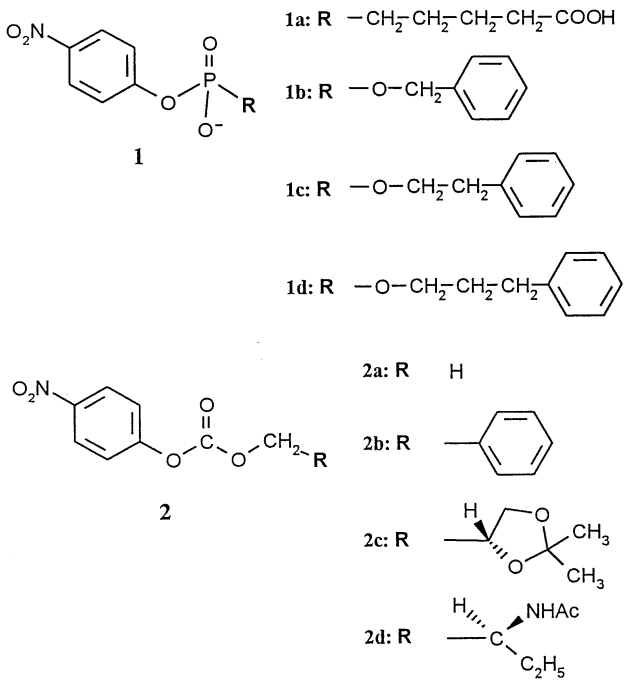
A Mechanistic Study for 4A1 Antibody-Catalyzed Hydrolysis : Detection of Acyl Intermediate

Yumiko Wada, Yukio Sudo,† and Mitsunori Ono*
Fuji ImmunoPharmaceuticals Corp., 125 Hartwell Avenue, Lexington, MA 02173, U. S. A.
†*Research Laboratories, Ashigara, Fuji Photo Film Co. Ltd., Minami-Ashigara, Kanagawa 250-01*

(Received July 1, 1997; CL-970513)

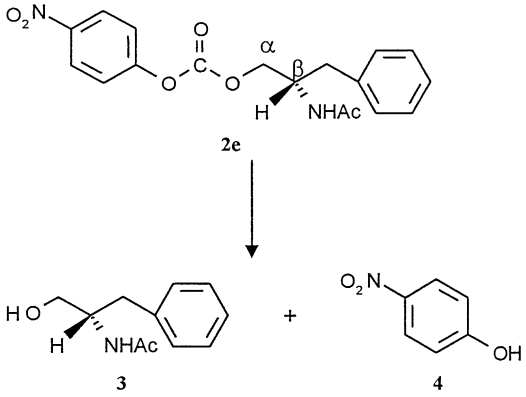
Recent study identified a hydrolytic abzyme 4A1, that has a significant rate enhancement in hydrolysis of carbonate substrates differing from the inducing hapten in structure around the scissile bond. Kinetic investigation suggested the existence of other affinity elements for potential substrate side chains. This paper presents further study of 4A1-catalyzed hydrolysis based on detection of an acyl intermediate in the hydrolytic reaction.

In practice, three or four weakly immunogenic aliphatic methylene carbons are used to connect a hapten with a carrier protein in order to preclude any steric interference from the carrier when hydrolytic catalytic antibodies (abzyme) are elicited.[1] In the preceding paper, we reported that the hydrolytic abzyme 4A1 elicited by hapten **1a**, shows a significant rate acceleration for carbonates different from the hapten structure in the corresponding carrier-proximal region.[2] The rate enhancement (kcat/kuncat) can be greater than 6.4 x 10^4 for substrate, such as **2c**, having an (*S*)-(+)-2,2-dimethyl-1,3-dioxolane-4-methoxy group. Kinetic characterization of this catalytic reaction[2] suggested that affinity element(s) for the substituent must exist in antibody 4A1. In this paper, we describe further study of the recognition site of 4A1 based on detection of an acyl intermediate in the hydrolytic reaction.



The relative importance of structural features affecting the hydrolytic activity of 4A1 were ranked by SAR study[3] using various carbonates. The results indicated that the potential substrates preferably bear a branch at the β-carbon vicinal to the α-methylene group (i.e., **2b-2d**); proton-accepting groups such as ether oxygen (i.e., **2c**) or carbonyl oxygen (i.e., **2d**); and hydrophobic groups. The importance of these features is indicated by the higher value of kcat/kuncat = 5.2 x 10^3 for **2b** as well as lower binding constants K_D = 1.6 x 10^{-5} M, 1.0 x 10^{-5} M and 1.5 x 10^{-5} M for the transition-state analogs **1b**, **1c** and **1d**, respectively.

Interestingly, a steep retardation occurred after the initial burst and then the liberation of *p*-nitrophenol **4** was saturated (Figure 1),[4] when substrate **2e** (200 μM),[5] introducing a benzyl moiety instead of an ethyl group (as in **2d**), was hydrolyzed by 4A1 (4.4 μM) at 30 °C in 10 mM tris/HCl buffer at pH 8.5.



The catalytic activity of the inactivated 4A1 treated with **2e** as well as the hapten binding affinity of the antibody was not

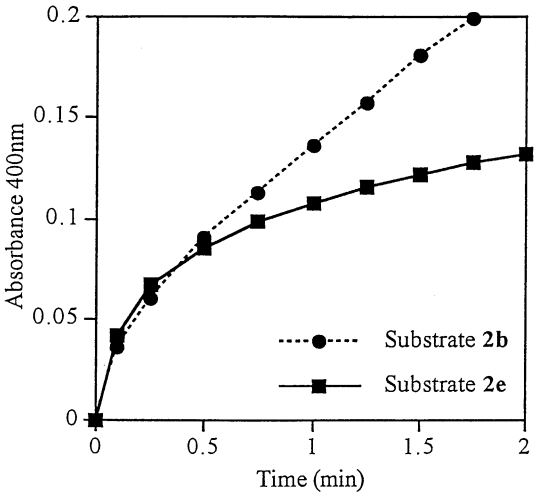


Figure 1. The partial time course for hydrolysis of **2b** and **2e**. The release of *p*-nitrophenol was measured with a absorbance at 400nm.

recovered after extensive dialysis at pH 3.0 in citrate buffer. However, when the inactivated 4A1 was incubated in 10% aqueous CH_3CN at 37 °C at pH 10.5 for 1 h followed by ultra centrifugation, quantitative release of a cleaved fragment (*S*)-(-)-*N*-acetyl phenylalaninol **3**[6] was observed by HPLC analysis.[7] Moreover, greater than 93% of the catalytic activity for **2c** and the binding affinity for **1a** were recovered in the 4A1 antibody incubated at pH 10.5. Since the kcat of the 4A1 antibody-catalyzed reactions exhibited a first order dependence on concentration of hydroxide ion at pH ranging from 7.5 to 9.5 without a significant change of Km values, the retardation observed with **2e** seems to be due to slow hydrolysis of an intermediate in a pH-dependent manner to liberate the fragment **3**, rather than irreversible inactivation.[8] Moreover, supporting data were obtained through 600 MHz ^{13}C-NMR study by using **2e** labeled with ^{13}C on the *N*-acetyl carbonyl carbon. Antibody 4A1 (73 μM) was reacted with the labeled **2e** (5 mM) for 1 h at 37 °C at pH 8.5 and then dialyzed with D_2O/150 mM NaCl at 4 °C for 24 h. As shown in Figure 2, the ^{13}C enriched carbonyl carbon in the inactivated 4A1 is clearly assigned to the peak at 176.2 ppm relative to that of intact 4A1 in D_2O.

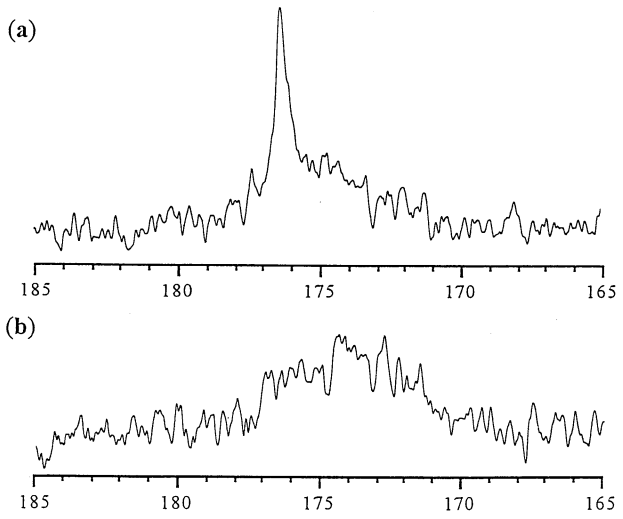


Figure 2. 600 MHz ^{13}C-NMR spectra of fragment **3** incorporated 4A1 (**a**) and intact 4A1 (**b**).

These findings support a mechanism of catalysis of 4A1 abzyme that involves rapid formation of an intermediate followed by slow hydrolysis of the intermediate.[9] Most likely, the intermediate is an acyl antibody resulting from nucleophilic attack by amino acid residue(s) in or near the combining site of **2e**.[10]

Covalent attachment of the acyl moiety of **2e** to a nucleophilic site in the binding site enabled a site-specific chemical modification of 4A1 for further understanding of the recognition sites in 4A1 antibody against **2e** and **2c**. The modification of arginine residue(s) in intact 4A1 antibody with phenylglyoxal[11] led to a 70% loss in catalytic activity for **2c**, but only a 14% loss for **2a**, suggesting that arginine(s) in the abzyme are part of the complementary pocket for the carrier-proximal region of **2c**. However, when the antibody was reacted with substrate **2e** producing the acyl-antibody intermediate, the treatment with phenylglyoxal followed by incubation at pH 10.5 to liberate fragment **3** resulted in only a 10% loss in catalytic activity for **2c** and 7% decrease in titer (ELISA binding to hapten **1a**). These data indicate that the 4A1 affinity site that binds the bulky side chain in **2e** overlaps with the recognition site for the carrier-proximal region of **2c**.

The antibody binding site combined with **2e** is converted to a much less active conformational state than that of **2c** against direct attack of hydroxide,[12] so that the hydrolytic reaction is retained as an acyl antibody intermediate. It is also worth noting that the antibody binds quite tightly to spacially extended β-acetylaminophenethyloxy moiety, nevertheless there is no corresponding portion in the inducing hapten.

Detection of an acyl antibody intermediate and chemical modification experiments have been presented in this paper to reveal that antibody 4A1 uses a multi-step kinetic sequence for the hydrolytic reaction. Considering the level of interest and effort being expended for mechanistic studies of antibody catalysis, the present findings provide an important tool for mechanistic understanding of the enzymatic nature of hydrolytic abzymes. Further efforts are underway to elucidate the primary structure of variable regions of antibody 4A1.

We are grateful to Mr. Mitsuo Yoshikane for carrying out the 600 MHz ^{13}C-NMR work and to Ms. Haruko Shimizu for technical assistance.

References and Notes

1 T. Nishima, A. Tsuji, and D. K. Fukushima, *Steroids*, **24**, 861 (1974); M. K. Shokat and P. G. Schultz, in "Methods in Enzymology," Academic Press, New York (1991), **203**, p.327.
2 Y. Wada, M. Yamamoto, I. Ito, and M. Ono, *Chem. Lett.*, preceding paper.
3 Y. Wada, manuscript in preparation.
4 The concentration of active site is related to an initial burst. It was estimated by extrapolation that about 1.8 mol of *p*-nitrophenol is released per mol of the antibody in the burst.
5 **2e**: mp 70-71 °C; $[\alpha]_D$ +9.7 (c 1.0, $CHCl_3$) ^1H-NMR ($CDCl_3$) δ 1.98 (3H, s, CH_3), 2.91 and 2.96 (each 1H, dd, J = 9 and 6 Hz, $-CH_2-$), 4.21 and 4.33 (each 1H, dd, J = 3 and 9 Hz, $-CH_2O-$), 4.50-4.62 (1H, m, -CH-), 5.71(1H, d, J = 5 Hz, -NH), 7.21-7.42 (5H, m, $-C_6H_5$), and 8.27 and 8.31 (each 2H, ABq, J = 5 Hz, $O_2N-C_6H_4-$). MS (FAB) m/z 359 (M+H).
6 **3**: mp 128 °C, $[\alpha]_D$ = + 23 (c 1.0, $CHCl_3$).
7 A linear gradient of 0%-100% aqueous CH_3CN at pH 7.5 was run over 20 min, monitoring absorbance at 210 nm. Under these conditions, a retention time of 16.2 min was observed for (*S*)-*N*-acethyl-phenylalaninol.
8 J. D. Stewart, J. F. Krebs, G. Siuzdak, A. J. Berdis, D. B. Smithrud, and S. J. Benkovic, *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 7404 (1994).
9 G. W. Zhou, J. Guo, W. Huang, R. J. Fletterick, and T. S. Scanlan, *Science*, **265**, 1059 (1994).
10 D. S. Tawfik, R. R. Zemel, R. A-Yellin, B. S. Green, and Z. Eshhar, *Biochemistry*, **29**, 9916 (1990).
11 K. D. Janda, M. I. Weinhouse, T. Danon, K. A. Pacelli, and D. M. Schloder, *J. Am. Chem. Soc.*, **113**, 5427 (1991).
12 J. Jacobs and P. G. Schultz, *J. Am. Chem. Soc.*, **109**, 2174 (1987).