).[28] The lipid-coated catalytic antibody was stable and shows the high catalytic activity in 20-60% DMSO (dimethyl sulfoxide)-water, although the native one was deactivated largely.

## Experimental Section

**Materials.** Chloramphenicol **2**, 2,2-dichloro-*N*-[2-hydroxyl-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetoamide, was purchased from Wako Pure Chemicals, Osaka and used without further purification. The monoester **1** as a substrate and the phosphonate **4** as a hapten were prepared according to the previous paper.[28] The **4** reacted with glutaric anhydride to bind with carrier protein such as keyhole limpet hemocyanin (KLH).[28]

**Preparation and Purification of Monoclonal Antibodies.** Although the monoester of chloramphenicol exists in a mixture of regioisomers at C-3 and C-1 (**1a** and **1b**), as shown in Figure 1, the one phosphonate **4** was designed as a hapten on the concept of transition-state stabilization,[29] and conjugated with KLH for use as antigens for production of antibodies. The immunizations and cell fusions were performed according to the previous paper.[28] A preliminary assay for the hydrolytic activity of purified antibodies was accomplished by HPLC detection and 6 of 12 antibodies were found to catalyze the hydrolysis at a significant rate above the uncatalyzed background reaction. We chose finally one antibody 6D9 as an active catalytic antibody from them.[28]

**Preparation of a Lipid-coated Catalytic Antibody.** A lipid-coated antibody was prepared as similar to the preparation of lipid-coated enzymes.[11-15] To an aqueous solution (1.0 ml, 0.05 M Tris buffer, pH 8.0) of antibody 6D9 (1 mg), acetone solution (0.3 ml) of synthetic glycolipids (**5**, 10 mg) was added dropwisely into the antibody aqueous solution at room temperature, and then kept under stirring for 20 h at 4 °C. Precipitates were centrifuged at 4 °C (5000 rpm, 5 min.) and lyophilized. To the supernatants, the same lipid solution was added twice and precipitates were also gathered. The obtained white powder (8 mg) of the lipid-coated antibody was insoluble in water or aqueous buffer solution but freely soluble in most organic solvents such as benzene, isooctane, chloroform, and dimethyl sulfoxide (DMSO).

The protein content in the lipid-coated antibody was obtained to be 10 ± 1 wt% from the elemental analysis (C, H, and N) and the UV absorption of aromatic amino acid residues in proteins at 280 nm in chloroform solution, respectively. The recovery amount of a lipid-coated antibody as proteins was 50-60 wt%.

Gel-permeation chromatography of the lipid-coated antibody 6D9 showed one peak at the estimated molecular weight of (1400 ± 50) kD in dichloromethane as eluent (detector, UV at 240 nm; molecular weight was calibrated using a standard polystyrene). Since the molecular weights of a native 6D9 and the glycolipid were *ca.* 150 kD and 621, respectively, the lipid-coated antibody is calculated to contain 2000 ± 100 lipid molecules per one antibody. It can be roughly estimated from the molecular area of the lipid (0.45 $nm^2$) and antibody (surface area: *ca* 300 $nm^2$) that *ca.* 660 ± 50 lipid molecules are required to cover the surface of an antibody as a monolayer. Thus, the catalytic antibody was found to be covered by 2-3 layers of lipid molecules. These values are consistent with each other and with the protein content (10 ± 1 wt%) in the complex obtained from elemental analyses and UV measurements.

**Catalytic Hydrolyses of Monoester of Chloramphenicol in Water-miscible Organic Solvents.** Since the ester substrate is hardly soluble in pure aqueous buffer solution, the hydrolysis reactions were performed in water-miscible organic solvents such as DMSO-water. Monoester of chloramphenicol **1** (5.9 µg, 55 µM) was solubilized in the mixture (0.2 ml) of DMSO (20-80 vol%) and Tris buffer solution (0.05 M, pH 8.0) and mixed with the same DMSO-buffer solution of a lipid-coated antibody (1 µM, 30 µg of protein) at 30 °C. In the case of catalytic hydrolysis by a native antibody, the similar procedure was performed. With the prescribed time interval, the production of chloramphenicol **2** and the disappearance of the monoester **1** were followed by HPLC (instrument, model CCPD, TOSOH Co., Tokyo; column, TSK GEL ODS-80T, φ 4.6 x 250 mm; elution, acetonitrile/$H_2O$/0.1% trifluoroacetic acid; flow rate,

1.0 ml min$^{-1}$; detector; UV at 278 nm). Identification and quantification of the substrates and the products were performed by comparing of the retention time and the peak area to those of the authentic samples, respectively.

## Results and Discussion

Typical time courses of hydrolyses of monoesters in Tris buffer solution (0.05 M, pH 8.0) containing 20 vol% DMSO at 30 °C are shown in Figure 2. Although the monoester substrate was hydrolyzed slowly and spontaneously in the rate of $v_{sp}$ = 9.4 nM min$^{-1}$, the hydrolysis rate was slightly increased by the addition of a native antibody 6D9 with a rate of $v_{cat}$ = 14 nM min$^{-1}$ and largely increased by the addition of the same protein amount of a lipid-coated antibody ($v_{cat}$ = 91 nM min$^{-1}$). Thus, the lipid-coated antibody showed 6 times larger catalytic activity than a native antibody, and the reaction was completely proceeded after 6 h in this condition. The catalytic hydrolyses by both a native and a lipid-coated antibody 6D9 (2 μM) were largely inhibited by the addition of 5 mM hapten **4** (remaining activity, >90%) demonstrating that the catalysis takes place against monoester **1b** in the antibody combining site. These results indicate that both lipid-coated and native antibody 6D9 act as multiple turnover catalysts without a severe product inhibition, probably because of the conformational change between a substrate and a product.[28]

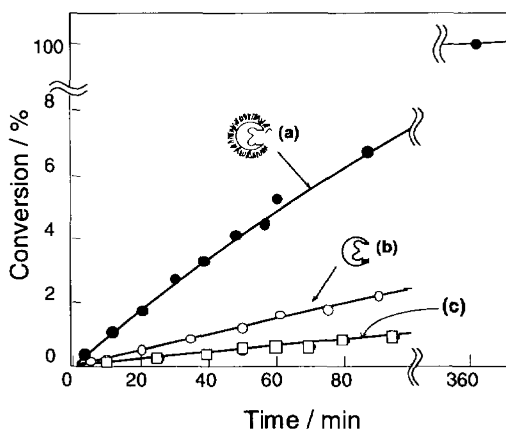


**Figure 2.** Typical time-courses of hydrolyses of ester **1** (55 mM) in 20% DMSO in Tris buffer solution (0.05 M, pH 8.0) at 30 °C. (●): catalyzed by a lipid-coated antibody 6D9 (1 μM, 30 μg of protein), (○): catalyzed by a native antibody 6D9 (1 μM, 30 μg of protein), and (□): spontaneous hydrolysis.

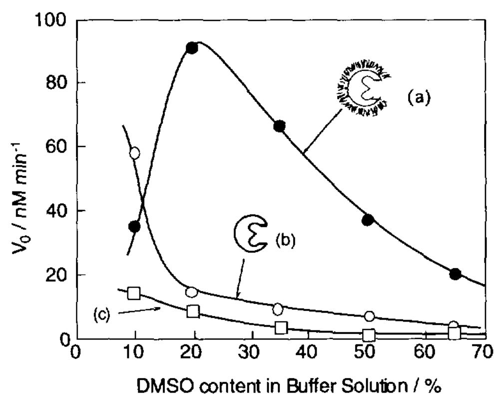


**Figure 3.** Effect of DMSO content in Tris buffer solution (0.05 M, pH 8.0) on hydrolysis reactions of esters **1** (55 μM) at 30 °C. (●): catalyzed by a lipid-coated antibody 6D9 (1 μM, 30 μg of protein), (○): catalyzed by a native antibody 6D9 (1 μM, 30 μg of protein), and (□): spontaneous hydrolysis.

Figure 3 shows the effect of DMSO content in buffer solution on the hydrolysis rates at 30 °C. In the range of 20-65 vol% of DMSO in buffer solution, both substrates and native or lipid-coated antibodies are soluble homogeneously, and the catalytic activity of the lipid-coated antibody was larger than the native antibody due to the denaturation of the native 6D9 in the high content of DMSO. Since the monoester substrate is hardly soluble in pure buffer solution, the spontaneous and catalytic hydrolysis reactions are hardly observed in pure buffer solution. In 10% content of DMSO, the lipid-coated antibody is relatively insoluble due to its lipophilic surface and the catalytic activity was reduced compared with that of the native one.

**Table I.** Hydrolysis Rates of Chloramphenicol Esters in 20% Water-miscible Organic Solvents in Tris buffer (50 mM, pH 8.0) at 30 °C[a]

| Organic Solvents | $v_{sp}$ / nM min$^{-1}$ | $v_{cat}$ / nM min$^{-1}$ | |
|---|---|---|---|
| | | Native antibody | Lipid-coated antibody |
| DMSO (dimethyl sulfoxide) | 6.5 | 14 | 84 |
| DMF (N,N-dimethylformamide) | 4.1 | 5.2 | 69 |
| Tetrahydrofuran | 4.8 | 2.6 | 64 |
| Dioxane | 4.5 | 5.7 | 55 |
| Acetonitrile | 4.4 | 6.9 | 29 |

[a] [substrates] = 50 μM, [catalyst] = 1 μM, 30 μg as protein

Table I shows effect of other organic solvents on the hydrolysis reactions. In all water-miscible organic solvents, the catalytic activity of the native antibody was largely reduced almost equal to the

spontaneous hydrolysis. The lipid-coated antibody seems to be more stable than native one and showed a large catalytic activity in most of water-miscible organic solvents.

Michaelis-Menten kinetics were studied in 20 vol% of DMSO in buffer solution at 30 °C. Figure 4 shows typical saturation behavior of initial rates when the concentration of monoester was changed to 150 μM in the presence of the native and the lipid-coated antibody 6D9. Linear Lineweaver-Burk plots of the steady data gave kinetic parameters of $K_m$ and $k_{cat}$, and the results are summarized in Table II. In 20% of DMSO content, the lipid-coated antibody showed $k_{cat}$ of 0.21 min$^{-1}$ and $K_m$ of 75 μM. Comparison of the $k_{cat}$ value with the rate constant of uncatalyzed reaction gives a 1200-fold acceleration rate. On the contrary, the native antibody showed the very low $k_{cat}/k_{sp}$ = 29, due to the large depression of $k_{cat}$ value compared with the small desperation of $K_m$ value. The native antibody seems not to be denatured in 10% content of DMSO and the obtained kinetic parameters ($K_m$ = 64 μM, $k_{cat}$ = 0.13 min$^{-1}$, $k_{cat}/k_{sp}$ = 1800)[28] are similar to those of the lipid-coated antibody in 20 vol% DMSO. The catalytic activity itself is not increased by lipid-coating. Thus, the lipid-coated antibody can maintain the catalytic activity even in water-miscible organic solvents due to the lipophilic coating lipid wall, although the native antibody is easily denatured in these solvents.

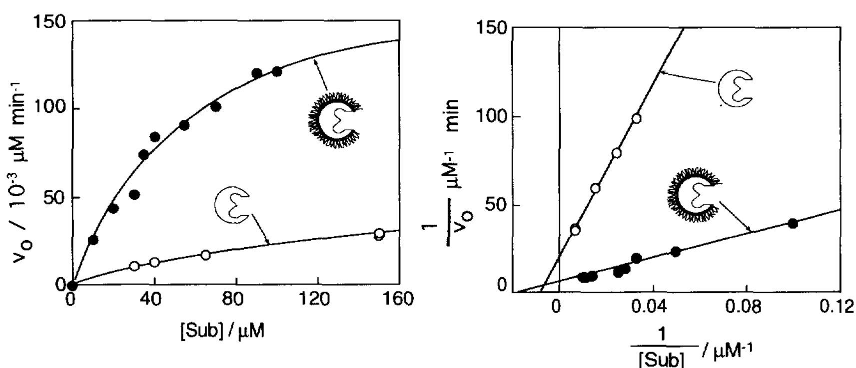


**Figure 4.** Michaelis-Menten kinetics of catalytic hydrolyses of ester **1** in 20 vol% DMSO in Tris buffer solution (0.05 M, pH 8.0) at 30 °C. (●): catalyzed by a lipid-coated antibody 6D9 (1 μM, 30 μg of protein) and (○): catalyzed by native anitibody 6D9 (1 μM, 30 μg of protein).

**Table II.** Michaelis-Menten Kinetic Parameters in Catalytic Hydrolysis of Esters **1** in 20 vol% DMSO in Tris buffer (50 mM, pH 8.0) at 30 °C[a]

| Catalysts | $K_m$ / μM | $k_{cat}$ / min$^{-1}$ | $k_{sp}$ / min$^{-1}$ | $k_{cat}/k_{sp}$ | $k_{cat}/K_m$ / $10^{-4}$ μM min$^{-1}$ |
|---|---|---|---|---|---|
| native antibody 6D9 | 120 | 0.0050 | 0.00017 | 29 | 0.42 |
| lipid-coated antibody 6D9 | 75 | 0.21 | 0.00017 | 1200 | 28 |

[a] [substrates] = 20 - 150 μM, [catalyst] = 1 μM, 30 μg as protein

Since catalytic antibodies are designed to catalyze the wide range organic reactions for lipophilic substrates, it is important to use antibodies in water-miscible or hydrophilic organic solvents. The physical stability is also expected to be increased by coating lipids. The lipid-coated antibody is easily prepared by mixing catalytic antibodies with lipids, which is solubilized in most of organic solvents and can catalyze the reactions without denaturation in organic solvents. We believe that the lipid-coating method can be widely applicable for improving the stability of catalytic antibodies as well as enzymes in organic solvents.

**Acknowledgments:** The research was supported partially by a grant-in-aid for science research from Ministry of Education, Science and Culture Japan, and a grant pioneering research project in biotechnology from Ministry of Agriculture, Forestry and Fisheries Japan.

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(*Received in Japan* 24 *April* 1995; *accepted* 22 *May* 1995)