Monitoring Catalytic Activity by Immunoassay: Implications for Screening

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Abstract: We have developed an immunoassay for screening large libraries of potential catalysts of a bimolecular Diels–Alder reaction. The procedure involves the reaction of immobilized dienophile with diene in solution and subsequent detection of the product with a monoclonal antibody as in a regular enzyme-linked immunosorbant assay (ELISA). The assay exhibits the expected dependence on time and diene concentration. A catalytic antibody accelerates the reaction in a concentration dependent manner despite immobilization of the dienophile, and its catalytic efficiency is comparable to that in free solution. The current study explores both the utility of this assay and criteria for its successful application to other reactions. Employment of this strategy, previously dubbed catELISA (Tawfik, *et al. PNAS* **1993**, *90*, 373), to monitor a bimolecular cycloaddition reaction supports its potential as a general approach to screening for catalysts.

Rational approaches to catalyst design, including catalytic antibodies, are being pursued in a number of research groups.[1] Many of these strategies entail the generation of large libraries of variants biased in some manner to contain catalysts (for example, by selecting molecules that bind transition-state analogs). To optimize the chances of identifying and isolating highly efficient catalysts, it is desirable to evaluate as many candidates from the biased libraries as possible. Consequently, the development of efficient and facile assays for screening large numbers of molecules for catalytic activity is of considerable importance.

The most common method of assaying catalytic activity relies on the release of a chromophore as the reaction proceeds. While this approach has been exploited successfully for screening in many instances,[2-4] the range of substrates that can be studied is relatively limited. Alternative, yet sensitive, means of product detection can obviate the need for chromophoric substrates. For example, Schwabacher and co-workers[5] have recently developed an assay for bimolecular reactions in which an easily detected tag, like biotin, becomes incorporated into the product during the reaction. A potentially more general approach has been reported by Green and co-workers[6] in which product is detected by specific antibodies as in a regular enzyme-linked immunosorbent assay (ELISA).[7,8] The authors used the assay, which they dubbed catELISA, to monitor the hydrolysis of an immobilized ester and identified nine catalytic antibodies from a total of 1570 hybridoma clones.

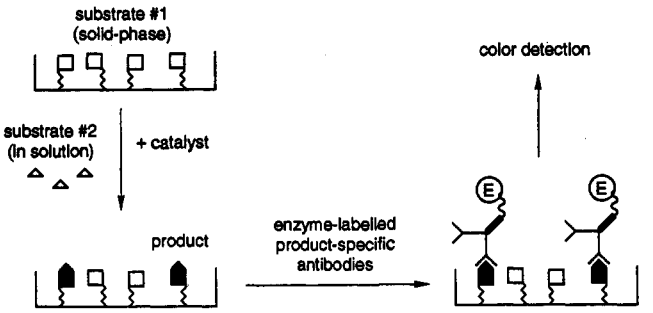

Figure 1. Strategy of the bimolecular catELISA. Substrate #1 is immobilized on a microtiter plate. Substrate #2 is then added in solution, along with the putative catalyst being screened. Catalytic activity results in formation of immobilized product, which is then detected using a product-specific antibody. An enzyme covalently linked to this antibody catalyzes a reaction with a chromophoric substrate.

The effectiveness of the catELISA approach has thus been demonstrated for a hydrolytic reaction in which strongly antigenic determinants on the substrate are removed during the reaction. In order to evaluate the generality of this assay, we sought to extend the strategy to synthetic, bimolecular reactions (Figure 1). For this study, we chose the Diels–Alder reaction of Figure 2, which represents a particularly promising aspect of rational catalyst design: the development of enzyme-like macromolecules that catalyze classes of reactions for which natural enzymes are unavailable. Application of the catELISA to this reaction enables us to assess the accuracy of the assay in estimating catalytic efficiency and to evaluate the factors required for successful implementation of this technique.

Materials and Methods

All chemicals, unless otherwise stated, were obtained from Aldrich and used without further purification. The catalytic antibody 1E9 was prepared and purified as previously described.[9] The substrate tetrachlorothiophene dioxide (TCTD) was prepared

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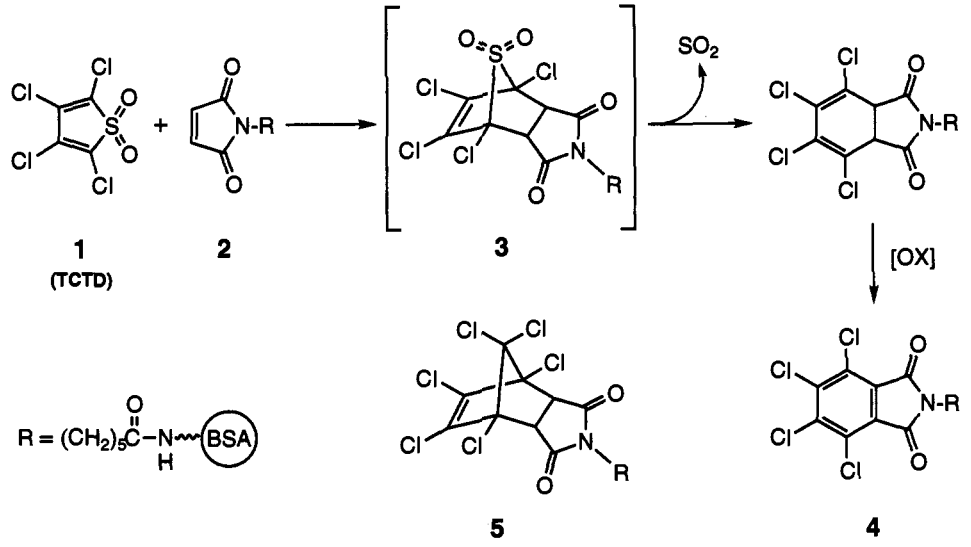

Figure 2. Diels–Alder reaction of tetrachlorothiophene dioxide (**1**) with *N*-derivatized maleimide (**2**). The transition state of the reaction (similar to **3**) is mimicked by the analog **5**.

according to the method of Raasch.[10] Flash chromatography was performed by the method of Still, Kahn, and Mitra[11] using 60-mesh silica gel. NMR spectra were obtained with Fourier transform spectrometers operating at the indicated frequencies and were referenced to the residual peak of the indicated solvent. Mass spectra were obtained on a VG ZAB-2VSE double-focusing high-resolution mass spectrometer.

Maleimide–Protein Conjugates. 6-Maleimidocaproic acid was synthesized as reported by Keller and Rudinger.[12] To a solution of 125 mg (0.59 mmol) of 6-maleimidocaproic acid and an excess of triethylamine in 10 mL of dichloromethane was added an excess of disuccinimidyl carbonate and the mixture stirred for 2 h under N_2 at room temperature. The succinimidyl ester was recovered by evaporation of solvent. Purification (silica gel, elution with 1:1 ethyl acetate/dichloromethane) gave 165 mg (90%). ^{1}H-NMR: ($CDCl_3$, 300 MHz) δ 6.67 (s, 2H), 3.50 (t, 2H, J = 7.0 Hz), 2.79 (s, 4H), 2.58 (t, 2H, J = 7.6 Hz), 1.79–1.69 (m, 2H), 1.65–1.55 (m, 2H), 1.44–1.34 (m, 2H). MS m/z (LSIMS$^+$): calcd 309.1087, obsd 309.1093.

The succinimidyl ester (20 mg, 65 μmol) was dissolved in 0.40 mL of 1,4-dioxane and added to 43 mg (0.65 μmol) of bovine serum albumin (BSA) in 10.6 mL of 100 mM $NaHCO_3$(aq), pH 8.0. After 2 h at room temperature, the BSA conjugate was purified on a Sephadex G-50 column, eluting with MBS-6.0 (20 mM MES (2-[*N*-morpholino]ethanesulfonic acid), 100 mM NaCl, pH 6.0). Amine titration[13] indicated 44 haptens/BSA molecule. The conjugate was stored at −70 °C.

Tetrachlorophthalimide–Protein Conjugates. To a solution of 1.0 g (3.5 mmol) of tetrachlorophthalic anhydride in 50 mL of ethyl acetate was added 500 mg (3.8 mmol) of 6-aminocaproic acid. After refluxing under argon for 1 h, 2.2 g (28 mmol) of acetyl chloride was added and the mixture refluxed for an additional hour. The mixture was filtered and the filtrate washed once with 1 N HCl(aq) and once with saturated NaCl(aq) and dried with $MgSO_4$. Evaporation of solvent gave 1.18 g (85%) of 95% pure 6-(*N*-tetrachlorophthalimide)caproic acid. Further purification was achieved by silica gel chromatography, eluting with ethyl acetate. ^{1}H-NMR: (DMSO-d_6, 300 MHz) δ 11.02 (s, 1H), 3.54 (t, 2H, J = 7.1 Hz), 2.18 (t, 2H, J = 7.3 Hz), 1.62–1.45 (m, 4H), 1.32–1.22 (m, 2H). ^{13}C-NMR: (DMSO-d_6, 500 MHz) δ 174.4, 163.5, 138.0, 128.4, 128.0, 38.1, 33.4, 27.3, 25.7, 24.0. MS m/z (LSIMS$^-$): calcd 397.9520, obsd 397.9462.

To a solution of 160 mg (0.40 mmol) of 6-(*N*-tetrachlorophthalimide)caproic acid and 121 mg (1.20 mmol) of triethylamine in 25 mL of dichloromethane was added 205 mg (0.80 mmol) of *N,N'*-disuccinimidyl carbonate and the mixture stirred for 2 h under argon at room temperature. The succinimidyl ester was recovered by evaporation of solvent. Purification (silica gel, elution with 1:1 ethyl acetate/dichloromethane) gave 170 mg (85%). ^{1}H-NMR: (DMSO-d_6, 300 MHz) δ 3.56 (t, 2H, J = 6.9 Hz), 2.78 (s, 4H), 2.65 (t, 2H, J = 7.2 Hz), 1.69–1.56 (m, 4H), 1.41–1.31 (m, 2H). ^{13}C-NMR: (DMSO-d_6, 500 MHz) δ 170.2, 168.9, 163.5, 138.0, 128.5, 127.9, 37.9, 30.0, 27.0, 25.4, 25.1, 23.8. MS m/z (LSIMS$^+$): calcd 494.9684, obsd 494.9695.

The succinimidyl ester (52 mg, 105 μmol) was dissolved in 0.80 mL of 1,4-dioxane, and 0.60 mL (76 μmol) of the resulting solution was added to 10 mg (0.15 μmol) of BSA in 5 mL of 100 mM $NaHCO_3$(aq), pH 8.0. In a parallel experiment, while 0.12 mL (15 μmol) of the ester solution was added to 10 mg (15 nmol) of thyroglobulin (TG) in 3 mL of 100 mM $NaHCO_3$(aq), pH 8.0. After 16 h at room temperature, the BSA and TG conjugates were purified by size exclusion chromatography (G-50 column) using PBS-7.5 (10 mM phosphate, 140 mM NaCl, pH 7.5). Amine titration[13] indicated 44 haptens/BSA molecule and 121 haptens/TG molecule.

Antibody Production. Mice (129 GIX$^+$ strain) were immunized with the TG conjugate of 6-(*N*-tetrachlorophthalimide)caproic acid emulsified in MPL + TDM emulsion (RIBI ImmunoChem Research, Inc.). Serum titer was determined with the BSA conjugate **4** by ELISA.[7,8] Hybridomas were prepared by fusion of SP2/0+ myeloma cells with the spleen cells of a hyperimmunized mouse.[14] Cell lines secreting IgG antibodies specific for the hapten were subcloned twice, and the four clones with the highest titers were propagated in mouse ascites using (BALB/c × 129 GIX$^+$)F_1 mice.[14] Monoclonal antibodies were purified from ascites fluid by ammonium sulfate precipitation followed by cation exchange chromatography (DE52 from Whatman). Protein was eluted from the gel with a NaCl step gradient (0, 50, 100, 150, 250, 500 mM) in 10 mM Tris·HCl (pH 8.0). IgG was eluted at 100 mM NaCl, concentrated, and dialyzed exhaustively against MBS-6.0. Protein concentration was determined by ultraviolet absorption at 280 nm assuming a molar extinction coefficient, ϵ_{280}, of 220 000 M^{-1} cm^{-1} and a molar mass of 160 000 g mol^{-1}.[7] The affinities of the purified antibodies for product **4** and substrate **2** were evaluated by ELISA, and the antibody 11A5 was identified as exhibiting the best discrimination between

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substrate and product. The antibody 11A5 was covalently linked to horseradish peroxidase (HRPO) using ImmunoPure activated peroxidase from Pierce. The conjugate (11A5-HRPO) was stored at –20 °C (long term) or 4 °C (short term).

CatELISA. Microtiter plates (96-well A/2 Costar ELISA plates) were coated with BSA–maleimide conjugate **2** (50 μL, 15 μg/mL in MBS-6.0) for 1 h at 37 °C, shaken dry, and blocked with BSA (100 μL, 3% in MBS-6.0) for 1 h at 37 °C. The plates were then rinsed with distilled water (dH_2O) and shaken dry. When the concentration of catalytic antibody 1E9 was varied, 25 μL of MBS-6.0 was added to each well, and a stock of 10 μM 1E9 in MBS-6.0 was serially diluted 1 in 2 across the plate. Then, a solution (25 μL) of TCTD in MBS-6.0 (6.7% CH_3CN) was added to each well and mixed by pipetting up and down several times. The reaction was left to proceed for 1 h at room temperature. When 1E9 concentration was kept constant and reaction time varied, all wells on the plate were filled with MBS-6.0 and the reaction was initiated at various times by replacing the buffer with a solution (50 μL) containing TCTD and 100 nM 1E9 in MBS-6.0 (3.3% CH_3CN). Following the reaction, the plates were rinsed thoroughly with dH_2O and incubated for 1 h at 37 °C with 11A5-HRPO diluted in binding buffer (10 mM phosphate, 2 M NaCl, 40 mM $MgSO_4$, 0.05% Tween-20). The concentration of active 11A5-HRPO used for this incubation was determined empirically (see text). The plates were rinsed extensively with dH_2O and developer was added (40 μL of 0.3 mg/mL 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS), 0.02% H_2O_2 in 0.1 M citrate, pH 4.0). Absorbance at 405 nm was recorded after about 30 min and the average absorbance (≈0.08) of several control wells (no TCTD during reaction incubation) subtracted.

Estimation of Dissociation Constants. Standard ELISAs[7,8] were performed by immobilizing antigen and titrating purified antibody by serial dilution. Bound antibody was detected using goat anti-mouse kappa-HRPO (FisherBiotech). Absorbance at 405 nm was recorded following color development. Since the absorbance is proportional to the fraction of antigen bound by antibody, the dissociation constant K_d was determined by fitting the data to the equation

$$A_{405nm} = c\frac{[Ag]_t + [Ab]_t + K_d - (([Ag]_t + [Ab]_t + K_d)^2 - 4[Ag]_t[Ab]_t)^{1/2}}{2[Ag]_t}$$

where c is the constant of proportionality, $[Ag]_t$ is the total antigen concentration, and $[Ag]_t$ is the total antibody concentration. The parameters c and K_d were abstracted by general curve-fitting techniques.[15] Analysis of this equation reveals that the estimate of K_d does not change significantly with $[Ag]_t$ if $[Ag]_t \ll K_d$. This regime was maintained for all determinations.

Results and Discussion

The strategy of the catELISA for a bimolecular reaction is outlined in Figure 1. Immobilizing one of the substrates on the surface of a 96-well microtiter plate can be achieved by covalently linking it to BSA, which adheres strongly to polystyrene surfaces. The choice of substrate to immobilize and the site of linker attachment is dictated by a need to maximize the antigenic distinction between the substrate and product while avoiding interference with the reaction and maintaining synthetic accessibility. Considering these criteria for the Diels–Alder reaction of Figure 2, the maleimide–protein conjugate **2** was synthesized. The phthalimide product of the reaction, **4**, was also prepared, and protein conjugates of the compound were used to elicit a product-specific monoclonal antibody (11A5). Horse radish peroxidase was covalently linked to 11A5 to yield the enzyme-labeled antibody, 11A5-HRPO.[16]

(15) KaleidaGraph (Abelbeck Software).

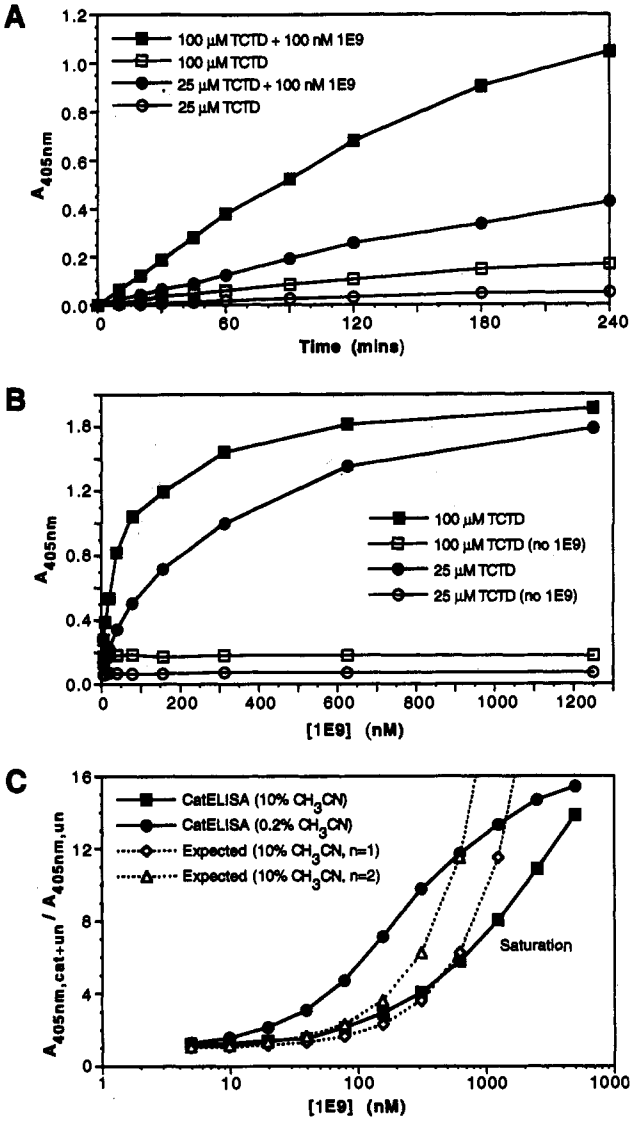

Figure 3. catELISA results. Each of the three experiments were performed separately; hence, the absolute absorbance values cannot be compared between experiments. In each case, the mean absorbance of several control wells in which TCTD was not added was subtracted: (A) dependence of the signal (A_{405nm}) on the length of the reaction; (B) dependence of the signal (A_{405nm}) on the concentration of 1E9 (antibody catalyst); (C) dependence of the ratio $A_{405nm,cat+un}/A_{405nm,un}$ on the concentration of 1E9. Experimental results from the catELISA performed with 10% acetonitrile and 0.2% acetonitrile are shown with solid lines. The expected results, based on published data,[9] for the reaction in 10% acetonitrile are shown with dashed lines. Expected results were calculated for $n = 1$ and $n = 2$ (see text), representing the upper and lower bounds of $A_{405nm,cat+un}/A_{405nm,un}$.

The catELISA was assessed by studying the time dependence of the reaction between tetrachlorothiophene dioxide (**1**, TCTD) and immobilized maleimide **2** in the presence or absence of antibody catalyst 1E9 (Figure 3A). The antibody 1E9, raised against the transition state analog **5** (TG conjugate), has previously been shown to catalyze the reaction of *N*-ethylmaleimide (**2**, *R* = ethyl) with TCTD in solution.[9] The signal (A_{405nm}), propor-

(16) The product can also be recognized by unmodified 11A5, which is detected in a subsequent step by a commercially available enzyme-linked goat antibody which recognizes the constant region of mouse antibody kappa chains. The direct linking of horse radish peroxidase to the detection antibody 11A5 increases the convenience of the assay by reducing the number of steps. Furthermore, since the catalytic antibody 1E9 is also a mouse antibody, direct detection decreases the "noise" in the data that is caused by small amounts of 1E9 bound to the plate.

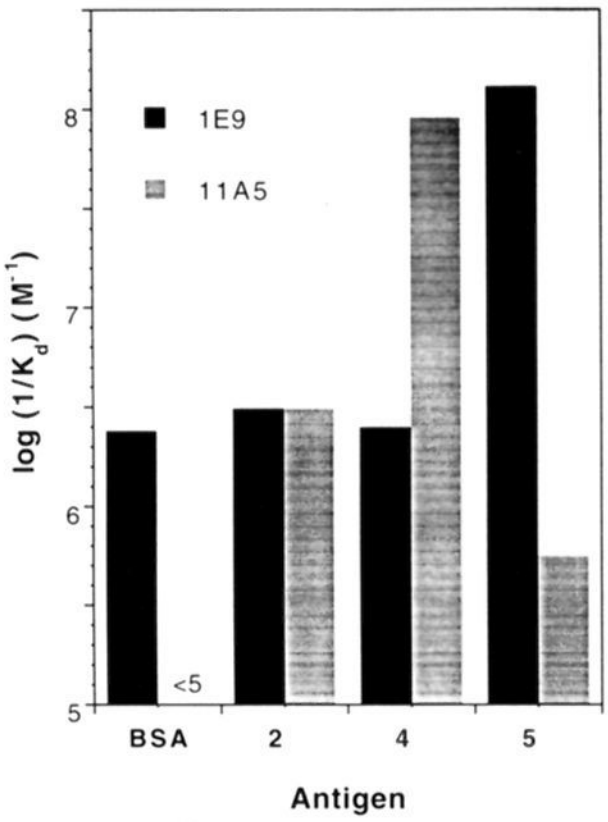

Figure 4. Affinities of the antibodies 1E9 and 11A5 for various antigens. The dissociation constants were determined as described in the Materials and Methods section.

tional to the amount of product **4** formed during the reaction, increased linearly with time for at least the first hour of the reaction, leveling off at longer reaction times. The addition of 1E9 resulted in an increased signal, whereas addition of non-catalytic antibodies had no discernible effect (data not shown). Furthermore, increasing the concentration of TCTD resulted in an appropriately higher signal. The dependence of the signal on catalyst concentration was also followed at different TCTD concentrations, maintaining a reaction time of 1 h (Figure 3B). The signal appeared to increase linearly with [1E9] at low concentrations, leveling off at higher concentrations. Again, the signal was appropriately larger with higher [TCTD].

Formation of product is expected to slow down as the reaction proceeds. However, the leveling off observed at long reaction times and high catalyst concentrations (Figure 3A,B) is not believed to arise from substrate depletion. Previously determined kinetic parameters for 1E9[9] predict the extent of the reaction to be too small under these conditions to account for the observed effect. Rather, it is believed that the leveling off results primarily from saturation of 11A5-HRPO binding, since the density of substrate or product moieties on a BSA molecule exceeds the physically attainable density of detection antibody due to size constraints. The affinity of 1E9 for BSA (Figure 4) may also contribute to the saturation phenomenon: the rate of the catalyzed reaction will cease to increase linearly when [1E9] approaches or exceeds the apparent dissociation constant of 1E9 for BSA (420 nM). It is important to note that the control afforded by varying both the reaction time and TCTD concentration enables saturation to be avoided, thereby maximizing the signal to background ratio at a given concentration of catalyst.

The accuracy of the catELISA can be assessed by deriving kinetic parameters from the results and comparing them to previously determined values. However, since the concentration of product cannot be determined explicitly and since saturation arises from factors not easily related to the extent of the reaction, the absolute rates of the catalyzed or uncatalyzed reactions cannot be determined from the data. Nevertheless, the rate of the catalyzed reaction *relative* to the uncatalyzed reaction can be determined. The signal (A_{405nm}) is proportional to the concentration of product formed during the course of the reaction. This can be described by the integrated rate law for a first-order rate equation since the reaction occurs under pseudo-first-order conditions (TCTD is present in large excess) and the concentrations of both substrates are maintained well below their K_m values. Thus,

$$\frac{A_{405nm,cat+un}}{A_{405nm,un}} = \frac{1 - \exp(-n(k_{cat}/K_{m(2)})[1E9]t - k_{un}[TCTD]t)}{1 - \exp(-k_{un}[TCTD]t)} \quad (1)$$

Here, $A_{405nm,cat+un}$ and $A_{405nm,un}$ are the signals observed in the presence and absence of added catalyst 1E9, $k_{cat}/K_{m(2)}$ is the specificity constant of 1E9, k_{un} is the second-order rate constant for the uncatalyzed reaction, and n is the number of effective active sites of the antibody IgG molecule. Although the antibody IgG molecule has two active sites, if the density of the BSA–substrate conjugate on the plate is extremely low, only one site can participate in catalysis at a given time and $n = 1$. If, on the other hand, the density is very high, both antibody binding sites may have access to substrate at all times and $n = 2$. Between these two extremes, $1 < n < 2$.

The ratio $A_{405nm,cat+un}/A_{405nm,un}$ was calculated from catELISA data covering a large range of catalyst concentrations, and the expected values were calculated from published data[9] using eq 1. Since n, the number of effective active sites on the antibody molecule, cannot be determined experimentally, expected values were calculated for $n = 1$ and $n = 2$ and the resulting upper and lower bounds plotted with the catELISA data (Figure 3C). The results from the catELISA, performed under the same conditions used to determine the published data, fall within the expected bounds at low 1E9 concentrations, exhibiting saturation at higher concentrations. In the original studies,[9] 10% acetonitrile was included to increase the solubility of TCTD in the aqueous buffer. Since the detection limit of the catELISA permits much lower concentrations of TCTD, the assay was also performed in 0.2% acetonitrile for comparison. If the value of k_{un} for the reaction in solution is used to calculate the corresponding value of $k_{cat}/K_{m(2)}$ by general curve fitting[15] of the catELISA data to eq 1, the apparent $k_{cat}/K_{m(2)}$ at 25 μM TCTD and 10% acetonitrile falls in the range 6.5–13 M^{-1} min^{-1} (bounds determined by $n = 1$ and $n = 2$). This compares to an expected value of 8.4 M^{-1} min^{-1}, calculated from the published data.[9] At 0.2% acetonitrile, $k_{cat}/K_{m(2)}$ falls in the range 30–59 M^{-1} min^{-1}, consistent with observations that 1E9 activity is increased at lower acetonitrile concentrations (K. Hill, unpublished data).

Substrate(s). In developing a screening procedure such as this, it is necessary to consider the requirements and limitations of each element of the assay. The Diels–Alder reaction studied here provides a representative system for evaluating the various features involved and some of the potential complications that must be addressed.

One problem that arises is that of substrate instability. For the reation under study, both substrates present complications. The diene, TCTD, is particularly subject to Michael addition, and buffers containing nucleophiles (such as Tris) must be avoided. Besides buffer molecules, nucleophilic groups on the catalyst and on the BSA molecules, such as the ϵ-amino groups of surface-accessible lysine residues, must be considered. Reaction of TCTD with these proteins can lead to reduction of TCTD concentrations, destruction of enzyme activity by reaction with residues near the active site, or cross-linking of detection antibody to BSA on the surface of the plate, yielding false positive signals. In the original characterization of 1E9,[9] such complications were avoided by reductive methylation of the amines on 1E9 and by reducing the pH to 6.0. In the catELISA assay, reducing the pH to 6.0 minimized these side reactions sufficiently to obtain reproducible signals so that modification of amines was not required. Although cross-linking of detection antibody to the plate becomes problematic at higher pH, this side reaction can be blocked by

acetylation of the BSA coating the plate with acetic anhydride (data not shown).

The maleimide substrate **2** is also susceptible to undesired side reactions: Michael addition of nucleophiles to the double bond and ring-opening by hydrolysis[12] compete with the Diels–Alder reaction. The half-life of **2** under the experimental conditions can be determined conveniently by catELISA, varying the time it is preincubated with buffer prior to reaction with TCTD. Even when the pH is reduced to 6.0, the half-life of the BSA–maleimide conjugate is under 5 h at room temperature in MES buffer. Nevertheless, we find that these competing reactions are effectively independent of catalyst concentration so that the signal to background ratio is unaffected by the instability of **2**.

Detection Antibody. Fundamental to any reaction assay is the ability to distinguish product from substrate. In the catELISA this is achieved through the remarkable binding specificity of antibody molecules. The *degree* of discrimination between product and substrate can be defined by considering a hypothetical experiment in which product and substrate are immobilized in separate wells of a microtiter plate at the same concentration. The detection antibody is then added to each well and allowed to bind. The ratio of the fraction of product bound by antibody to the fraction of substrate bound by antibody, $F_{p/s}$, provides a good index of discrimination between product and substrate. From standard equilibrium equations, we know that the fraction of antigen bound by an antibody depends on the total antigen concentration ($[Ag]_t$), the total antibody concentration ($[Ab]_t$), and the dissociation constant of the antibody for the antigen ($K_{d,Ag}$). Thus, an expression for $F_{p/s}$ as a function of these three parameters can be derived:

$$F_{p/s} = \frac{[Ab]_t + [Ag]_t + K_{d,p} - (([Ab]_t + [Ag]_t + K_{d,p})^2 - 4[Ab]_t[Ag]_2)^{1/2}}{[Ab]_t + [Ag]_t + K_{d,s} - (([Ab]_t + [Ag]_t + K_{d,s})^2 - 4[Ab]_t[Ag]_t)^{1/2}} \quad (2)$$

Here, $K_{d,p}$ and $K_{d,s}$ are the dissociation constants of the detection antibody for the product and substrate, respectively. Analysis of eq 2 reveals that the maximum value of $F_{p/s}$ is $K_{d,s}/K_{d,p}$, approached as $[Ag]_t$ and $[Ab]_t$ both tend to 0 (a derivation of this statement is provided as supplementary material). Furthermore, to achieve >85% of the maximum discrimination, both $[Ag]_t$ and $[Ab]_t$ must be less than 10% of $K_{d,p}$. This is an extremely important aspect to consider when determining the conditions of the assay. For example, if the concentration of antibody used to detect the product is too high (e.g., close to $K_{d,s}$), very little discrimination between product and substrate will be observed, even if $K_{d,p} \ll K_{d,s}$. In practice, $[Ag]_t$ and $[Ab]_t$ can be optimized empirically.

Assuming $[Ag]_t$ and $[Ab]_t$ are chosen such that $F_{p/s} \approx K_{d,s}/K_{d,p}$, the ability to identify catalysts in a library of variants depends on both $K_{d,s}/K_{d,p}$ and the *extent* of the reaction. Since confident identification is limited by the precision of the data (estimated to be about 10% for enzyme immunoassays),[8] the signal resulting from product binding must be >10% of the background resulting from substrate binding. If the detection antibody offers only a small value of $K_{d,s}/K_{d,p}$, longer reaction times will be needed to increase the extent of the reaction. Large values of $K_{d,s}/K_{d,p}$ obviate the need for longer reaction times. For the reaction studied in this paper, long reaction times were inaccessible due to substrate instability. Thus, an antibody with a large value of $K_{d,s}/K_{d,p}$ was required. Dissociation constants of 1E9 and 11A5 were estimated for substrate **2** and product **4**, as well as for BSA and for the transition state analog **5** (see Figure 4). The apparent values of $K_{d,s}$ = 320 nM and $K_{d,p}$ = 11 nM yield $F_{p/s,max}$ = 29. This corresponds well to the observed $F_{p/s}$ of the 11A5-HRPO conjugate under the conditions of the assay and was more than sufficient to permit unequivocal detection of catalysis.

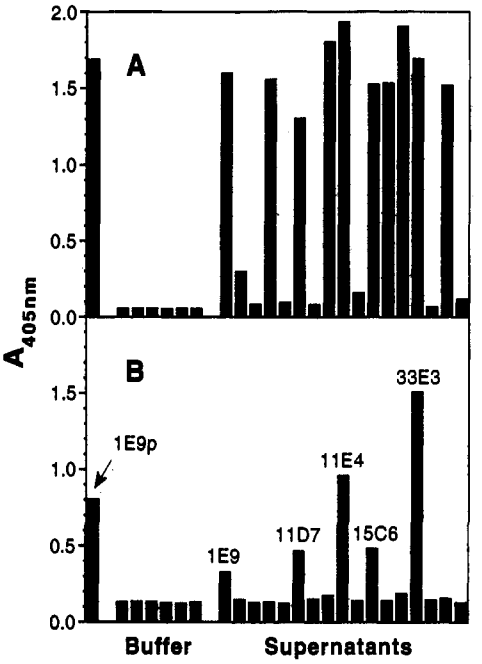

Figure 5. (A) Standard ELISA results with immobilized transition state analog **5**: lane 1, 100 nM purified 1E9; lanes 3–8, MBS-6.0 buffer (negative controls); lanes 10–26, hybridoma supernatants, diluted 50% with binding buffer. Bound antibody was detected with a commercial goat anti-mouse antibody covalently linked to horseradish peroxidase. (B): catELISA results with immobilized substrate **2**. The reaction was initiated by diluting the samples 50% with TCTD in 60 mM acetate + 40 mM NaCl (pH 5.0), giving a final concentration of 100 μM TCTD. The reaction was allowed to proceed for 1 h at room temperature. Product was subsequently detected with 11A5-HRPO. The lanes of part B correspond to those of part A. Hybridoma supernatants displaying significant catalytic activity are labeled.

The high value of $F_{p/s}$ in our system was obtained by using the monoclonal antibody 11A5. Since production of monoclonal antibodies is time consuming and costly, it would be advantageous, when possible, to use polyclonal serum to detect the product. This has proved sufficient for at least one reaction.[6] However, we have found that the use of polyclonal serum is often not possible. If the substrate and product have strongly antigenic elements in common, polyclonal serum is likely to contain antibodies that recognize these common functionalities. We have shown for a phosphate ester hydrolysis reaction involving a phosphotyrosine-containing pentapeptide substrate that polyclonal serum offered no detectable discrimination between substrate and product (J. Shin, unpublished). The observed $F_{p/s}$ of the serum was increased somewhat by passing the serum through a column of excess immobilized substrate. However, even after this purification step, the value of $F_{p/s}$ was less than 2, seriously compromising the reliability of the assay.

Utility of the catELISA. The data of Figure 3 demonstrate the successful application of the catELISA to a bimolecular, cycloaddition reaction. Our results, together with those of Tawfik *et al.*,[6] support the potential of this assay as a general approach to screening for catalysts, complementing and extending other strategies reported to date.[3,5] In our example, control over the extent of the reaction is achieved by varying both the time of the reaction and the concentration of the second substrate added in solution, enabling conditions to be chosen that optimize the signal to background ratio by avoiding saturation of product formation or detection. Under nonsaturating conditions, the catELISA provides a remarkably accurate estimate of the efficiency of the catalyst being assayed, despite immobilization of one of the substrates.

Applying these results, the catELISA was used to screen hybridoma supernatants from the original fusion that yielded

1E9. Supernatants were tested for the ability to bind the transition state analog **5** by regular ELISA (Figure 5A) and for the ability to catalyze the Diels–Alder reaction of Figure 2 by catELISA (Figure 5B). The results of Figure 5B clearly demonstrate that the catalytic antibody 1E9 can be distinguished from other, noncatalytic antibodies and from negative controls (no antibody) by catELISA. Higher signals over background have been noted for other samples of 1E9 supernatant, likely reflecting higher antibody concentrations in these samples. Interestingly, four new catalysts were identified, each displaying significant catalytic activity (Figure 5B). All four of these new catalysts bind the transition state analog **5** (Figure 5A), consistent with the strategy used to elicit the catalysts. Notably, the previously untested 33E3 is substantially more active than 1E9 at comparable concentrations, as estimated by ELISA titration. Characterization of these antibodies is currently in progress.

The success of this screen can be attributed to the remarkably low limit of catalytic activity that can be identified unambiguously by catELISA. Two factors affect this limit: the precision of the data and the minimum amount of product that can be detected. Estimating the precision to be about 10%,[8] the rate of the catalyzed reaction could in principle be as low as 10% of the rate of the background reaction and still allow identification of a catalyst. If the background reaction is extremely slow, product detection may instead define the lower limit of catalytic activity that can be identified. However, 10–0.1 fmol of antigen can often be detected in a microtiter plate well by enzyme immunoassay, depending on the affinity of the antibody for the antigen.[8] This exquisite level of detection means that in most cases, including the reaction under study, it is the precision of the data that determines the level of activity that can be observed. Since catalytic activity is a function of both the efficiency and concentration of the catalyst, high concentrations of the species being assayed enable relatively inefficient catalysts to be found, while lower concentrations result in more restricted searches. In the case of the Diels–Alder reaction, the data of Figure 3 predict that catalysts as efficient as 1E9 can, in principle, be detected at concentrations as low as 2 nM. In practice, other factors increase the variation in the data, and a more realistic lower limit for 1E9 would be about 10 nM. Less efficient catalysts would require higher concentrations, while more efficient catalysts could be detected at even lower concentrations.

The ability to detect catalysts at such low concentrations has important practical implications for screening large libraries of variants. As demonstrated in Figure 5B, catalytic activity can be identified in hybridoma supernatants, where antibodies are typically expressed at concentrations of 30–300 nM.[7] However, it should also be possible to exploit the catELISA for identification of highly active catalysts in libraries of Fab molecules selected using phage display methods[17] and expressed in microorganisms. This technology lends access to much larger sizes of libraries, increasing the probability of finding more active catalysts. Furthermore, the catELISA is not restricted to screening antibodies. Other proteins or even nonproteinaceous catalysts might be identified using this powerful approach. We are currently using the assay described in this paper to screen libraries of Fab molecules, single-chain Fv species, and randomized oligoribonucleotides.

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Supplementary Material Available: Derivation of eq 2 and limit of $F_{p/s}$ as $[Ab]_t$ and $[Ag]_t$ approach 0 (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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