ring of molecule A and N3A over uridine ring B ($-1 + x, y, z$). Atom C2B is stacked approximately right above C4A and C4B above C2A. The C···D dimer stacks atoms O4, C4, N3, and C2 of molecule C approximately right above atoms C2, N3, C4, and O4 of molecule D ($-1 + x, y, z$). The pattern in AZT is intermediate between these two but involves molecules A and B ($x, y, 1 + z$). In all three cases the distance between the planes is about 3.2 Å. No short intermolecular contacts other than hydrogen bonds are observed in the structure of CS-87.

Conclusions

Low-temperature data collection has allowed accurate determination of conformational parameters, including complete resolution of a disordered azido substituent. Relatively large variations are observed in the conformations of molecules that have only very small differences in intramolecular steric interactions. However, correlations can be observed between the most important intermolecular contacts (hydrogen bonding between bases and with the 5'-hydroxyl group) and the variations in molecular conformations. Pairs of molecules with identical base pairing (AZT, the A-B dimer of CS-85) have identical conformations in the glycosyl link and the furanose ring geometry. The alternate base pairing observed in the second dimer of CS-85 and the chain hydrogen bonding in CS-87 is correlated with differences in the internal conformational features. The latter molecules also have the least planar uridine rings. The preferred conformation of the azido group is trans to the C2'-C3' bond. However, deviation from this position is correlated with an increased C2'-endo conformation of the furanose ring. On the other hand, tendency toward C3'-envelope conformations appears to be correlated with a χ angle close to 180° and a trans conformation for the γ angle.

The large variation in packing arrangements appears to be the result of several different factors, including hydrogen bonding, base stacking, and effects of molecular conformation. One would assume that strongest type interaction possible in these structures would be the base pairing involving two hydrogen bonds and stacking of the uridine rings. Both interactions are observed in the structures of AZT and CS-85 but not in the structure of CS-87, which lacks the substitution at the 5 position. The geometries of the uridine rings are essentially identical. The only difference, a small deviation from planarity appears to be correlated with the use of O4 in hydrogen bonding and not with the substitution at C5. Therefore, this substitution does not greatly alter the electron density distribution in the uridine ring but may well enhance dipole–dipole interactions that promote base stacking and base pairing. These intermolecular interactions are strong enough to compensate for the destabilizing effect of the use of ether oxyens as acceptors for hydrogen bonds while carbonyl oxygen atoms remain unused. Crystallization of all three compounds have been attempted from a large range of solvents and at different temperatures. Crystals have been obtained from several of these conditions, but all have been isomorphous with the structures reported here. This indicates that the observed structures are very stable low-energy structures achieved by a combination of low-energy molecular conformations and favorable intermolecular interactions.

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Supplementary Material Available: Tables of anisotropic thermal parameters, hydrogen atom coordinates and thermal parameters and bond distances and angles (9 pages); listing of observed and calculated structure factor amplitudes (43 pages). Ordering information is given on any current masthead page.

Antibody Catalysis Approaching the Activity of Enzymes†

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Abstract: Antibodies were raised to phosphonate esters that model a carboxyl esterolytic transition state. Twenty monoclonal antibodies were screened by a direct assay for hydrolysis of the carboxylic ester deduced from the structure of the transition-state analogue. Five of these were found to be esterases. The transition-state analogue is a specific inhibitor of the activity. One antibody accelerates the hydrolysis of a related substrate with k_{cat} of 20 s^{-1} and K_m of 1.5 mM at pH 8.0. This represents an acceleration of several million times above the spontaneous rate of hydrolysis. The kinetic constants for two substrates and the inhibition constant suggest the better binding of transition states than ground states to the antibody. The pH dependence of the activity can be explained by the titration of an amino acid side chain with a pK_a of about 8.9. Activity is abolished by protein nitration that is specific for tyrosyl groups, and the nitration of two or more groups is prevented by the presence of the phosphonate ligand. Thus, the residues that constitute the combining site of the antibody may act analogously to residues in the active site of enzymes. These results demonstrate that an esterolytic antibody can be relatively efficient as compared with similar enzymes.

The binding of ligands to antibodies is mediated by the same fundamental interactions used by enzymes to bind substrates. This analogy gives cause to speculate that the immune system might be manipulated to yield antibodies with catalytic activity. Recent reports have provided the first experimental evidence for this possibility. We have obtained esterolytic antibodies with a predicted specificity by using a presumed transition-state mimic as part of an immunogen.[1,2] A subsequent report has shown that antibodies elicited to a similar transition-state analogue may also catalyze carbonate hydrolysis.[3] These findings begin to define the properties of a new class of catalyst that has many of the advantages and characteristics of enzymes. For example, lactonization of a δ-hydroxy ester is catalyzed by an antibody with

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(1) Tramontano, A.; Janda, J. D.; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6736.
(2) Tramontano, A.; Janda, J. D.; Lerner, R. A. *Science (Washington, D.C.)* **1986**, *234*, 1566.
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Table I. Kinetic Constants and Rate Factors for the Antibody (50D8) Catalyzed Hydrolysis of **7** and **8**[a]

subst	K_m, mM	V_{max}, $\mu M\ s^{-1}$	k_{cat}, s^{-1}	k_{uncat},[b] $10^6\ s^{-1}$	k_{cat}/k_{uncat}
7	1.5	1.40	20	3.2	6.25×10^6
8	0.15	0.14	2	16.3	1.2×10^5

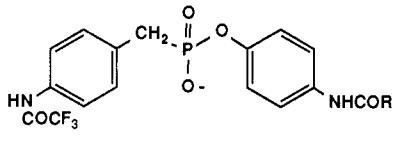
[a] Rates determined spectrophotometrically by measuring initial linear absorbance change at 290 nm in 100 mM Tricine buffer, pH 8.0. Antibody (70 nM) was preincubated at 25 °C, and the reaction was initiated by addition of an aliquot of stock solution of ester in DMF to give initial concentrations of 0.20–5.00 mM and a constant 3% total DMF (v/v). [b] Determined as in (a) in Tricine buffer (25–200 mM, pH 8.0), in the absence of antibody and extrapolated to zero buffer concentration.

very high enantioselectivity.[4] To date, the rates observed with these catalysts are modest by enzymatic standards. We now report that the rates of esterolysis by an antibody are comparable to those found for common esterolytic enzymes.

The use of phosphonyl derivatives related to known substrates as peptidase inhibitors[5] is based on the premise of enzyme transition-state complementarity.[6] The phosphonyl moiety in these ligands occupies the position of the scissile amide, where is imparts the stereoelectronic features of the hydrolytic intermediates or transition states. We used this strategy to design analogues for the transition state in ester hydrolysis. Monoclonal antibodies were prepared to such substances, and some of these were identified by their specific reaction with a potential ester substrate that releases a fluorescent product. This ester acts as a suicide substrate, but the same antibody was then shown to be a catalyst for hydrolysis of phenyl esters having greater analogy to the antigenic ligands. When antibodies from the original pool were directly assayed for their ability to hydrolyze the more stable phenyl esters, new catalytic activities were identified. We describe here the kinetic properties of one of the catalysts, which illustrates similarity in efficiency and kinetic behavior to enzymatic catalysts.

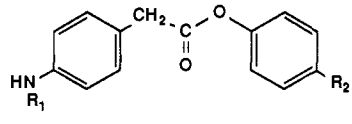
Results

Esterolytic Assay. Twenty monoclonal antibodies (mAb) were selected for their high affinity for carrier-coupled **1** in a heterogeneous immunoassay, propagated in quantity, and purified as previously described.[1] These also bind to the carrier-free ligand



1 R = $(CH_2)_4COON(COCH_2)_2$
2 R = CH_3

2. An HPLC assay for esterolysis of **3** detected a significant activity in five mAb's that were derived from an immunization with the protein conjugate of **1** ($t_{1/2}$ <240 min at pH 8.0; [mAb] = 0.5 μM; [3] = 10 μM). This reaction is completely inhibited



3 R_1 = $COCF_3$, R_2 = $NHCOCH_3$
4 R_1 = $COCH_3$, R_2 = $NHCOCH_3$
5 R_1 = $COCH_3$, R_2 = $NHCOCF_3$
6 R_1 = $COCF_3$, R_2 = H
7 R_1 = $COCH_3$, R_2 = $NHCO(CH_2)_3COOH$
8 R_1 = $COCF_3$, R_2 = $NHCO(CH_2)_3COOH$

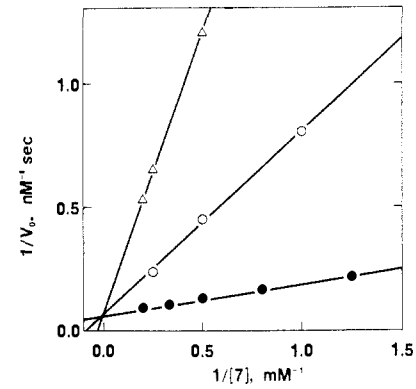
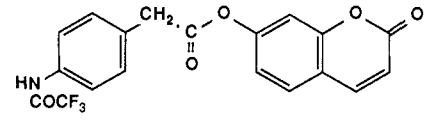


Figure 1. Double-reciprocal plot of the rate of antibody (50D8) catalyzed hydrolysis of **7** as described in Table I in the absence (●) of inhibitor and inhibited by 300 nM (○) and 600 nM (△) of **2**.

by the presence of 2.0 μM phosphonate **2**. The other mAb's had negligible or inhibitory effect on the spontaneous rate of hydrolysis of **3**.[7] Substrate specificities of the two more efficient antibodies (mAb's 50D8 and 57G4) were determined by HPLC with various related aryl esters **4–9** and the carbonate **10**. Both antibodies



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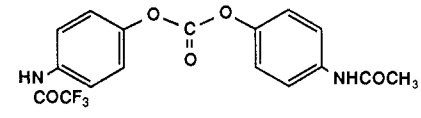

10

showed a preference for the aryl *p*-acetamidophenylacetyl ester **4** over the more hapten congruent *p*-(trifluoroacetamido)-phenylacetyl ester **3** ($t_{1/2}$ 33 min vs $t_{1/2}$ 175 min with mAb 50D8). The "inverted" ester **5** was also hydrolyzed, though not as rapidly as **4**.[8] The phenyl ester **6** appeared to be only a poor substrate. The observed rate of hydrolysis of coumarin ester **9** by mAb 50D8 was comparable to that of ester **4**, though its spontaneous hydrolysis rate was also fast. At pH 7.0 the rate of hydrolysis of **9** by mAb 50D8 is much slower. The hydrolysis at pH 8.0 of *p*-nitrophenyl acetate (0.25 mM), or the carbonate **10** (20 μM), was not accelerated by the mAb's 50D8 or 57G4.

Kinetics and Competitive Inhibition. The initial rate of hydrolysis of **4** with mAb 50D8, measured spectrophotometrically, increased linearly with substrate concentration up to the limit of ester solubility (>0.4 mM)! Therefore, we determined kinetic constants with the more soluble hemiglutarate derivative **7**, which proved to be a remarkably good substrate for 50D8.[9] The maximum rate (V_{max}) was computed from nonlinear regression analysis or from double-reciprocal plots of the initial rate data (Figure 1). The turnover rate (k_{cat}) indicates a rate enhancement greater than 10^6-fold over the uncatalyzed hydrolysis rate extrapolated to zero buffer concentration at pH 8.0 (Table I). The phosphonate ligand **2** was an effective inhibitor. Since the conditions for these kinetics may result in "mutual depletion",[10] the inhibition was shown to be competitive by the method of Henderson (Figure 2).[11] From this data a K_i is estimated at 50 ± 5 nM. The initial rates showed

(4) Napper, A.; Benkovic, S. J.; Tramontano, A.; Lerner, R. A. *Science (Washington, D.C.)* **1987**, *237*, 1041.
(5) See: Bartlett, P. A.; Kezer, W. B. *J. Am. Chem. Soc.* **1984**, *106*, 4282 and references therein.
(6) Fersht, A. R. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman: New York, 1985; pp 311–346.
(7) These mAb's were obtained by immunization with the same or a similar hapten.[1]
(8) The interchange of the trifluoroacetamide and acetamide substituents from their corresponding positions in the hapten **2** might allow this substrate to bind with an inverted orientation of the ester bond.
(9) Ester **7** was soluble at 10 mM in water. At the same initial concentration (0.30 mM) it is hydrolyzed by mAb 50D8 ca. 3 times faster than is **4**.
(10) Webb, J. L. *Enzyme and Metabolic Inhibitors*; Academic: New York, 1963; Vol. 1.
(11) Henderson, P. J. *Biochem. J.* **1972**, *127*, 321.

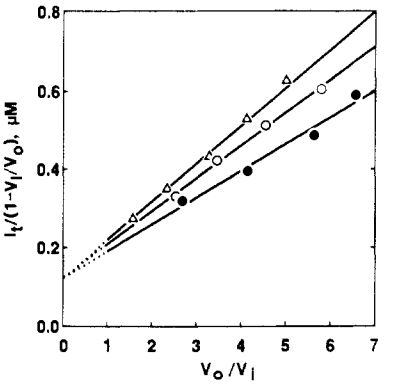

Figure 2. Henderson plot of the inhibition of antibody (50D8) catalyzed hydrolysis of **7** by **2** at pH 8.0 and 25 °C: I_t, total concentration of inhibitor; V_0, initial rate in the absence of **2**; V_i, respective rate in the presence of **2**. Substrate concentrations (mM): 1 (●); 2 (○); 4 (△).

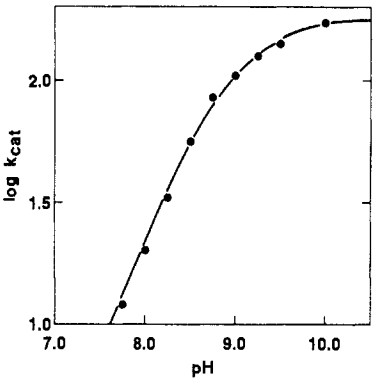

Figure 3. pH–rate profile for antibody (50D8) catalyzed hydrolysis of **7**. Reactions were performed as described in Table I and in the Experimental Section. k_{cat} was determined from the initial rate data at five concentrations of **7** for each pH. The curve shown was calculated by nonlinear least-squares fitting of the data to eq 1 and converting to the logarithmic form.

good linearity and did not suggest the accumulation of an intermediate.

pH–Rate Profile. The catalyzed rate of hydrolysis is found to vary significantly with hydrogen ion concentration. Log k_{cat} plotted vs pH (Figure 3) increases linearly up to about pH 8.5 and then shows downward curvature. The value of K_m does not vary appreciably over this range. The reproducibility of the data suggests these variations in rate above pH 9 are not due to irreversible denaturation of the protein. A pK_a of 8.87 ± 0.05 is calculated from the best fit of the data to a theoretical curve.

Chemical Modification of Antibody. The esterolytic activity of mAb 50D8 was unaffected by treatment with diethyl pyrocarbonate in 100-fold molar excess to protein. Similar treatment with tetranitromethane resulted in a time-dependent loss of about 96% of activity over 1 h. A comparison of the concentration of nitrotyrosine, as determined by the specific absorbance of the modified protein at 428 nm, with the protein concentration suggests the modification of 8.5 ± 0.1 mol of tyrosyl groups per mol of protein.[12] Identical treatment of the protein except in the presence of inhibitor **2** in 7-fold molar excess to protein resulted in nitration of only 6.1 ± 0.1 tyrosyl groups.

The esterolytic activity was also diminished, by about 35%, on treatment of 50D8 with 10 mM (10^5 mol equiv) of iodoacetamide. Neither of the two antibodies were inactivated by **9** or other labile esters, as are other esterolytic mAb's that appear to be covalently modified at the combining site by this reagent.[1,2]

Preparation of Esterolytic Fab. A limited digest of the antibody, an IgG of the γ_1 isotype, and purification by protein A–Sepharose chromatography yielded the Fab fragment as judged by polyacrylamide gel electrophoresis. This fraction retained activity in the esterolysis assay and was otherwise kinetically equivalent to the intact IgG. These observations are consistent with the activity residing in the combining site of the antibody.

(12) Sokolovsky, M.; Riordan, J. F.; Vallee, B. L. *Biochemistry* **1966**, *5*, 3582.

Discussion

The activity of the esterolytic antibody 50D8 is in the range reported for known esterolytic enzymes. Its turnover rate is only 10–10^2 times slower than those of carboxylesterase from pig liver with its good substrates and perhaps 10^3 times slower than acetylcholinesterase hydrolysis of similar substrates. The rate is 10^2–10^4 times faster than α-chymotrypsin's rate of hydrolysis of many reactive esters and nearly equal to that enzyme's fastest esterolyses.[13] Unlike these well-known enzymes that hydrolyze a variety of substrates, antibody-catalyzed esterolysis is more discriminate and can distinguish two very similar esters such as **4** and **6**. The specificity is highlighted by the failure to accelerate the hydrolysis of a labile ester such as *p*-nitrophenyl acetate. Though fluorogenic substrates can be useful for the assay of hydrolytic enzymes, an assay at pH 7.0 using ester **9** did not detect the activity of the catalysts reported here, though the mAb 50D8 will catalyze hydrolysis of **9** more efficiently at pH 8. Thus, we are alerted to the possibility of significant pH dependence of antibody catalysts. The more stable ester **4** is better suited as a substrate in the general assay. We also find that these and other anti-phosphonate antibodies have high chemical specificity for esters, while others report anti-phosphonate antibody-catalyzed hydrolysis of labile carbonates only.[3]

The relatively weak substrate binding (K_m of 1.5 mM) and the good binding of the transition-state analogue (K_i ~50 nM) may reflect the effective stabilization of the transition states vs ground states. By our estimate the differential is greater than 10^4, and this may account for the efficiency of the antibody as a catalyst.[14] A difference of 1 order of magnitude in K_m for substrate **7** vs **8** reflects a reduced affinity, perhaps as a consequence of interactions in the complex where an acetyl group is in a position that is preferentially occupied by a trifluoroacetyl group as defined by the hapten **1**. The interaction may be less important to the binding of activated states than to the binding of ground states. This would suggest a strategy for the design of catalytic antibodies and their appropriate substrates. A substrate may bear a conservative relation to the hapten (**4** as compared with **1**), but the absence of a functional group (as in **6**), or a significant change in structure around the scissile bond (as in **10**), is not acceptable.

Accumulating evidence suggests that catalysis by antibodies can result from the same types of chemical processes found in enzymatic catalysis. Amino acid residues in the complementarity-determining region, which probably define the active site, might participate in the reaction. This may be detected by the effect of pH or specific chemical modification of amino acids on the catalytic activity. The rate profile with varying pH for the mAb 50D8 indicates dependence of activity on the basic form of a group with pK_a of about 9. This is consistent with typical pK_a's for the titration of a tyrosyl or cysteinyl group in proteins.[15] The activity is also quenched by protein chemical modification that is specific for tyrosine residues. Antigen binding protects the protein from modification at more than two of the nitratable tyrosines.[16]

Earlier observations on the chemical modification of the mAb 6D4 implicated a histidine residue as being important for esterolytic activity.[1,2] Preliminary results on the pH dependence of the catalyst mAb 6D4 are consistent with that interpretation. The inherent hydrolytic activity of MOPC167 antibody is also sensitive to tyrosine modification.[17] However, the pH dependence did not

(13) Stoops, J. K.; Horgan, D. J.; Runnegar, M. T. C.; deJersey, J.; Webb, E. C.; Zerner, B. *Biochemistry* **1969**, *8*, 2026.
(14) A good enzyme is one that binds its substrates poorly while binding the respective transition states tightly (see ref 6, p 324).
(15) Cleland, W. W. *Adv. Enzymol.* **1977**, *47*, 273.
(16) Cloning and sequence determination of antibody 50D8 are in progress. The completed sequencing of the heavy chain revealed four tyrosine residues in the complementarity determining domain: Bowdish, K.; Hicks, J. B., manuscript in preparation.

detect the titration of an active-site group, presumably because the spontaneous hydrolysis of the carbonate substrate is very rapid above pH 8 and the catalyzed reaction is relatively slow.

Generally, the antibody molecule can be regarded as are other protein catalysts, and the participation of amino acid side chains as general acids or bases or as nucleophiles is possible. Our results strongly implicate a functional participation of the tyrosine or histidine side chains in chemical catalysis by antibodies. An alternate explanation would assign the protonated or deprotonated amino acid a role in assisting the binding of the transition state. Additional kinetic studies may cast light on this issue, though direct approaches, as by site-directed mutagenesis,[18] are also being considered.

A broad mechanism for an antibody-catalyzed reaction is defined implicitly if the antigen for the antibody is, in fact, a transition-state analogue. Here the mechanism should involve direct attack of water or hydroxide on the ester group. It will be of interest to examine this assumption experimentally. There is significant latitude for the catalytic effects on this mechanism due to the chemical composition of the antibody combining site. We note several distinctions between the catalytic properties of the esterase reported here and one described previously that was elicited to a similar hapten. These differences may reflect variety in the precise mechanisms available to antibodies. Many aspects of these new catalytic entities remain to be resolved. Further studies are expected to reveal other analogies to enzymatic catalysis.

Experimental Section

General Procedures. ^{1}H NMR spectra were recorded on a Bruker WP100 spectrometer. Chemical shifts are reported (δ) from tetramethylsilane. Melting points are uncorrected. The pH values of buffer solutions were measured on a Fisher Model 825 MP pH meter. Reversed-phase liquid chromatography (HPLC) was performed on a Hitachi Model 655A chromatography system with an analytical RP-C18 column (Vydac 218TP54) unless otherwise stated. Immunoassay measurements were made on a Bio-Tek ELISA microplate reader.

Materials. Thin-layer chromatography (TLC) precoated silica plates Kieselgel 60 were obtained from E. M. Science. Buffers were reagent grade (Sigma), and solutions were prepared in distilled, deionized water. Adjuvants and proteins for immunizations are commercially available. Anti-mouse immunoreagents were obtained from Organon Teknika (West Chester, PA). Reagents for protein modification were obtained from Sigma and used as such.

4′-[[(Succinimidyloxy)carbonyl]valerimido]phenyl [[4-(Trifluoroacetamido)phenyl]methyl]phosphonate (1). General procedures for preparation of **1** have been described.[1] The crude product was isolated as a white powder and used as such: ^{1}H NMR ($(CD_3)_2SO$) δ 1.68 (m, 4 H), 2.34 (m, 2 H), 2.75 (m, 2 H), 2.90 (s, 4 H), 2.95 (d, J = 21 Hz, 2 H), 6.6–7.5 (m, 8 H), 8.2 (br, 2 H).

4′-Acetamidophenyl [[4-(Trifluoroacetamido)phenyl]methyl]-phosphonate (2) was prepared as described in ref 1 and purified by preparative TLC, eluting with dichloromethane/methanol (19:1): ^{1}H NMR ($(CD_3)_2SO$) δ 1.75 (s, 3 H), 2.95 (d, J = 21 Hz, 2 H), 6.75 (d, J = 9 Hz, 2 H), 7.0–7.5 (m, 6 H).

4′-Acetamidophenyl 4-(Trifluoroacetamido)phenylacetate (3). Trifluoroacetic anhydride (2.1 mL, 15 mmol) was added slowly to a suspension of 4-aminophenylacetic acid (0.76 g, 5.0 mmol) and sodium carbonate (1.12 g, 10.6 mmol) in acetonitrile/water (20:1, 15 mL) at 4 °C. A solution of 1 N hydrochloric acid (20 mL) was added, and the mixture was extracted with ethyl acetate (2 × 40 mL). The organic solution was dried ($MgSO_4$) and concentrated to give 0.99 g (80%) of 4-(trifluoroacetamido)phenylacetic acid: ^{1}H NMR (CD_3CN) δ 3.60 (s, 2 H), 7.35 (d, 2 H), 7.6 (d, 2 H), 9.3 (br, 1 H). A solution of the acid (0.5 g, 2.0 mmol) in thionyl chloride (4 mL) was stirred at 40 °C for 3 h. Volatile components were removed in vacuo, leaving the acid chloride as a yellow solid: ^{1}H NMR (CD_3CN) δ 4.30 (s, 2 H), 7.35 (d, 2 H), 7.65 (d, 2 H), 9.3 (br, 1 H). Triethylamine (0.31 mL, 2.2 mmol) was added to a solution of the acid chloride (0.53 g, 2.0 mmol) and 4-acetamidophenol (0.30 g, 2.0 mmol) in dichloromethane (5.0 mL). Ethyl acetate (50 mL) was added, and the solution was washed with 0.5 N hydrochloric acid (2 × 10 mL), dried ($MgSO_4$), and concentrated. Silica chromatography (dichloromethane/ethyl acetate, 9:1) afforded 0.55 g (72%) of **3**: ^{1}H NMR ($CDCl_3$) δ 2.20 (s, 3 H), 3.90 (s, 2 H), 7.0–7.6 (m, 8 H), 8.0 (br, 2 H).

4′-Acetamidophenyl 4-Acetamidophenylacetate (4). A mixture of acetic anhydride (1.9 mL, 20 mmol), 4-aminophenylacetic acid (1.06 g, 7.0 mmol) and sodium carbonate (2.1 g, 20 mmol) in acetonitrile/water (10:1, 20 mL) was stirred for 30 min. A solution of 1 N hydrochloric acid (30 mL) was added and the mixture extracted with ethyl acetate (2 × 40 mL). The organic solution was dried ($MgSO_4$) and concentrated to give 1.2 g (81%) of 4-acetoamidophenylacetic acid: ^{1}H NMR (CD_3CN) δ 2.3 (s, 3 H), 3.9 (s, 2 H), 7.0–7.6 (m, 4 H). The acid (0.59 g, 3.0 mmol) was dissolved in trifluoroacetic anhydride (4.0 mL). After 15 min, volatile components were removed under reduced pressure. The residue was dissolved in dichloromethane (5 mL) and treated with 4-acetamidophenol (0.45 g, 3.0 mmol) and triethylamine (0.56 mL, 4.0 mmol) at 0 °C. A similar workup as for **3** provided 0.90 g (92%) of **4**, which was crystallized from acetonitrile. **4**: mp 225–227 °C; ^{1}H NMR ($CDCl_3$) δ 2.20 (s, 3 H), 2.25 (s, 3 H), 3.90 (s, 2 H), 6.95–7.55 (m, 8 H), 7.8 (br, 1 H), 8.0 (br, 1 H).

4′-(Trifluoroacetamido)phenyl 4-Acetamidophenylacetate (5). According to the procedure used for preparation of **4**, the trifluoroacetyl mixed anhydride was treated with 4-(trifluoroacetamido)phenol and triethylamine to provide **5** in 75% yield: ^{1}H NMR (CD_3CN) δ 2.05 (s, 3 H), 3.86 (s, 2 H), 7.1–7.8 (m, 8 H), 8.3 (br, 1 H), 9.2 (br, 1 H).

Phenyl 4-(Trifluoroacetamido)phenylacetate (6). According to the procedure used for **3** the acid chloride and phenol were combined to produce 6 in 95% yield: ^{1}H NMR ($CDCl_3$) δ 4.0 (s, 2 H), 7.0–7.7 (m, 9 H), 8.5 (br, 1 H).

4′-(4″-Carboxybutyramido)phenyl 4-Acetamidophenylacetate (7). According to the procedure described for **4**, the trifluoroacetyl mixed anhydride was combined with 4-nitrophenol to provide 4′-nitrophenyl 4-acetamidophenylacetate in 94% yield: ^{1}H NMR ($CDCl_3$) δ 2.15 (s, 3 H), 3.80 (s, 2 H), 7.15–7.60 (m, 6 H), 8.25 (d, 2 H), 9.3 (br, 1 H). The nitrophenyl ester was reduced by catalytic hydrogenation (1 atm H_2) over 10% palladium on charcoal in ethanol containing 0.5% concentrated hydrochloric acid. The mixture was stirred rapidly for 8 h, diluted with ethyl acetate, and washed with 5% aqueous sodium bicarbonate and then with brine. The organic solution was dried ($MgSO_4$) and concentrated to give crude 4′-aminophenyl ester: ^{1}H NMR (CD_3CN) δ 2.05 (s, 3 H), 3.80 (s, 2 H), 6.5–6.8 (m, 4 H), 7.2–7.6 (m, 4 H). This was dissolved in dry tetrahydrofuran together with 1 equiv of glutaric anhydride and triethylamine (1.2 equiv). After 1 h, the mixture was diluted with ethyl acetate and washed with 0.5 N hydrochloric acid and then with brine. Solvent was removed, and the solid was dissolved in water at pH 7.5 (adjusted with 5% Na_2CO_3 solution). The aqueous solution was washed once with ethyl acetate and then acidified to pH 2 with 1 N hydrochloric acid. The precipitate was collected and crystallized from 5% aqueous acetonitrile to obtain **7**: mp 199–200 °C; ^{1}H NMR ($(CD_3)_2SO$) δ 1.4–2.3 (m, 6 H), 1.8 (s, 3 H), 3.6 (s, 2 H), 7.2 (d, J = 8.5 Hz, 2 H), 7.3 (d, J = 8.5 Hz, 2 H), 7.5 (d, J = 8.5 Hz, 2 H), 7.6 (d, J = 8.5 Hz, 2 H).

4′-(4″-Carboxylbutyramido)phenyl 4-(Trifluoroacetamido)phenylacetate (8) was prepared analogously to **7**, via 4′-nitrophenyl 4-(trifluoroacetamido)phenylacetate: ^{1}H NMR ($CDCl_3$) δ 2.15 (s, 3 H), 3.80 (s, 2 H), 7.15–7.60 (m, 6 H), 8.25 (m, 2 H). This was treated as described above to provide **8** as a white solid after recrystallization from acetonitrile: mp 208–209 °C; ^{1}H NMR (CD_3CN) δ 2.05 (m, 2 H), 2.25 (m, 2 H), 2.65 (m, 2 H), 3.80 (s, 2 H), 6.8–7.3 (m, 6 H), 7.5 (d, 2 H), 8.0 (br, 1 H).

2′-Oxo-2*H*-1′-benzopyran-7′-yl 4-(Trifluoroacetamido)phenylacetate (9). The procedure described for **3** was used with 7-hydroxycoumarin as the phenol to obtain **9** as a white solid after silica chromatography, eluting with dichloromethane/ethyl acetate (9:1): ^{1}H NMR (CD_3CN) δ 4.0 (s, 2 H), 6.4 (d, J = 10 Hz, 1 H), 7.1–7.7 (m, 7 H), 7.9 (d, J = 10 Hz, 1 H), 9.2 (br, 1 H).

4′-Acetamidophenyl 4-(Trifluoroacetamido)phenyl Carbonate (10). *p*-Acetamidophenol (0.44 g, 2.9 mmol) was added to a solution of carbonyldiimidazole (0.49 g, 3.0 mmol) in tetrahydrofuran (5 mL). A precipitate of *p*-acetamidophenyl imidazoyl carbonate formed and was separated by filtration: ^{1}H NMR (CD_3CN) δ 2.1 (s, 3 H), 7.2–7.8 (m, 6 H), 8.3 (m, 1 H), 8.5 (br, 1 H). The solid was added to a solution of *p*-(trifluoroacetamido)phenol (0.60 g, 2.9 mmol) and triethylamine (0.40 mL, 2.9 mmol) in chloroform (10 mL), and the solution was refluxed for 24 h. The solvent was removed, and the product was isolated by silica chromatography (ethyl acetate): ^{1}H NMR (CD_3CN) δ 2.20 (s, 3 H), 7.25 (d, J = 10 Hz, 2 H), 7.35 (d, J = 10 Hz, 2 H), 7.60 (d, J = 10 Hz, 2 H), 7.70 (d, J = 10 Hz, 2 H), 8.4 (br, 1 H), 9.3 (br, 1 H).

Antibody Production and Assays. Protein conjugates were prepared and used to immunize mice (129G1X$^+$) as described.[1] Spleen cells from these mice were fused to myeloma cells (SP 2/0), and hybridoma cultures were obtained by standard procedures.[19] Cells producing anti-**1** mAb

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were selected by an immunoassay (ELISA)[20] against a conjugate of **1** with bovine albumin. Antibody of IgG class was obtained by propagating selected clones in mouse (Balb/c 129G1X$^+$) ascites. Purified[1] mAb was dialyzed against phosphate buffer (20 mM, pH 7.0), and its protein concentration was determined by the BCA method (Pierce). An aliquot of the antibody stock solution, calculated to give 0.5 μM protein, was added to a solution of the ester or carbonate (10 μM) and an internal standard (acetophenone, 20 μM) in phosphate buffer (50 mM, pH 8.0). The mixture was kept at 23 °C and analyzed by HPLC as previously described.[2]

Kinetic Measurements and Data Analysis. Esterolysis rates were measured by absorbance changes at 290 nm on a Cary 14DS that is digitally interfaced to an AT+T personal computer as supplied by AVIV Corp. (Lakewood, NJ). Reactions were performed in 1.00 mL of buffer solution in a quartz cuvette maintained at 25.0 ± 0.1 °C by a water-jacketed cell holder equilibrated to a Lauda RM6 circulating water bath.

The initial linear rate was measured at <3% hydrolysis of total substrate. The observed rate was corrected for the uncatalyzed rate of hydrolysis in the absence of antibody. Kinetic parameters V_{max} and K_m were determined by nonlinear least-squares fitting of the initial rate vs substrate concentration to a hyperbolic curve described by the Michaelis-Menten equation. The inhibition constant K_i was extrapolated from a replot of the slopes of the linear Henderson plots vs total substrate concentration. The variation of initial rates as a function of pH was measured in borate (50 mM) at pH above 8.5 and in Tricine (100 mM) otherwise. The pK_a that describes the pH dependence of k_{cat} vs hydrogen ion concentration was determined by fitting the data to eq 1, where

$$k_{cat}(H) = k_{cat}K_a/(K_a + [H^+]) \quad (1)$$

$k_{cat}(H)$ is the turnover rate at a given pH, k_{cat} is the limiting k_{cat} at high pH, K_a is the ionization constant for a single titrable amino acid side chain, and $[H^+]$ is the hydrogen ion concentration.

Chemical Modification of mAb 50D8. An aliquot of tetranitromethane in dioxane (200 mM), calculated to give 2.0 mM (100 equiv), was added to a solution of antibody (20.0 μM) in Tris buffer (100 mM, pH 8.0) at 23 °C. The activity was checked periodically by addition of an aliquot of this solution to a solution of **7** (0.60 mM) in borate buffer (50 mM, pH 9.2) and the initial rate determined spectrophotometrically. After 1 h the mixture was separated by HPLC on size-exclusion gel (TSK gel G2000SW, Phenomenex), eluting with Tris buffer (100 mM, pH 8.0). The protein was collected and concentrated, and its specific absorbance at 428 nm and protein concentration was determined. In a second experiment antibody was preincubated with **2** (0.14 mM) for 30 min and then subjected to tetranitromethane treatment as before.

A similar procedure was used for treating the protein (6.0 μM) with iodoacetamide (10.0 mM) of diethyl pyrocarbonate (0.60 mM), and activity was monitored as a function of reaction time.

Production of Fab Fragment. A mixture of antibody (10 mg) and immobilized papain on 6% beaded agarose (0.5 mL of commercial slurry; Pierce) in 100 mM phosphate, 20 mM cysteine hydrochloride buffer (1.0 mL) was adjusted to pH 7.0 ± 0.2 and shaken for 8 h at 37 °C. Reaction progress was checked by SDS-Page[21] (10–15% gel), using the Pharmacia pHast system. Immobilized enzyme was removed by filtration through a 50-μm filter, and the filtrate was passed through a prepacked protein A-Sepharose column with the recommended buffers (Pierce "immunopure" kit). The esterase activity of the effluent was assayed by the HPLC method described.

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Specific Ring Stacking Interaction on the Tryptophan–7-Methylguanine System: Comparative Crystallographic Studies of Indole Derivatives–7-Methylguanine Base, Nucleoside, and Nucleotide Complexes[1]

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Abstract: For a comparative interaction study between the protonated or methylated guanine base in nucleic acid and the tryptophan residue in protein, three model complexes, 7-methyl-9-ethylguanine (m^{7}GuA)–indole-3-acetic acid (IAA), 7-methylguanosine (m^{7}GuO)–indole-3-acetic acid (IAA), and 7-methylguanosine 5′-monophosphate (m^{7}GMP)–tryptamine (TRP), were subjected to X-ray crystal analyses. All crystals formed extensive stacking layers consisting of alternating guanine and indole rings. These ring–ring interactions are all characterized by nearly parallel alignments of both aromatic rings and by interplanar spacing in the vicinity of 3.4 Å. However, these stacking interaction modes were significantly affected by the ribose and phosphate groups, and the overlapping area of both rings was in the order of m^{7}GMP ≥ m^{7}GuO > m^{7}GuA. In particular, some stacking pairs in m^{7}GuO–IAA and m^{7}GMP–TRP complexes showed the existence of partial π charge transfer from the indole ring to the guanine base in their ground states. The present results clearly show the importance of the tryptophan residue in protein for the selective recognition of the protonated or methylated guanine base in nucleic acid. Furthermore, the results shed light on the recognition mechanism of cap binding protein for the mRNA cap structure.

The ability of a protein to recognize a specific sequence of bases along a strand of DNA or RNA is a fundamental biological process, which is guaranteed by the specific interactions between their constituent chemical growth groups, as well as by the complementary spatial structure between these macromolecules. Therefore, information concerning the interaction geometries is of great significance for understanding the mechanism of selective recognition.

Aromatic amino acids can bind with nucleic bases via π–π stacking interaction.[3] Among them, tryptophan has the strongest binding ability because of the best π-electron donation character

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