Antibody-Catalyzed Michael Reaction of Cyanide with an α,β-Unsaturated Ketone

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Catalytic antibodies have been produced for more than 50 reactions involving conversion of either macromolecular or simple chemical substrates to products.[1] The majority of these reactions are either unimolecular or ester hydrolysis reactions, and the number of antibodies which catalyze reactions resulting in formation of carbon–carbon bonds is relatively small.[2] Bond formation between carbon atoms is a keystone of synthetic organic chemistry, and the Michael addition of weak acids to electron-deficient double bonds is among the most useful of such reactions.[3] We describe here the production and initial characterization of an antibody which catalyzes the reaction of hydrogen cyanide with an unsaturated ketone substrate. It is the first report, to our knowledge, of an antibody which catalyzes bimolecular carbon–carbon bond formation by 1,4-addition to a simple activated olefin,[4] and the transition state analog (TSA) strategy used may be extended to other Michael-type reactions.

The 1,4-addition of hydrogen cyanide to α,β-unsaturated ketones was studied by Agami et al.,[5] who concluded from the kinetics of the reaction and from the Hammett effect of aromatic substituents that the reaction proceeds along the pathway shown in Figure 1. The transition state was postulated to resemble the enolate structure (**3**, **4**), and the pH dependence of the reaction demonstrated that the cyanide ion was the likely reactant.

For catalysis to occur, the substrate must bind to anti-TSA antibodies and therefore must resemble the TSA to some extent. However, since the differential in binding energy between substrate (ground state) and transition state should lower $\Delta G^{\ddagger}$, the antibodies should have higher affinity for TSA than for substrate. To minimize product inhibition, the anti-TSA antibodies should have low affinity for the product. The conversion of the β- and γ-carbon atoms from trigonal in the TSA to tetrahedral in the product should result in a significant change in the geometry of the molecule. We anticipated that this geometric change would be accompanied by sufficient change in relative affinities so as to minimize product inhibition.

We synthesized the α,β-unsaturated ketone **7** ("enone"; Figure 2) for use as a substrate in the antibody-catalyzed reaction. Phenyl groups were introduced at the 1- and 2-positions of the propenone chain since aromatic substituents at these positions enhance the reaction rate.[6]

For the TSA we prepared the enol ether **9a** as shown in Figure 2. The double bond in the TSA mimics the incipient double bond in the presumed transition state, and the enol ether oxygen provides an electron-rich center as a surrogate for the enolate-like oxygen of the transition state. The terminal hydroxyl group provides a convenient point for attachment of protein to form conjugates for immunization and screening, and the two aromatic rings give bulk and rigidity to the molecule, favoring enhanced immunogenicity. Molecular modeling studies indicated that the *E*- and *Z*-isomers of the proposed TSA differed little in energy, and in practice the synthetic procedure resulted in a mixture containing both isomers. The mixture was used to make protein conjugates (**9c,d**) for production and screening of anti-TSA antibodies. For further characterization of selected anti-TSA antibodies, the *E*- and *Z*-isomers were separated by HPLC and identified by ^{1}H-NMR techniques (data not shown).

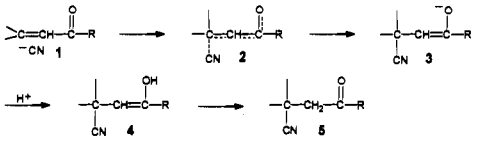

Figure 1. Reaction pathway for hydrocyanation of enones. The transition state is postulated to resemble the enol form of the product (**3**, **4**) more closely than it does the reactants.

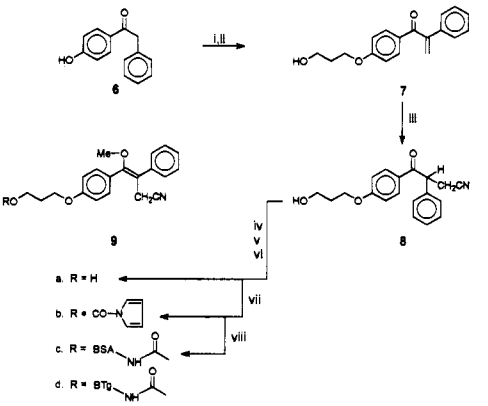

Figure 2. Synthesis of substrate (**7**), product (**8**), transition state analog (**9a**), and protein conjugates (**9c,d**). Reaction conditions: (i) 3-bromopropanol, K_2CO_3, acetone, (ii) formaldehyde, piperidine and acetic acid (cf. ref 12), (iii) KCN, NH_4Cl, *N,N*-dimethylformamide, water, 100 °C (cf. footnote 4), (iv) Ac_2O, triethylamine, methylene chloride, 4-(dimethylamino)pyridine, (v) potassium *tert*-butoxide in hexamethylphosphoramide; methyl trifluoromethanesulfonate (cf. ref 13), (vi) K_2CO_3, methanol, (vii) carbonyldiimidazole, tetrahydrofuran, (viii) protein (BSA = bovine serum albumin, BTg = bovine thyroglobulin), aqueous $NaHCO_3$, dioxane. For binding and kinetic studies, **7** was purified by preparative HPLC to a single peak on analytical HPLC. Enol ether **9a** was obtained as an approximately equal mixture of *E*- and *Z*-isomers (as determined by ^{1}H-NMR and HPLC, data not shown). The mixture was carried through subsequent steps. Protein conjugates were purified by size exclusion chromatography (Bio-Gel P-6). Ratios were determined by differential UV spectrophotometry[14] at 265 nm in water. All synthetic compounds exhibited ^{1}H-NMR spectra consistent with the assigned structures.

The product of the catalyzed reaction is the cyanomethyl ketone **8** (Figure 2). The initial product presumably would be the enol or enolate, but this would tautomerize rapidly to the more stable ketone, which would be expected to be the predominant species in solution.

Female BALB/c mice were immunized with TSA–protein conjugates. Sera from all immunized mice exhibited high titers of anti-TSA antibodies in ELISA, and hybridomas were derived using spleen cells from selected mice.[7] Twenty-eight cloned hybridomas producing anti-TSA antibodies were injected into Pristane-primed mice. Antibodies were purified from ascites by ammonium sulfate precipitation and affinity chromatography

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(4) A special case of this reaction—the addition of cyanide to 6-hydroxy-3*H*-xanthen-3-one, in which the product is stabilized as an aromatic compound—has been catalyzed by an anti-fluorescein antibody (Janjic, N.; Tramontano, A. *Biochemistry* **1990**, *29*, 8867–8872).
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on protein A. Antibody purity was assessed by polyacrylamide gel electrophoresis in the presence of SDS.

Protein A-purified antibodies were tested at concentrations of 5−20 μM for their ability to enhance the initial velocity of the reaction of potassium cyanide with the enone substrate at 37 °C, pH 7.0. Product formation was measured by HPLC using a Zorbax RX-C8 reverse phase column with an isocratic mobile phase of methanol/water (65;35 v/v). Product concentrations were determined by UV measurements at 285 nm using an external standard curve. Linear regression analysis was used to determine the apparent initial velocity. The uncatalyzed (background) velocity was determined in the absence of antibody and used to correct the observed velocity. Four antibodies were identified which clearly enhanced the reaction rate. Addition of TSA to the reaction mixture in the presence of antibody markedly diminished the reaction velocity, thus indicating the involvement of the antibody binding site in the observed enhancement (data not shown).

Kinetic analysis was carried out on antibody 5G4. Initial velocities were determined by HPLC analysis of the product over the first 5−10% of the reaction, and the reaction was shown by regression analysis to be essentially linear over this period. The identity of the product of the reaction catalyzed by 5G4 was confirmed by mass spectrometry.

The reaction was analyzed kinetically as a random bimolecular reaction by studying the rate of the reaction over 30−200 μM enone and 23.7−593 μM cyanide ion[8] [CN^-]. A random bimolecular kinetic model (eq 1) was fitted to the data by nonlinear regression analysis.[9] This gave values of V_{max} = 26.6 ± 2.4 (SE) μM h^{-1}, K_{enone} = 64.3 ± 8.6 μM, and K_{CN^-} = 141 ± 11 μM. These parameters were used to calculate the lines for the reciprocal plots shown in Figure 3. The catalytic rate constant, k_{cat}, was calculated from V_{max} and the antibody concentration to be 1.28 h^{-1}. On the basis of the observed background rate constant, k_{uncat}, of 3.78 × 10^{-5} μM^{-1} h^{-1}, these data suggest an effective molarity (k_{cat}/k_{uncat}) of 0.03 M, a rate enhancement of 240 for the encounter of the enone with antibody−cyanide complex, and a rate enhancement of 530 for the encounter of cyanide with the antibody−enone complex.

$$V_0 = V_{max}[enone][CN^-]/(K_{enone}K_{CN^-} + [enone]K_{CN^-} + K_{enone}[CN^-] + [enone][CN^-]) \quad (1)$$

The catalytic reaction was inhibited by the TSA. A K_i value of 11.2 ± 1.6 μM was obtained by varying the concentration of TSA (20−100 μM), CN^- (59−119 μM) and enone (50−200 μM) and fitting eq 2 to the resulting initial velocity values, with V_{max}, K_{CN^-}, and K_{enone} having the previously calculated values.[10] No inhibition was observed in the presence of 100 μM product with initial concentrations of 100 μM enone and 119 μM CN^-.

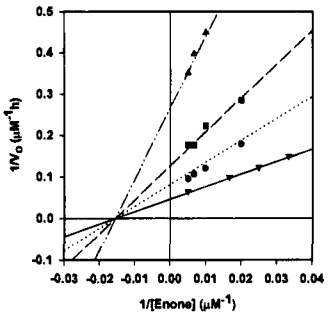

Figure 3. Reciprocal plots for reaction of enone with 593 (▼), 119 (●), 59 (■), and 24 (▲) μM CN^- catalyzed by 21 μM antibody 5G4.F11C in Tris buffer at 37 °C, pH 7.0. Parameter values obtained from fitting eq 1 to the data were used to draw the lines shown.

$$V_0 = V_{max}[enone][CN^-]/(K_{enone}K_{CN^-} + [enone]K_{CN^-} + K_{enone}[CN^-] + [enone][CN^-] + (K_{enone}K_{CN^-}[TSA]/K_i)) \quad (2)$$

In one experiment to assess the stability of the antibody, the 5G4-catalyzed reaction was allowed to proceed until 2.5 molecules of product had been produced per molecule of antibody.

Antibody/substrate combinations such as the one described here may be useful for protection against cyanide exposure, but would require antibodies with higher catalytic turnover rates. The introduction of catalytic groups (either by modifying the existing catalytic antibodies or by using the "bait and switch" approach[11]) to generate new antibodies that could facilitate proton transfer should greatly enhance the reaction at physiological pH. Higher turnover rates may also be attained by modification of the enone substrate. However, if a catalytic antibody/substrate system is developed for in vivo use, the pharmacological and pharmacokinetic properties of the enone substrate must also be examined in order to achieve a balance between the catalyzed reaction with cyanide and undesirable reactions with endogenous nucleophiles.

The approach used here may be applicable to other weak carbon acid Michael donors.

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Supporting Information Available: Experimental details for synthesis of substrate, product, hapten, and conjugates, antibody purification, and kinetic experiments (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(7) Spleen cells from immunized mice were fused at a ratio of 2:1 with the P3×63-Ag8.653 cell line. Immediately after fusion, hybridomas were plated out in 96-well microtiter plates. A total of 3000 wells were seeded with fused cells, and growth of hybridomas was observed in 2329/3000 wells. Supernatants from 593/2304 hybridomas were positive for binding to TSA−protein conjugate, and 401/593 showed inhibition by free TSA in competitive inhibition immunoassays. Selected hybridomas were cloned by limiting dilution. Research using animals was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86−23, revised 1985).

(8) The concentration of cyanide ion at pH 7.0 and 37 °C was estimated from the Henderson-Hasselbalch equation using the pK_a of HCN at 25 °C (9.21) and the ΔH for ionization (43.5 kJ mol^{-1}) to be 0.01186 of the nominal concentration of KCN. The pK and ΔH values were taken from the following: *Handbook of Biochemistry and Molecular Biology*, 3rd ed.; Physical and Chemical Data, Vol. I; CRC Press: Boca Raton, FL, 1976.

(9) Equation 1 was adapted from Segel: Segel, I. H. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*; John Wiley & Sons: New York, 1975; pp 273 ff. A random bimolecular model incorporating an interaction coefficient (α) for binding of the two substrates and an ordered bimolecular model were examined but were eliminated by *F*-test considerations (Motulsky, H. J.; Rasnas, L. A. *FASEB J.* **1987**, *1*, 365−373). The program used for fitting the model was RS/1, PC Version Release 4, BBN Software Products Corporation, Cambridge, MA. A weighting factor of $1/(V_0)^2$ was used.

(10) Equation 2 was adapted from Segel (Segel, I. H. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*; John Wiley & Sons: New York, 1975; pp 283 ff) by assuming that TSA binding prevented binding of either substrate. A model that assumed only partial interference with the binding of cyanide was not applicable.

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