Mechanistic Studies of a Tyrosine-Dependent Catalytic Antibody

Mark T. Martin,*,‡ Andrew D. Napper,‡ Peter G. Schultz,§ and Anthony R. Rees‡,∥

IGEN, Inc., 1530 East Jefferson Street, Rockville, Maryland 20852, and Department of Chemistry, University of California, Berkeley, California 94720

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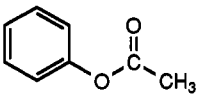
ABSTRACT: A pre-steady-state multiple-turnover kinetic burst is observed during hydrolysis of phenyl acetate by the catalytic antibody, 20G9. The burst is caused by partial product inhibition by phenol ($K_{i,app}$ = 2.5 μM), which lowers both k_{cat} and K_M by almost an order of magnitude without affecting k_{cat}/K_M. The acid limb of the steady-state k_{cat} pH profile of native 20G9 has a pK_a of 9.6, suggesting a catalytic role for tyrosine. Additional evidence for an essential tyrosyl residue is that mild treatment of 20G9 with tetranitromethane nitrates a single tyrosine per equivalent of antigen binding sites and the mononitrated derivative has <5% of the native activity. Near-UV absorbance spectroscopy suggests that the alternative substrates *N*-carbobenzoxyglycine *O*-phenyl ester (ZG-OPh) and *N*-acetylglycine *O*-phenyl ester (AcG-OPh) acylate multiple tyrosines on the antibody. Neither ZG-OPh nor AcG-OPh are measurably catalyzed once appreciable acylation has taken place. Antibody acylated by ZG-OPh is inactive toward phenyl acetate hydrolysis, but can be reactivated by hydroxylamine. The data and derived kinetic rate equations are consistent with an acyl mechanism for phenyl acetate hydrolysis in which phenol inhibits by binding to a covalent *O*-acetyltyrosyl intermediate, slowing deacylation. Although the data are consistent with such a mechanism, they do not rule out other plausible, yet less unifying mechanisms of phenol inhibition; the observed burst could conceivably result from partial mixed phenol inhibition or from phenol-induced nonproductive substrate binding. Because antibodies often use tyrosines in antigen binding, tyrosyl catalytic antibodies may be commonly encountered in the future.

Man-made catalytic antibodies tend to be less efficient catalysts than their natural counterparts, the enzymes (Powell & Hansen, 1989; Shokat & Schultz, 1990). In order to make catalytic antibodies better in the future, it is important to understand the mechanisms and structures of those that exist today. Here, we describe a mechanistic investigation of phenyl acetate (**1**) hydrolysis by 20G9, a catalytic antibody raised to a phenyl phosphonate hapten (**2**) (Durfor et al., 1988) which was designed as a stable analogue of the tetrahedral transition state of phenyl acetate hydrolysis (for structures, see Chart I). We chose to study this particular antibody because its hydrolytic time course is remarkable in having a pre-steady-state kinetic burst. Our results demonstrate that the burst is caused by partial product inhibition by the aromatic leaving group, phenol. The kinetic details of the burst, along with observations that an essential tyrosine on the antibody is acylated by substrate analogues, provide insight into the mechanism of phenyl acetate hydrolysis. The data suggest an inhibition mechanism in which phenol binds to a covalent *O*-acetyltyrosyl intermediate, slowing hydrolytic deacylation. Although the data are entirely consistent with this mechanism, they do not allow us to rule out two alternative inhibition mechanisms; in one, phenol is a mixed partial inhibitor of phenyl acetate hydrolysis, and in the other, phenol causes nonproductive binding of substrate.

Chart I



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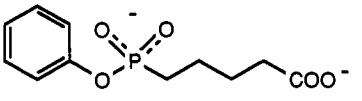

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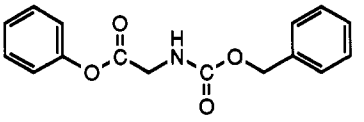

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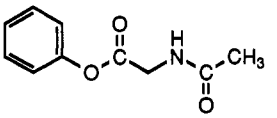

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Materials and Methods

Monoclonal antibody was prepared from ascites fluid by lipid extraction, $(NH_4)_2SO_4$ precipitation, and chromatography on a protein A column, and then on a DEAE-Sephacel column. The purified antibody was determined to be >99% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Active antibody concentration was determined by active site titration using the equation $[E_t] = [I_t]/(1 - V_p/V_a)$ (Henderson, 1972), where V_a and V_p are the respective velocities of phenyl acetate hydrolysis in the absence and presence of a tight-binding inhibitor, I_t, and $[E_t]$ is the active site concentration. For this equation to be valid $[E_t]$ must be $>100K_i$. The steady-state activity of an estimated 1.0 μM antibody was

* To whom correspondence should be addressed.
‡ IGEN, Inc.
§ University of California.
∥ Present address: Department of Biochemistry, University of Bath, Bath, U.K.

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measured toward phenyl acetate in the absence of inhibitor and in the presence of at least four different concentrations of hapten, **2** (K_i = 2.2 nM) (Blackburn et al., 1990).

Kinetic assays were carried out by monitoring absorbance changes at 270 nm during phenyl acetate (Sigma Chemical Co., St. Louis, MO) hydrolysis using a Hitachi U-3200 recording spectrophotometer equipped with a Perkin-Elmer thermoelectric cell holder and digital temperature controller. Unless otherwise indicated, all experiments were performed at 25 ± 0.1 °C at pH 8.8 in 10 mM Tris and 140 mM NaCl. Contribution of background (uncatalyzed) substrate hydrolysis was routinely corrected for by including, in a reference cuvette, the same solution as in the assay cuvette, except antibody. The change in the molar extinction coefficient ($\Delta\epsilon$) for phenyl acetate hydrolysis at pH 8.8 used in this work is 1425 M^{-1} cm^{-1}. Data reported for pH values other than 8.8 have been corrected for pH-dependent changes in $\Delta\epsilon$.

To obtain initial and final velocities of bursts (V_0 and V_f, respectively), progress curves of 20–25 min were fitted to eq 2 in the text using a least-squares nonlinear regression computer program (Duggleby, 1981) run on an AT&T 6300 personal computer. Kinetic parameters were obtained from direct computer fitting (Duggleby, 1981) of substrate concentrations and velocities to the Michaelis–Menten equation. In the determinations of k_{cat} and K_M, initial substrate concentrations ranged from 50 to 600 μM and the antibody concentration was 0.79 μM.

Antibody (0.88 or 8.8 μM) was reacted with tetranitromethane (Sigma Chemical Co., St. Louis, MO) (1, 10, or 100 equiv) in 50 mM Tris, pH 8.0, for 45 min at room temperature. Immediately following the reaction, the reaction mixture was either dialyzed or passed over a NAP-10 Sephadex G-25 column (Pharmacia, Uppsala, Sweden) to remove nitroformate and any unreacted TNM from the antibody solution.

HPLC separation and quantitation of substrate and products was carried out on a Waters HPLC system (Millipore Corp., Milford, MA) using a Vydac 218TP54 C_{18} reverse-phase column (The Separations Group, Hesperia, CA). Samples were eluted isocratically in a mixture of 25% methyl alcohol (JT Baker, Phillipsburg, NJ) and 75% 25 mM citric acid (EM Science, Cherry Hill, NJ) at a flow rate of 0.5 mL/min. Substrate and product elution were monitored at 254 and 222 nm with a Waters Model 480 wavelength detector.

N-Carbobenzoxyglycine phenyl ester (ZG-OPh, **3**) and *N*-acetylglycine phenyl ester (AcG-OPh, **4**) were from a previous study (Blackburn et al., 1990). Absorption spectroscopy of the effects of these compounds on 20G9 was carried out using the Hitachi spectrophotometer described above at 25 °C with a spectral band-pass of 1.0 nm.

Results and Discussion

Phenyl acetate hydrolysis by 20G9 is characterized by a multiple-turnover pre-steady-state burst (Figure 1, upper curve). Addition of more antibody after the reaction has reached steady state results in only a much smaller sized burst, indicating that the brust is not due to a slow substrate binding or a conformational change in the antibody. If more phenyl acetate is added during the steady state, no burst is observed, demonstrating that the brust is not a result of any slow physical change in the substrate upon dilution into the assay cuvette.

Partial inhibition by products was investigated. Acetate (1.0 mM) has no effect on the burst (under conditions where the burst is virtually complete by the time 6 μM product is formed), but 12.5 μM phenol virtually eliminates the burst (Figure 1, lower curve), indicating that product phenol partially inhibits catalysis. As would be expected, addition of lower

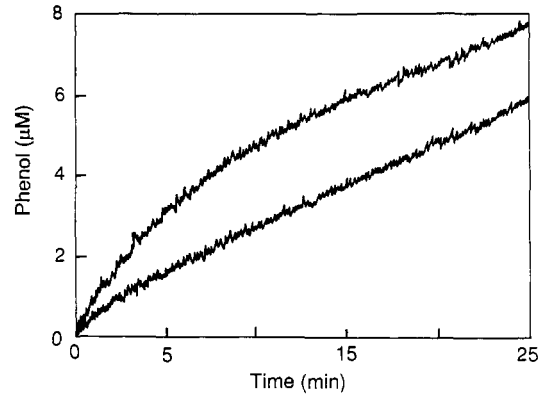


FIGURE 1: Hydrolysis of 200 μM phenyl acetate by the monoclonal catalytic antibody 20G9 (0.79 μM active site concentration) initiated in the absence (upper curve) and presence (lower curve) of 12.5 μM phenol.

concentrations of phenol (1–10 μM) only partially eliminates the burst.

The rate of activity decline during the burst was found to be a pseudo-first-order phenomenon such that an excellent description of the brust is provided by

$$V_t = (V_0 - V_f) \exp(-k_{obsd}t) + V_f \quad (1)$$

where k_{obsd} is the observed apparent first-order rate constant of formation of the less active antibody species, V_0 is the initial velocity, V_t is the velocity at any time, and V_f is the steady-state velocity (Frieden, 1970). To obtain V_0 and V_f for bursts, progress curves were fitted to the integrated form in eq 1:

$$[P] = V_f t + [(V_0 - V_f)(1 - \exp(-k_{obsd}t))]/k_{obsd} + d \quad (2)$$

where d is an offset factor to allow for curves not passing through the origin. In practice, eq 2 provided superimposable theoretical fits to the experimental progress curves throughout the entire burst.

Michaelis–Menten parameters were determined for both initial and steady-state velocities (±SE). For V_0 k_{cat} = 4.9 (±0.5) min^{-1} and K_M = 300 (±70) μM, and for V_f, k_{cat} = 0.54 (±0.05) min^{-1} and K_M = 36 (±1.0) μM. Virtually no change occurred in k_{cat}/K_M upon phenol binding. Initially, k_{cat}/K_M is 1.6 × 10^4 M^{-1} min^{-1}, and with phenol bound it is 1.5 × 10^4 M^{-1} min^{-1}. Since 20G9 is a bivalent IgG class antibody, k_{cat} refers to catalytic turnovers per individual binding site rather than per molecule.

An apparent inhibition constant for phenol, $K_{i,app}$, was obtained from progress curves. The value of $K_{i,app}$ was estimated as the product concentration at which

$$V_t = V_f + (V_0 - V_f)/2 \quad (3)$$

The mean value for $K_{i,app}$ from eight progress curves ([S_0] = 50–200 μM) is 2.5 (±0.3) μM.

A pH profile for the steady-state velocity of 20G9-catalyzed hydrolysis of phenyl acetate shows an acid limb pK of 9.6 (Figure 2). The data have been corrected for pH-dependent changes in the $\Delta\epsilon$ of phenyl acetate hydrolysis at 270 nm. Steady-state rate determinations at different initial substrate concentrations demonstrated that K_M is constant over this pH range (data not shown), and the rate profile shown reflects changes in k_{cat}. An acid limb pK of 9.6 most likely results from the ionization of a catalytically essential tyrosine, active only in the phenolate form.

An alternative possiblity that the acid limb k_{cat} pK could be that of the inhibitory phenol was ruled out by the burst behavior at the different pH values. Over the entire pH range from 7.2 to 11.0 the product concentration formed during the

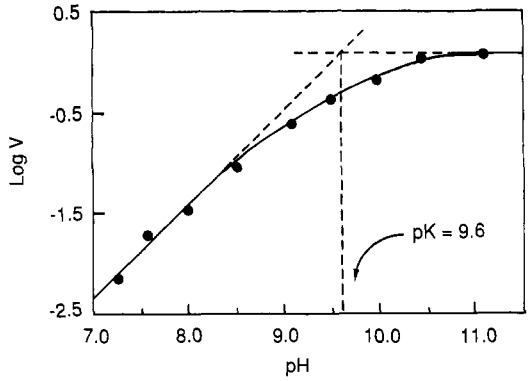


FIGURE 2: pH–log V rate profile of the steady-state hydrolysis of phenyl acetate ([S_0] = 250 μM) by 20G9 (0.88 μM). Assays were carried out at the desired pH in constant ionic strength MTEN buffer (50 mM MES, 25 mM Tris, 25 mM ethanolamine, 100 mM NaCl) (Ellis & Morrison, 1982).

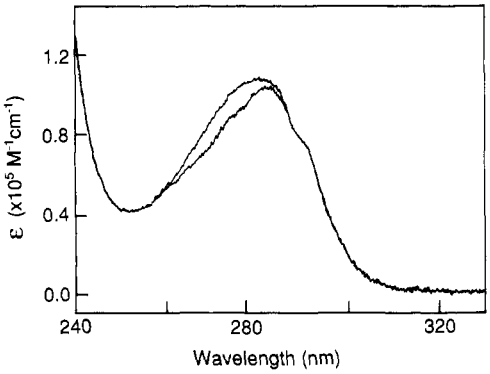


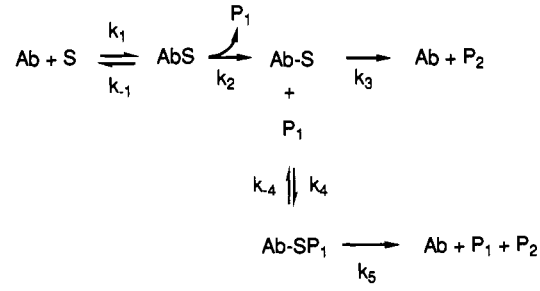
FIGURE 3: Effect of ZG-OPh on the near-UV absorbance spectrum of 20G9. The upper spectrum is of the native antibody (0.88 μM). The lower spectrum, which has been corrected for absorbance contributions from substrate and products, is the protein spectrum of 20G9 after 4-h incubation with ZG-OPh (100 μM) at 25 °C.

course of the burst does not change. This shows that the phenol–antibody dissociation constant is unaffected by phenol ionization. Also, the ratio of V_0/V_f is independent of pH, indicating that the degree of catalytic inactivation caused by phenol is the same irrespective of the ionization form of phenol present. These data indicate not only that is phenol ionization not responsible for the acid limb catalytic pK but that the ionization state of phenol has no effect on $K_{i,app}$; both phenol and phenolate inhibit equally.

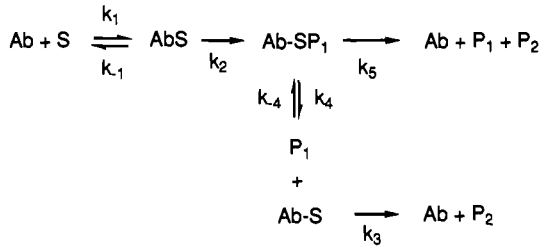
Catalytic tyrosines in enzymes can sometimes be selectively nitrated by mild treatment with tetranitromethane (TNM) (Means & Feeney, 1971). Room temperature reaction of 8.8 μM 20G9 with 1, 10, and 100 equiv of TNM resulted in the nitration of 0.5, 1.0, and 1.5 equiv of tyrosines/equiv of antigen binding sites, respectively, as determined by absorbance of the modified antibody at 428 nm (ϵ = 4100 M^{-1} cm^{-1} at pH 9.0) (Means & Feeney, 1971) following gel filtration. The stoichiometrically nitrated 20G9 (nitrated with 10-fold excess of TNM) retained less than 5% of the native activity over the entire pH range from 7.2 to 11.0.

We used the alternative ester substrates, *N*-carbobenzoxyglycine *O*-phenyl ester (**3**, ZG-OPh) and *N*-acetylglycine *O*-phenyl ester (**4**, AcG-OPh) to gain additional insight to the kinetic mechanism of 20G9. Treatment of 0.88 μM antibody with 100 μM ZG-OPh resulted in only transient hydrolysis (estimated at 4.3 turnovers) of this substrate. Reverse-phase HPLC analysis of product formation confirmed that the antibody did not substantially hydrolyze 100 μM ZG-OPh. Stable acylation of 8.3 tyrosyl residues by ZG-OPh was demonstrated by the near-UV absorbance decrease at 278 nm

Scheme I



Scheme II



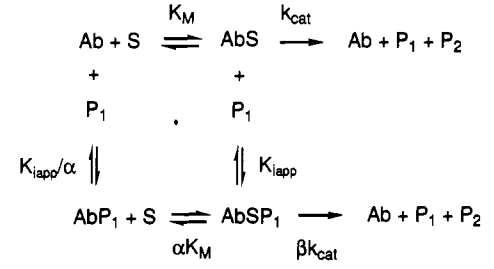
(ϵ = −1160 M^{-1} cm^{-1}) in the protein absorbance spectrum (Figure 3) (Riordan et al., 1965). Following quick gel filtration (NAP-10 Sephadex G-25 column, Pharmacia, Uppsala, Sweden), the acyl-20G9 was found to be completely inactive toward phenyl acetate hydrolysis. Brief treatment with 84 mM hydroxylamine not only restored the protein absorbance spectrum to its original form, but after a second gel filtration to remove hydroxylamine, the antibody was found to be reactivated toward phenyl acetate hydrolysis. These experiments show that although the substrate analogue ZG-OPh acylates multiple tyrosines and hence is not active site specific, it does acylate an essential active site tyrosine, resulting in loss of catalytic activity toward phenyl acetate.

While 20G9 was found only to transiently hydrolyze 100 μM ZG-OPh, the antibody completely hydrolyzed 200 μM AcG-OPh without any acylation detectable by absorbance spectroscopy. However, at 2.0 mM AcG-OPh extensive acylation was seen and analysis of the rate of product formation by reverse-phase HPLC demonstrated that catalysis had essentially stopped. Following quick gel filtration to remove excess AcG-OPh, taking at most 15 min, the AcG-OPh-acylated antibody was fully active toward phenyl acetate hydrolysis. It appears that the active site acetylglycine *O*-tyrosyl ester is less stable than the corresponding carbobenzoxyglycine *O*-tyrosyl ester.

The order of ability to form stable acyltyrosines in the active site of 20G9 thus appears to be ZG-OPh > AcG-OPh > phenyl acetate. Spectroscopic evidence indicates that ZG-OPh and AcG-OPh, but apparently not phenyl acetate, acylate multiple tyrosines on the surface of 20G9. One reason for these differences may be the inherent reactivity of the substrates; the uncatalyzed rates of hydrolysis of ZG-OPh (5.2 × 10^{-3} min^{-1}) and AcG-OPh (7.8 × 10^{-3} min^{-1}) are about an order of magnitude faster than the rate of phenyl acetate hydrolysis (6.1 × 10^{-4} min^{-1}).

The combined observations that phenol binding causes identical decreases in both k_{cat} and K_M and that the mechanism may involve an acyltyrosyl intermediate suggest that either of the mechanisms shown in Schemes I and II is occurring. In the schemes, Ab is antibody, S is substrate, AbS is the Michaelis complex, Ab–S is the acyl antibody, Ab–S·P_1 is the complex of phenol with the acyl intermediate, P_1 is phenol, and P_2 is acetate. The key feature of both mechanisms is that phenol inhibits by binding to an acyl intermediate, slowing

Scheme III



hydrolytic deacylation. Although Scheme II is somewhat simpler than Scheme I because it does not require release and reassociation of phenol, Scheme I cannot be ruled out in light of earlier findings that multiple phenols can bind to 20G9, each with distinct kinetic consequences (Martin et al., 1991).

Derivation of rate equations for k_{cat} and K_M for the mechanisms represented by Schemes I and II by the method of King and Altman (1956) demonstrate that they are kinetically virtually identical. The equations for Scheme I are

$$k_{cat} = \frac{k_2(k_3k_5 + [P]k_4k_5 + k_3k_{-4})}{[P]k_4(k_2 + k_5) + k_3k_5 + k_3k_{-4} + k_2k_5 + k_2k_{-4}} \quad (4)$$

$$K_M = \frac{[P]k_4k_5(k_{-1} + k_2) + k_3(k_{-1}k_5 + k_2k_{-4} + k_2k_5 + k_{-1}k_{-4})}{k_1([P]k_4(k_2 + k_5) + k_3k_5 + k_3k_{-4} + k_2k_5 + k_2k_{-4})} \quad (5)$$

and those for Scheme II are

$$k_{cat} = \frac{k_2(k_3k_5 + [P]k_4k_5 + k_3k_{-4})}{[P]k_4(k_2 + k_5) + k_3k_4 + k_3k_{-4} + k_2k_5 + k_2k_{-4}} \quad (6)$$

$$K_M = \frac{[P]k_4k_5(k_{-1} + k_2) + k_3(k_{-1}k_5 + k_2k_{-4} + k_2k_5 + k_{-1}k_{-4})}{k_1([P]k_4(k_2 + k_5) + k_3k_5 + k_3k_{-4} + k_2k_3 + k_2k_{-4})} \quad (7)$$

In agreement with our observations, k_{cat} and K_M both vary with phenol concentration until phenol saturates the antibody, at which point the expressions for both k_{cat} and K_M become independent of phenol concentration (and the kinetic differences between the two mechanisms disappear):

$$k_{cat} = k_2k_5/(k_2 + k_5) \quad (8)$$

$$K_M = K_S[k_5/(k_2 + k_5)] \quad (9)$$

It is also noteworthy that the specificity constant, k_{cat}/K_M, which is identical for both mechanisms, is equal to k_2/K_S at all phenol concentrations. (Our kinetic observations demand that k_{cat}/K_M be independent of phenol concentration.)

Although the results are entirely consistent with an acyltyrosyl mechanism in which phenol inhibits deacylation by binding to the acetyl intermediate, at this time we cannot disprove two alternative phenol inhibition mechanisms. Both alternative mechanisms are plausible, but neither incorporate all of the current data and they do not specifically involve an acyl intermediate or tyrosine.

One of the alternatives is that the product phenol is a partial mixed inhibitor of phenyl acetate hydrolysis (Scheme III) (Segel, 1975). In this mechanism, the overall effect of phenol binding would be to lower K_M and k_{cat} by an identical factor ($\alpha = \beta$ in Scheme III). In the case of 20G9, α would equal 0.12 and β would equal 0.11. As can be seen in Scheme III, binding of phenol or substrate to the antibody would reciprocally promote the other's binding, as reflected by a decrease in $K_{i,app}$ or K_M values in each other's presence. This seems reasonable if, for example, the aromatic ring of bound phenol were to π-stack with that of bound phenyl acetate to add to the binding energies of each. In this mechanism, phenol would facilitate substrate binding, demonstrated by a decrease in K_M, but slow the chemical catalytic step(s), reflected by a decrease in k_{cat}. This might occur if, for example, bound phenol physically blocked the attack of water or hydroxide, or if product dissociation became rate-limiting in the presence of phenol. Although this mechanism can be interpreted as evidence for transition-state stabilization as predicted by the hapten design (Martin et al., 1991), it does not take into consideration the current data suggesting the existence of an acyltyrosyl intermediate.

Another possible mechanism that would also result in identical decreases in K_M and k_{cat} would be nonproductive binding of substrate (Bender & Kezdy, 1965; Fersht, 1974). In this case, phenol binding would cause some change in the antigen binding site to allow phenyl acetate to become a competitive inhibitor of its own hydrolysis. The physical consequence of phenol binding would thus be to create a new (nonproductive) substrate binding mode that spatially overlaps the original productive mode without disturbing its inherent catalytic properties. The kinetic consequence of this mechanism would be that phenol binding would cause identical decreases in the k_{cat} and K_M of phenyl acetate hydrolysis. Nonproductive binding is not a particularly attractive mechanism in this case due to the delicate balance of physical requirements; phenol binding would have to cause physical changes in the antibody binding site great enough to create a competitive binding mode for substrate yet small enough not to perturb either the K_M (binding) or k_{cat} (catalysis) of phenyl acetate hydrolysis in the original mode.

In conclusion, we show that hydrolysis of phenyl acetate by 20G9 is partially inhibited by the product phenol and that an essential tyrosine is present in or near the antibody binding site. Although we cannot disprove other mechanisms of product inhibition, the data are most suggestive of a mechanism in which phenol inhibits the antibody by binding to an acetyltyrosyl intermediate, inhibiting deacylation. The possibility of acyltyrosyl catalytic antibody intermediates in acyl-transfer reactions has been previously proposed (Tramontano et al., 1986), and kinetic evidence for acyl antibody intermediates has been recently described (Benkovic et al., 1990; Janda et al., 1991). Considering the generally high concentration of tyrosines in antibody combining sites (Davies & Padlan, 1990; Glockshuber et al., 1991), tyrosine-dependent mechanisms similar to the acyl mechanism described here may be common among catalytic antibodies. If so, successful future strategies for creating catalytic antibodies could be based on reaction mechanisms devised to specifically exploit the chemistry of the tyrosyl phenolic side chain (or a chemically modified derivative thereof). Haptens prepared to resemble the transition states of these mechanisms could then be used to elicit efficient antibodies capable of catalyzing efficient tyrosine-dependent reactions.

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Use of Monoacetyl-4-Hydroxyaminoquinoline 1-Oxide To Probe Contacts between Guanines and Protein in the Minor and Major Grooves of DNA. Interaction of *Escherichia coli* Integration Host Factor with Its Recognition Site in the Early Promoter and Transposition Enhancer of Bacteriophage Mu†

G. B. Panigrahi and Ian G. Walker*
Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1
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ABSTRACT: Monoacetyl-4-hydroxyaminoquinoline 1-oxide (Ac-HAQO) reacts with DNA to form adducts at the C8- and N^2-positions of guanine and with the N^6-position of adenine. Only the N^2-guanine adduct blocks the 3′-5′ exonuclease action of phage T4 DNA polymerase. Piperidine treatment cleaves the DNA at sites bearing C8-guanine adducts. The N^2-position of guanine lies in the minor groove of DNA, whereas the C8-position of guanine occupies the major groove. We have taken advantage of these characteristics to employ Ac-HAQO in conjunction with either T4 DNA polymerase or piperidine in a footprinting technique to probe the interaction of the *Escherichia coli* integration host factor (IHF) with its binding site. We show that when IHF binds to its recognition site both the N^2- and C8-positions of guanines are protected from modification by AcHAQO. In addition, the binding of IHF to DNA was prevented when either an N^2- or a C8-AQO adduct was present in the binding site. When dimethylsulfate was used as the footprinting reagent, IHF protected against methylation of the N3 position of adenine in the minor groove but not the N7 position of guanine in the major groove. The difference in results obtained with the two reagents is ascribed to their relative sizes. Both DMS and AcHAQO are excluded by IHF from the minor groove, but only the larger AcHAQO molecule is excluded from the major groove.

DNA footprinting studies with chemicals have provided valuable information about the contacts between proteins and DNA during specific interactions. Dimethylsulfate can be used to identify the guanines that interact with protein in the major groove and the adenines that interact with protein in the minor groove. Until this time there was no reagent which allowed one to determine contacts between guanines and protein via the minor groove. The present study describes the use of monoacetyl-4-hydroxyaminoquinoline 1-oxide (Ac-HAQO) to determine the contacts between guanines and protein in both the minor and major grooves of DNA. Ac-HAQO reacts with native, double-stranded DNA to form three purine adducts, two with guanine and one with adenine. The relative amounts of the N^2-guanine, the C8-guanine, and the N^6-adenine adducts formed are 50%, 30%, and 10%, respectively (Galiegue-Zouitina et al., 1985, 1986). However, preferential formation of the C8-guanine adduct is observed when Ac-4HAQO is reacted with denatured single-stranded DNA (70% of total modification; Galiegue-Zouitna et al., 1984). We exploited this difference in reactivity to study the inhibitory effect of the purine adducts on the 3′-5′ exonuclease activity of phage T4 DNA polymerase. It was found that only the N^2-guanine adduct halted the action of this enzyme (Panigrahi & Walker, 1990). When the digestion products of the T4 enzyme on the modified DNA are examined by denaturing polyacrylamide gel electrophoresis, a G-ladder is obtained,

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* To whom correspondence should be addressed.

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