Table I. Secondary ^{18}O Isotope Effects on the Alkaline Phosphatase Catalyzed Hydrolysis of Glucose 6-Phosphate[a]

fractnal reactn	pH	isotope effect calculated from residual glucose 6-phosphate[b]	isotope effect calculated from glucose product[c]
0.4626	8	0.99893	
		0.99805	
		0.99897	
0.3907		0.99789	
			0.99830
			0.99841
mean	8	0.9984 ± 0.0004	
0.5501	6	1.00317	
		1.00320	
0.5691		1.00410	
			1.00338
mean	6	1.0035 ± 0.0004	

[a] The isotope effects are for three ^{18}O in the nonbridge positions of the phosphate group but have not been corrected for the lack of isotopic purity of the starting materials.[5] [b] Calculated from the expression $\log (1-f)/\log [(1-f)(R_s/R_o)]$, where f is the fraction of reaction and R_s and R_o is the ^{13}C content of the CO_2 from C-1 in the residual glucose 6-phosphate and initial glucose 6-phosphate, respectively. [c] Calculated from the expression $\log (1-f)/\log (1-fR_p/R_o)$, where R_p is the ^{13}C content of the CO_2 from C-1 in the glucose product.

effect on the rate of hydrolysis caused by ^{13}C at C-1, any discrimination between the two species of glucose 6-phosphate results from the ^{18}O substitution. This discrimination is measured by separating residual glucose 6-phosphate and glucose product after approximately 50% hydrolysis and degrading them separately to ribulose 5-phosphate and CO_2 by the action of glucose 6-phosphate and 6-phosphogluconate dehydrogenases (the glucose was phosphorylated to glucose 6-phosphate by hexokinase and MgATP first). The hydrolysis experiments were carried out in 50 mM buffer containing 500 μM each $MgCl_2$ and $ZnCl_2$. The mass ratio in the isolated CO_2 was measured with an isotope ratio mass spectrometer.

The observed isotope effects were 0.9984 ± 0.0004 at pH 8 and 1.0035 ± 0.0004 at pH 6 (see Table I). When these values are corrected for the isotopic purity of the starting materials and the cube root taken,[5] isotope effects of 0.9994 (pH 8) and 1.0012 (pH 6) were obtained for single ^{18}O substitution. Since the dianion of glucose 6-phosphate is the true substrate for alkaline phosphatase, the value at pH 6 must be corrected for the proportion of glucose 6-phosphate which is a dianion. This is done by dividing 1.0012 by the appropriate portion of the ^{18}O equilibrium isotope effect on deprotonation (1.0030).[7] This gives a value for $^{18}(V/K)$ of 0.9982 at pH 6. The value at pH 8 remains 0.9994. Both of these values may contain a contribution from an equilibrium isotope effect on binding to the enzyme; however, the ratio between the two values (0.9988 ± 0.0006) should reflect the effect of making catalysis more rate limiting by changing the pH. The fact that this ratio is inverse strongly supports a dissociative mechanism for phosphoryl transfer catalyzed by alkaline phosphatase.[9]

This conclusion is consistent with the fact that phosphorylated thiols are excellent substrates for alkaline phosphatase.[10] Such phosphorylated thiols are poor substrates for kinases, however, suggesting that there may be different mechanisms for the two classes of enzymes. Experiments are currently under way to differentiate between these possibilities.

Acknowledgment. This work was supported by NIH Grant GM 18938.

(4) Having the mass ratio close to natural abundance (1.1%) minimizes errors from contaminating CO_2 during the analysis.

(5) The equation used to calculate $^{18}(V/K)$ (the ^{18}O isotope effect for single ^{18}O substitution) at each pH was[6] $1 + W/(^{13}(V/K) - [(1-B)Z/(BX)][W - {}^{13}(V/K)])^{1/3} - 1[1 + 1 - Y)/3]$ where W = observed isotope effect (0.9984 at pH 8 and 1.0035 at pH 6), X = fraction of ^{13}C in the 1-[^{13}C]glucose = 0.99, Y = fraction of $^{18}O_3$ in the [^{13}C,$^{18}O_3$]glucose 6-phosphate = 0.85, Z = fraction of ^{13}C in the 1-[^{12}C]glucose = 0.0001, B = fraction of [^{13}C,$^{18}O_3$]glucose 6-phosphate in the final mixture (natural abundance = 0.0111) = 0.01175, and $^{13}(V/K)$ = the ^{13}C isotope effect at C-1 from analogous alkaline phosphatase hydrolysis experiments with natural abundance glucose 6-phosphate. Because of the broad range of substrate specificity displayed by alkaline phosphatase, this number is assumed to be 1.00.

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(9) A dissociative mechanism does not necessarily imply free metaphosphate as an intermediate. It is more likely that the bond order along the reaction coordinate is low, while that to the nonbridge oxygens is enhanced. See ref 1 for a graph of calculated secondary ^{18}O isotope effects as a function of transition-state structure.

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A Semisynthetic Catalytic Antibody

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Received October 27, 1988

The immune system is unique in its ability to generate highly selective binding sites to a vast array of structurally diverse molecules.[1] It has been demonstrated that the high binding affinity and specificity of antibodies can be exploited in the design of selective catalysts for acyl transfer,[2] pericyclic,[3] photochemical,[4] and redox reactions.[5] In each case, monoclonal antibodies were generated against haptens designed to generate combining sites which acted either to stabilize a transition state, as an entropic trap, or contained a catalytic amino acid side chain. Alternatively, it should be possible to introduce a wide variety of natural or synthetic catalytic groups directly into antibody-combining sites either by generating a cofactor binding site or by selective derivatization of the antibody-combining site.[6] Recently, we reported a general method whereby cleavable affinity labels could be used to site-specifically incorporate a nucleophilic thiol into antibody-combining sites.[7] We now report the first example of a semisynthetic antibody that incorporates a catalytic group in the antibody-combining site. Selective derivatization of a thiol-containing antibody with imidazole affords a selective catalyst for ester hydrolysis. Derivatization of antibodies with other groups such as transition-metal complexes or cofactors should enable us to generate semisynthetic antibodies with a variety of novel functions.

The antibody MOPC 315 binds substituted 2,4-dinitrophenyl (DNP) ligands with association constants ranging from 5×10^4 to 1×10^6 M^{-1}.[8] Lys 52H, which is proximal to the combining site of MOPC 315, has been selectively modified with a unique thiol through the use of cleavable affinity labels (Figure 1).[7,9] Importantly, this thiol acts as a unique handle for the subsequent introduction of catalytic groups into the antibody-combining site.

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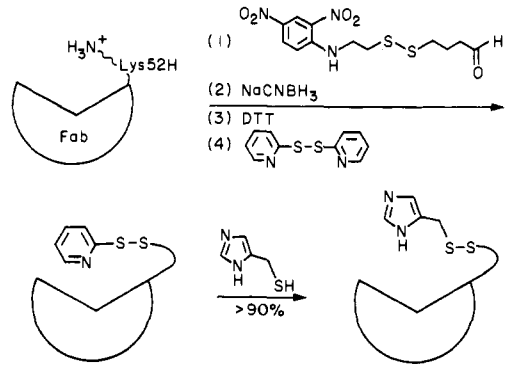

Figure 1. Modification of the Fab fragment of MOPC 315 to introduce an imidazole at the binding site.

In order to derivatize this modified antibody with imidazole, the thiopyridyl disulfide adduct of the antibody[7] (2.5 mg, 0.05 μmol) was treated with 4-mercaptomethylimidazole[10] (0.5 μmol) in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS) at 20 °C. Incorporation of imidazole was assayed by monitoring thiopyridone release spectrophotometrically at 343 nm (ϵ = 7060 M^{-1} cm^{-1}).[11] After 30 min, the reaction was complete, with quantitative release of thiopyridone. The mixture was dialyzed exhaustively against PBS and once against assay buffers. To verify imidazole incorporation, a sample of the imidazole–antibody adduct (1.0 mg) was reduced with 20 mM dithiothreitol and subjected to C18 analytical reverse phase high performance liquid chromatography.[12] A peak in the elution profile was observed with identical retention time to that for an authentic sample of 4-mercaptomethylimidazole.

The hydrolysis of coumarin esters **1a–d** (Figure 2a) by the semisynthetic antibody was assayed in the presence and absence of 1 μM derivatized Fab in 0.1 M sodium phosphate, pH 7.0, at 30 °C. The release of free coumarin was quantitated fluorometrically, exciting at 355 nm and measuring emission at 455 nm. Antibody rates were corrected by subtracting the rate of cleavage in the absence of antibody. From an Eadie–Hofstee plot of initial rate data (Figure 2a), the kinetic constants, K_m and k_{cat}, for the hydrolysis of ester **1b** were determined to be 2.2 ± 0.2 μM and 0.052 ± 0.005 min^{-1}, respectively (pH 7.0). Multiple (>10) turnovers were observed with no loss of catalytic activity. The hydrolysis reaction is competitively inhibited by *N*-DNP-glycine with a K_i of 4 ± 1 μM (pH 7.0).[13] This value is almost identical with the K_D (5 μM) for the binding of *N*-DNP-glycine to underivatized MOPC 315 (determined by fluorescence quenching in PBS).[8] The catalytic activity of the imidazole-derivatized antibody shows a pH dependence consistent with a titratable residue with pK_a 7.5 ± 0.3 (Figure 2b), which is similar to the pK_a (7.5) of 4-methylimidazole.[14] Moreover, the catalytic activity of the semisynthetic antibody is completely destroyed upon treatment with diethylpyrocarbonate, an imidazole-specific reagent.[15,16] The binding affinity of the antibody for *N*-DNP-glycine, as determined by fluorescence quenching, remained unchanged.[8] These data are consistent with the presence of a

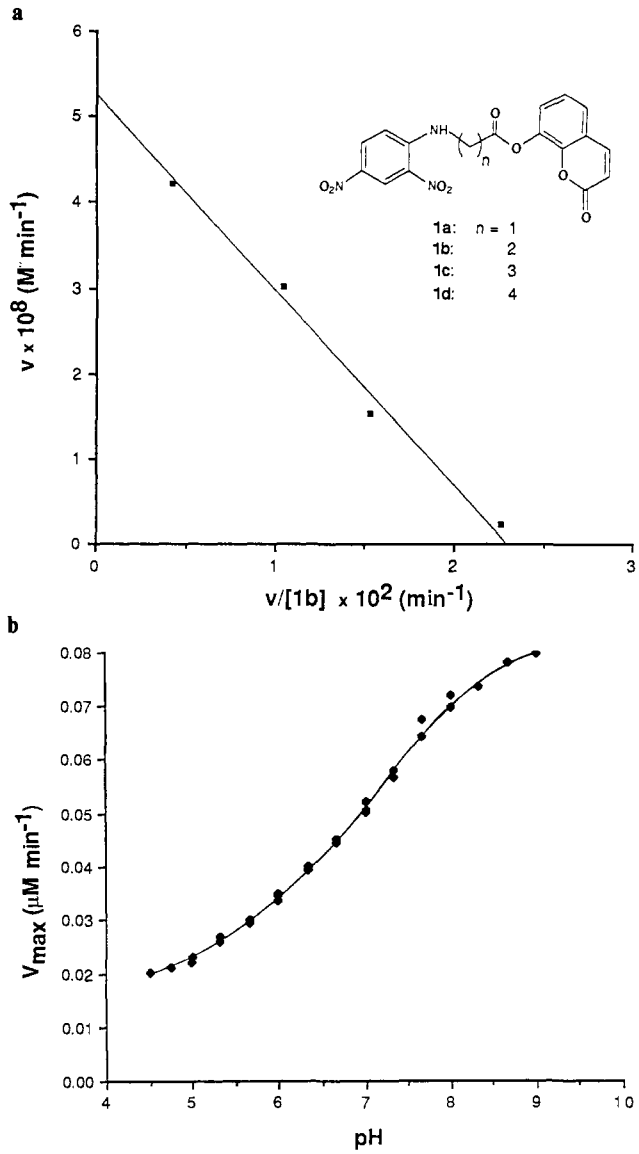

Figure 2. (a) Eadie–Hofstee plot for the hydrolysis of ester **1b** by imidazole-derivatized MOPC 315 Fab at pH 7.0. Inset: ester substrates. (b) Dependence of V_{max} for the antibody-catalyzed reaction on pH.[19]

catalytic imidazole acting either as a general base or directly as a nucleophile in the hydrolysis of ester **1b**. We are currently carrying out further studies to determine the mechanism of the imidazole-assisted catalysis. The rate of the antibody-catalyzed reaction depended on the length of the substrate: at pH 7.0, k_{cat}(**1a**) = 0.008 ± 0.002 min^{-1}; k_{cat}(**1b**) = 0.052 ± 0.005 min^{-1}, k_{cat}(**1c**) = 0.011 ± 0.002 min^{-1}; k_{cat}(**1d**) ≤0.001 min^{-1}. The rate decreased with shorter or longer esters (vs **1b**), presumably due to steric constraints in the binding site and higher entropic barriers, respectively. The second-order rate constant for the antibody-catalyzed reaction, k_{cat}/K_m, was compared to the rate constant for the reaction catalyzed by 4-methylimidazole at pH 7.0.[17] The ratio $[k_{cat}/K_m]/k_{4\text{-methylimidazole}}$ gives an acceleration factor of (1.1 ± 0.2) × 10^3 for hydrolysis of **1b** by the antibody.[18] A greater

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(12) A linear gradient of 0–20% acetonitrile in aqueous 0.1 M triethylammonium acetate, pH 7.5, was run over 20 min, monitoring absorbance at 230 nm. Under these conditions, a retention time of 4.6 min was observed for 4-mercaptomethylimidazole.

(13) Competitive inhibition was demonstrated by a Dixon plot of 1/v vs [*N*-DNP-glycine] at 1 μM Fab and two concentrations of **1b**, 1.0 μM and 3.0 μM, in 0.1 M sodium phosphate, pH 7.0, at 30 °C.

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(16) To an assay mixture consisting of the semisynthetic antibody (1.0 μM) and ester **1b** (10 μM) in 0.1 M sodium phosphate, pH 6.0, at 30 °C was added diethylpyrocarbonate (final concentration 1 mM). Within 13 min, the reaction rate had slowed to the rate in the absence of antibody. The pH of the mixture did not change in the course of the experiment.

(17) The second-order rate constant, $k_{4\text{-methylimidazole}}$, was determined at 3 μM **1b** and 5–50 mM 4-methylimidazole, pH 7.0. From the slope of velocity vs [4-methylimidazole], a value of (2.1 ± 0.1) × 10^1 M^{-1} min^{-1} was obtained.

(18) At increasing pH, the unmodified Fab begins to accelerate the cleavage of ester **1b**: at pH 7.0, k_{Fab} ≤0.005 min^{-1}; at pH 8.0, k_{Fab} = 0.017 ± 0.003 min^{-1}; at pH 9.0, k_{Fab} = 0.055 ± 0.005 min^{-1}. This reaction of the unmodified Fab is a single-turnover event, presumably involving modification of an amino acid side chain near the binding site. However, this stoichiometric reaction is not observed in the presence of the imidazole-derivatized antibody, where the reactive antibody side chain (perhaps the derivatized Lys) is probably inaccessible.

acceleration factor may be obtainable with a shorter, less flexible tether between the catalytic group and the binding pocket.

These experiments are a first step toward the development of selective catalysts which combine the high binding affinity and specificity of the immune system with the diverse, efficient catalytic groups available from synthetic chemistry.

Acknowledgment. We acknowledge support for this work from the National Institutes of Health (Al 24695-02) and General Motors (S.J.P.).

Supplementary Material Available: The synthesis and characterization of compounds **1a–d** (2 pages). Ordering information is given on any current masthead page.

(19) Sodium acetate (0.1 M) was used as the buffer in the range of pH 4.5–6.0, morpholineethanesulfonic acid (0.1 M) in the range of pH 5.0–7.0, sodium phosphate (0.1 M) in the range of pH 6.0–8.0 and tris·HCl (0.1 M) in the range of pH 7–9. These experiments were carried out at 30 °C in the presence of 1 μM modified antibody and 20 μM ester **1b**.

^{13}C NMR Evidence for an Enzyme-Induced Lossen Rearrangement in the Mechanism-Based Inactivation of α-Chymotrypsin by 3-Benzyl-*N*-((methylsulfonyl)oxy)succinimide

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Received November 2, 1988

There has been considerable interest in recent years in the development of mechanism-based inhibitors and their subsequent utilization as enzyme probes and as potential therapeutic agents.[1,2] A novel type of mechanism-based inhibitor is one that generates an electrophilic species via an enzyme-induced *rearrangement*. The one and only example reported so far[3] involves an enzyme-induced allyl sulfoxide–allyl sulfenate ester 2,3-sigmatropic rearrangement. We now present evidence that 3-benzyl-*N*-((methylsulfonyl)oxy)succinimide **1** and related compounds[4] inactivate α-chymotrypsin and human leukocyte elastase (HLE), an enzyme of considerable clinical interest,[5,6] via an enzyme-induced Lossen rearrangement and according to the mechanism depicted in Scheme I.[7]

In earlier biochemical studies[8] we demonstrated that compound **1** is a time-dependent irreversible inactivator of α-chymotrypsin and HLE and that the inactivation involves the active site. The chemical competence of the steps shown in Scheme I was also established. Thus, reaction of equivalent amounts of **1** and $NaOCH_3/CH_3OH$ (room temperature/1 h) resulted in the formation of a mixture of two isomeric Lossen rearrangement products.[4] Furthermore, amino acid-derived isocyanates such as, L-norvaline methyl ester isocyanate, for example, have been shown to inactivate HLE and chymotrypsin rapidly and irreversibly.[9,10]

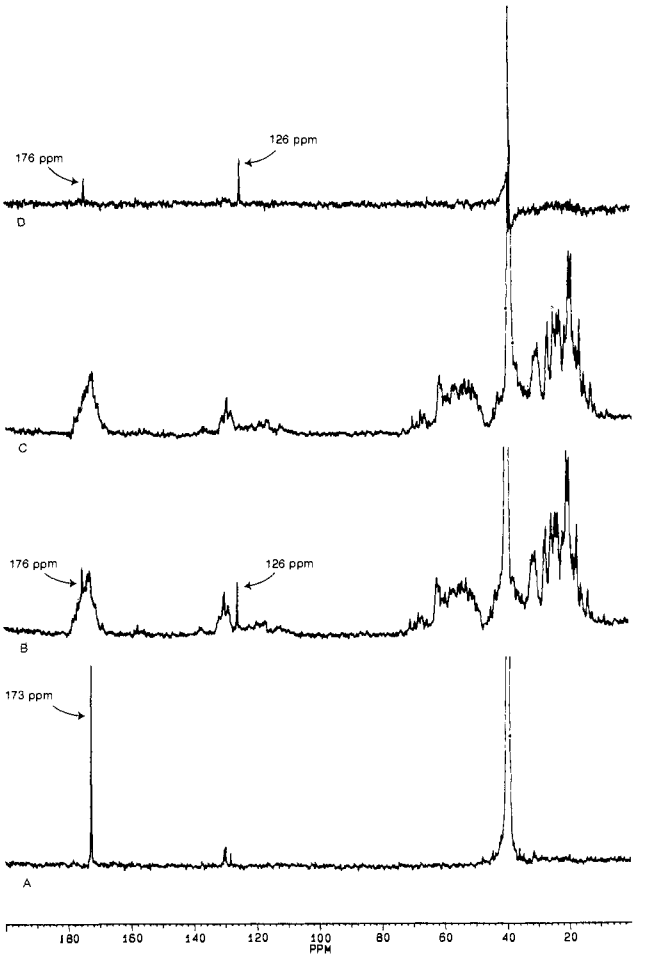
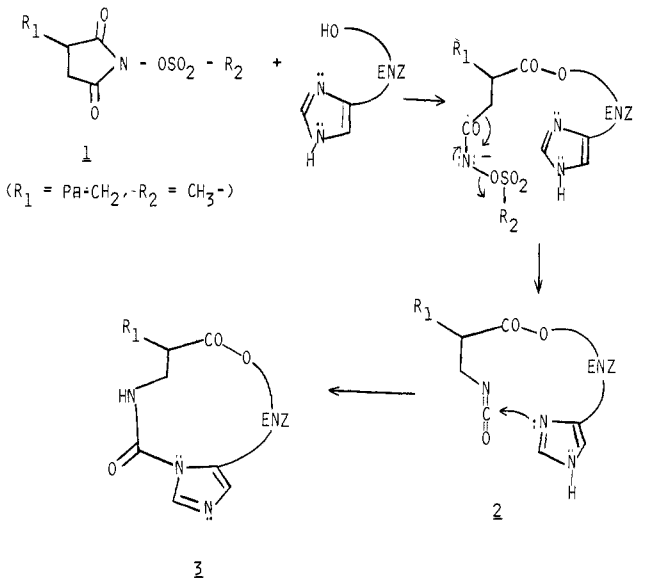


Figure 1. ^{13}C NMR spectra of labeled **1** and chymotrypsin. A: 2 mM **1** in D_2O (7.5% DMSO); B: 2 mM **1** plus 2 mM chymotrypsin in D_2O (7.5% DMSO); C: 2 mM unlabeled **1** plus 2 mM chymotrypsin in D_2O (7.5% DMSO); D: difference spectrum of B and C. All spectra were run on a Bruker 500 MHz instrument using the following conditions: 54° pulse, 0.6 s repetition period, 14 000 scans, broad band ^1H decoupling, and 20 Hz line broadening. In all spectra the large multiplet at 39.5 ppm is due to DMSO.

Scheme I



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