0273-2289/00/83/0195/$13.50

Catalytic Antibodies for Complex Reactions

Hapten Design and the Importance of Screening for Catalysis in the Generation of Catalytic Antibodies for the NDA/CN Reaction

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Abstract

Success in generating catalytic antibodies as enzyme mimics lies in the strategic design of the transition-state analog (TSA) for the reaction of interest, and careful development of screening processes for the selection of antibodies that are catalysts. Typically, the choice of TSA structure is straightforward, and the criterion for selection in screening is often binding of the TSA to the antibody in a microtiter-plate assay. This article emphasizes the problems of TSA design in complex reactions and the importance of selecting antibodies on the basis of catalysis as well as binding to the TSA. The target reaction is the derivatization of primary amines with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide ion. The desired outcome is selective catalysis of formation of the fluorescent derivative in preference to nonfluorescent side-products. In the study, TSA design was directed toward the reaction branch leading to the fluorescent product. Here, we describe a microtiter plate-based assay that is capable of detecting antibodies showing catalytic activity at an early stage. Of the antibodies selected, 36% showed no appreciable binding to any of the substrates tested, but did show catalytic activity in deriving one or more of the amino acids screened. In contrast, only two out of 77 clones that showed binding did not show catalysis. Thus, in this complex system, observation of binding is a good predictor of the presence of catalytic activity, and failure to observe binding is a poor predictor of the absence of catalytic activity.

Index Entries: Catalytic antibody; transition-state analog; NDA/CN reaction; screening; enzyme mimics.

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Introduction

Catalytic Antibodies for Complex Reactions

Catalytic antibodies, first reported simultaneously by the research groups of Lerner *(1)* and Schultz *(2)* in 1986, have revolutionized the concept of designed catalysts *(3)*. Catalytic antibodies can be induced by immunization against haptens resembling the transition state (resulting in transition-state analogs or TSAs) of the target reaction. The antibody-producing cells resulting from immunization are then immortalized as hybridomas that are subsequently used to select monoclonal antibodies (MAbs) as potential catalysts. Two crucial steps *(4–6)* are required to obtain successful catalytic antibodies with large turnover numbers and high rate enhancements: 1) the proper design of the TSA hapten and 2) effective selection processes for the catalytic antibodies.

For simple reactions, proper hapten design seems straightforward. However, it has been suggested that the design of transition-state analogs as haptens is more problematic than previously thought *(7)*, even for simple reactions. For complex reactions involving parallel and serial steps, there are a multiplicity of transition states to consider in design, and clearly the choice becomes a complicated matter *(8)*.

The selection process has often relied on the binding characteristics of potentially catalytic antibodies to a protein conjugate of the TSA hapten or on antibody binding to a "short TSA" *(4)*. The clones selected in the binding assay are expanded, and MAbs are prepared and purified. Usually, only a few of the selected MAbs are catalytic, thus making the entire process very tedious and time-consuming, with a low yield. Therefore, selection for catalysis at an early stage in the production process of MAbs is advantageous and cost-effective. The obviously more efficient selection for catalytic activity *(6,9–12)* is now a well-established approach, but the correlation of catalytic activity with TSA affinity remains elusive in general. Of course, it is well-recognized that efficient catalysts bind to the transition state preferentially and with high affinity *(13)*, and catalytic antibodies thus should bind a good TSA strongly. Yet one of the unresolved issues is how thoroughly the structure of a TSA can be distinguished in antibody binding from the structure of a reactant-state or product-state analog (binding of which should be anticatalytic), and whether there may be substantial kinetic barriers to TSA binding even when equilibrium affinity is high.

The NDA/CN Reaction

The target reaction in our study is the NDA/CN reaction *(14)* in which primary amines react with naphthalene-2,3-dicarboxaldehyde and cyanide ion to generate the fluorescent 2-cyanobenzoisoindole or CBI derivative (*see* 1 in Fig. 1, which indicates the stoichiometry and a few characteristics of the reaction). The NDA/CN reaction is of major analytical importance in the detection and quantitation of amino acids and small peptides of bio-

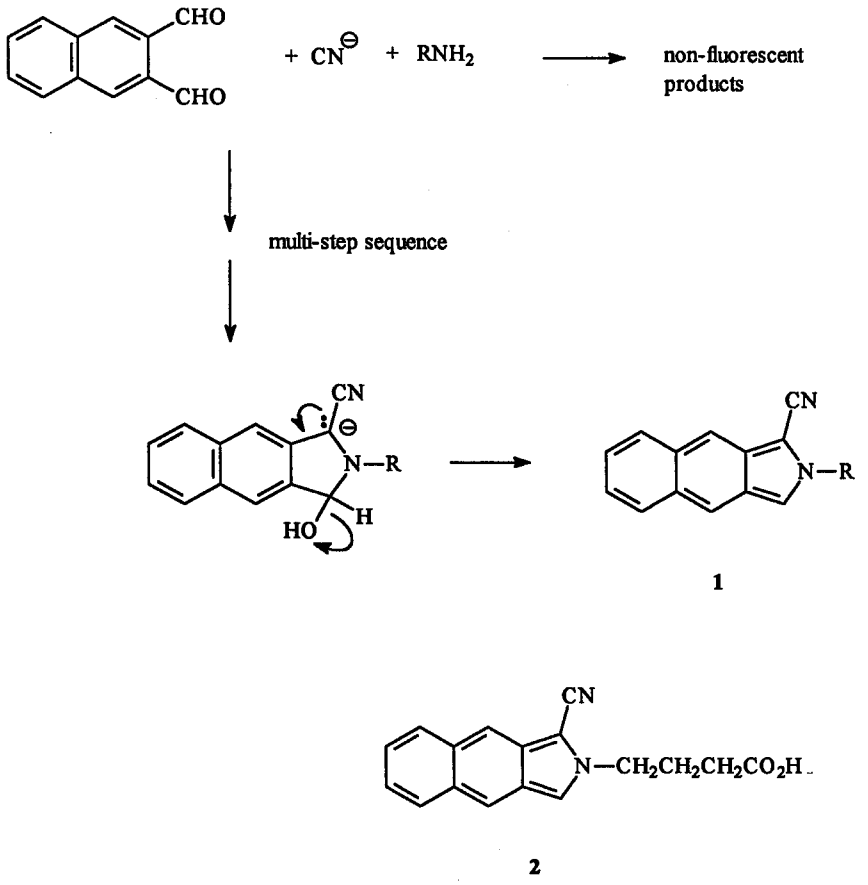

Fig. 1. Sketch of the stoichiometry and mechanism of the NDA/CN reaction of primary amines, indicating the branching pathways that lead in the early part of the mechanism to nonfluorescent products, and the formation (probably through an E_1cB process) of the fluorescent CBI product **1** in the late part of the mechanism. Also depicted is structure **2**, selected as the hapten for generation of catalytic antibodies intended to enhance the rate of the fluorogenic reaction over that of the nonfluorogenic side reactions.

logical interest at physiologically relevant levels. The CBI derivatives are relatively stable in solution, exhibit very high quantum efficiencies, possess useful spectroscopic and electrochemical properties, and are very efficient energy acceptors in the aryl oxalate–hydrogen peroxide chemiluminescent reaction *(15)*. However, a major drawback of the reaction is the formation of nonfluorescent side products, resulting in a lower sensitivity. Thus, a particular role for a catalytic antibody is to suppress side reactions by *selective catalysis* of the fluorogenic reaction as opposed to the reactions forming nonfluorescent side products.

Issues in design of the hapten will be discussed elsewhere. However, the rationale for hapten selection dictates that the hapten should model the transition state(s) of the fluorogenic reactions more closely than those

of the nonfluorogenic reactions in order to achieve the desired selective catalysis of the fluorogenic process. Generally, nonfluorescent products are formed in reactions that diverge from the fluorogenic pathway before generation of the direct precursor of the fluorescent product **1** (the transformation is probably an E_1cB reaction *(15)* (*see* Fig. 1). Thus, the fluorescent product itself is expected to be a better simulator of the fluorogenic transition states than of the transition states of the earlier, divergent pathways. The hapten selected was 4-(1′-cyanobenz[*f*]isoindoloyl)butyric acid (*see* 2, Fig. 1).

Methods and Materials

Instrumentation

The initial screening for catalytic antibodies during the supernatant stage employed a V_{max} microplate reader (Molecular Devices, Menlo Park, CA) in the kinetic mode at 405 nm with data analysis using SOFTmax® software, Version 1.01. Concentration-dependence studies used a Model 7620 Microplate Fluorometer (Cambridge Technology, Inc., Watertown, MA), with excitation and emission wavelengths at 420 and 485 nm, respectively (software: Fluororeader Series 7600, Version 4.0). Reverse-phase HPLC experiments were performed on a Shimadzu HPLC system (Columbia, MD) equipped with two LC-6A high-pressure pumps, an SCL-6B System controller, and an SIL-6B Autoinjector. Fluorescence was monitored by a Shimadzu RF-535 Fluorescence HPLC monitor (λ_{ex} 420 nm; λ_{em} 490 nm), and absorbance was measured by a Shimadzu SPD-6AV UV-VIS spectrophotometric detector. The data were recorded on a Shimadzu model C-R4A Chromatopac.

Preparation of 4-(1′-Cyanobenz[f]isoindoloyl)butyric Acid, 2

To a solution of 368 mg of 2,3-napthalenedicarboxaldehyde in 60 mL of dry methanol, 98 mg of NaCN was added. A solution of 206 mg of 4-aminobutyric acid in 4 mL of water was added immediately, and the resulting solution was stirred for 1 h. Evaporation of the solvent under vacuum, followed by titration of the residue with 10 mL of ether, produced 457 mg of sodium 4-(1′ cyanobenz[*f*]isoindoloyl)butyrate as an orange solid. The sodium salt was dissolved in 50 mL of water, and the water solution was extracted twice with 20-mL portions of ether and acidified to pH 1 with 1 M hydrochloric acid. The acidified solution was extracted with ether, and the ether solution was dried over $MgSO_4$. Evaporation of the solvent produced 404 mg of crude product as an orange solid, mp 174–182°C. Recrystallization from 9 mL of acetic acid afforded 362 mg of the pure product as golden yellow crystals, mp 208–209°C. IR (KBr): 3157 (br), 2195, 1725, 1457, 1402, 1167, 1130, 867, 741, and 711 cm^{-1}; ^1H NMR (DMSO): 12.2 (br 5, lH), 8.46 (s, 1H), 8.22 (s, 2H), 7.91 (t, 2H), 7.28 (m, 2H), 4.58 (t, 2H), 2.25 (m, 4H); ^{13}C NMR: 173.3, 131.5, 130.1, 129.4, 128.7, 128.0, 125.1, 123.8, 123.6, 120.9,

119.7, 115.0, 114.0, 49.8, 30.4, 25.9; MS(EI): 278 (M+), 206, 205, 192 (base peak), 165, 164, 138; exact mass: calculated 278.1055, found 278.1053.

Preparation of the TSA–Protein Conjugates

Keyhole limpet hemocyanin (KLH, Sigma, St. Louis, MO) and purified protein derivative (PPD, provided by Lederle Laboratories, NY) were used as the protein carriers in the conjugation of **2** (TSA) for subsequent immunizations. Bovine serum albumin (BSA) (Sigma) was used in the conjugation of **2** for screening. Compound **2** was coupled through the carboxylate group by initial activation with water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, Pierce, IL) according to the enhanced carbodiimide-coupling method *(16)* using sulfo-*N*-hydroxysuccinimide (Sulfo-NHS, Pierce, IL) as the enhancer. The molar ratio of KLH: **2** was 1:100. One milligram of **2** was dissolved in 1 mL of 0.01 *M* phosphate buffer (PB), pH 7.4, containing no azide. EDAC was added to a final concentration of 0.1 *M*. After stirring for a few minutes, Sulfo-NHS was added dropwise to achieve 1.25-fold the TSA concentration. Finally, 1 mg of the carrier protein was added and the solution was made up to 2 mL. The reaction was allowed to proceed for 24 h at room temperature. The order of the addition of the reagents is critical to obtain a successful conjugate. The reaction mixture was dialyzed against 0.1 *M* phosphate-buffered saline (PBS, 0.1 *M* PB +0.15 *M* NaCl), pH 7.4, to remove the excess EDAC, Sulfo-NHS, and **2**. The PPD–**2** conjugate and BSA–**2** conjugate were prepared in a similar manner, using a molar-ratio protein: **2** of 1:10.

Preparation of MAbs

Two sets of four 6-wk-old female Balb/c mice were used for immunizations. The PPD–**2** conjugate was employed at 30 μg/immunization. One week prior to the first immunization of the PPD–**2** conjugate, the mice were sensitized with 50 μg of bacillus Calmette-Guerin (BCG, RIBI Immunochem Research Inc., MT) via the intramuscular (im, 50 μL) route. The rest of the immunization protocol was as follows: the first immunization was intraperitoneal (ip, four sites with 100 μL/site) in complete Freund's adjuvant (CFA, Gibco Laboratories, NY) at 3:1 v/v (adjuvant:conjugate) ratio. The second immunization was intravenous (iv, 30 μg in 50 μL) in sterile saline at a ratio of 2:1 v/v (saline:conjugate) after 2 wk. Biweekly immunizations ip (four sites with 100 μL/site) in incomplete Freund's adjuvant (IFA, Gibco Laboratories, NY) were given until a desired circulating antibody concentration (1:1000 dilution) specific to the TSA was attained in the serum. A v/v ratio of 3:1 (IFA:conjugate) was maintained. Four days after the final immunization, the spleen of an immunized mouse was removed using standard surgical procedures, and the spleen cells were fused with the murine myeloma cell line sp2/0 (American Type Culture Collection, Rockville, MD) according to Galfré and Milstein *(22)*. After 2wk, the supernatants were screened for specific antibody production, using enzyme-linked immunosorbent assay (ELISA) as

described here. The KLH–**2** conjugate was administered at a dose of 10 μg/mouse, according to a similar procedure with the elimination of the sensitization with BCG.

Hybridomas that secrete specific antibodies and those that exhibit catalytic activity were selected, expanded in culture medium, and cloned by the limited dilution method described by Goding *(17)*. Seven MAb-secreting cell lines were selected, and ascites was produced in Balb/c mice, using 2,6,10,14-tetramethylpentadecane (Pristane, Sigma). The immunoglobulin fraction in the ascites fluid was precipitated with saturated ammonium sulfate (33%), according to Tijssen *(18)*. The immunoglobulins were further purified using anion-exchange chromatography on diethylaminoethyl (DEAE)-Sephacel (40–160 μm in wet form) (Pharmacia, Piscataway, NJ) *(17)*, employing a sodium-chloride gradient to isolate the IgG fractions. The purified MAbs were aliquoted into 200-μL samples and stored at 4°C until used.

*Screening Procedures: ELISA Method 1—Screen for Binding to the BSA–**2** Conjugate*

The ELISA plates were coated with 100 μL of BSA–**2** conjugate and BSA (10 μg/mL in 0.1 *M* carbonate buffer, pH 9.35) (plate-coating buffer) overnight at 4°C. The plate was washed four times with 0.01 *M* PBS containing 0.05% Tween 20 (Sigma) (wash buffer), and patted dry on a stack of paper towels; 150 μL/well of wash buffer containing 0.2% BSA (ELISA diluent buffer) was added and incubated for 1 h at 37°C to block the remaining active sites on the plastic surface. The plates were washed four times with wash buffer and patted dry. Serum or culture supernatant (1 μL/well) was added and incubated for 1 h at 37°C. The plates were washed four times with wash buffer and patted dry. Horseradish peroxidase (HRP)-labeled goat antibody against mouse (goat anti-Mo HRP, Organon Teknika Corp., Durham, NC) immunoglobulin (100 μL, 1:10,000 dilution) was added and incubated at 37°C for 1h more. The plates were washed four times with Nanopure water (Barnstead, Nanopure II Sybron, Barnstead, MA) and patted dry. Then, 100 μL of HRP substrate [tetramethylbenzidine (TMB) and hydrogen peroxide, KP Laboratories, MD] was added, and after 20 min the enzyme reaction was quenched with 1 *M* HCl. The absorbance was read at 450 nm using a 96-well plate reader (V_{max}, Molecular Devices, CA). In ELISA experiments using supernatant, serum from an immunized mouse served as the positive control and serum from an unimmunized mouse was used as the negative control. Positive selection for binding was made if the range for the observed signal was between threefold the negative-control signal up to the positive-control signal.

*Screening Procedures: ELISA Method 2—Screening for Binding to NDA and **2***

The ELISA method of Jitsukawa et al. *(19)* was used with the following modifications. Equal concentrations of **2** and BSA (50 μg/mL) were

made up in plate-coating buffer, mixed, and diluted to 5 mL using the same buffer; 50 μL of this solution was added to each well in an ELISA plate and incubated at 37°C overnight or longer to completely evaporate the liquid. The same procedure was carried out with NDA (Oread Laboratories, Lawrence, KS) and with BSA as control. The subsequent steps were exactly the same as in the ELISA method *1*. Positive selection for binding was made if the range for the observed signal was between threefold the negative-control signal up to the positive-control signal.

Affinity Determinations for MAbs

The affinity constants were determined using ELISA methods similar to those of methods 1 and 2, as outlined here. The ligands (BSA–**2** conjugate, **2**/BSA or NDA/BSA) were used as the solid-phase antigens. The purified antibodies were added in serial dilution. All other steps were carried out according to method 1. The affinity constant was estimated from the dilution at 50% binding as described by Tijssen *(18)*.

Screening of Supernatants for Catalytic Activity

Catalytic activity was tested for five amino acids (L-serine, *N*-α-acetyl-L-lysine, L-asparagine, L-glutamine, and L-leucine) in a 96-well ELISA plate with the plate reader in kinetic mode. The fluorogenic NDA/CN reaction was followed by monitoring the formation of the CBI derivative over a period of 10 min at 405 nm. The pH of the system was kept at 7.4 in order to minimize the (still large) uncatalyzed rate of this reaction. The data up to 2 min were used for initial-rate calculations (thereafter the absorbance rise became nonlinear). After 20 min, assuming that the NDA/CN reaction with the amino acid was complete, the absorbance was read again at 405 nm. This measurement is indicative of the total amount of product formed.

Each well contained 30 μL of supernatant, 30 μL of amino acid (5 m*M*), 50 μL of NaCN (100 m*M*), and 50 μL of NDA (10 m*M*). The total volume in the well was made up to 200 μL with 0.01 *M* PBS, pH 7.4. Reagents were added according to the following sequence: buffer, supernatants, amino acid, NaCN, and, finally, NDA. The reactions were carried out at room temperature. NDA stock solutions (100 m*M*) were made weekly in acetonitrile and stored at 4°C in amber glass vials. NaCN stock solutions (1 *M*) were made daily, using Nanopure water. The stock solution of NaCN was diluted to the desired concentration (100 m*M*) in 0.01 *M* PBS, pH 7.4, at the time of use. Amino-acid stock solutions (10 m*M*) were prepared weekly in 0.01 *M* PBS, pH 7.4, and stored at 4°C. Extreme precautions were taken when cleaning the glassware used for solution preparation. Control experiments with supernatant alone, amino acid alone, and NDA and CN alone were also carried out in the same plate. The supernatants were screened by this method, both at the 96-well-plate stage initially and then at the 24-well-plate stage. The experiments were confirmed using a Model 7620 fluorescence-plate reader (Cambridge Instruments, Inc., Watertown,

MA). The purified MAbs were used and the experiments were done using black 96 well plates from Dynatech Laboratories, Chantilly, VA. At the MAb stage, candidates were selected as catalytically active if the reaction rates were at least 5–10% higher than control levels, and the reproducibility of individual rate measurements was below 3%.

Methylation of MAb 6D1 02E8

The primary-amine groups of MAb 6D1 02E8 were methylated with the formaldehyde procedure of Dottavio-Martin and Ravel *(20)*. The procedure was as follows: 0.1 mL of a 37% formaldehyde solution (Fisher Scientific, Fair Lawn, NJ) was added to 1 mg of MAb in 10 m*M* PB, pH 7.4, followed by 100 mL of a freshly prepared solution of $NaBH_3CN$ (6 mg/mL in 10 mM PB, pH 7.4) (Pierce, Rockford, IL). The reaction mixture was incubated at 25°C for 1 h, and was shaken gently at 15-min intervals. The reaction volume was increased to 1 mL, and the solution was dialyzed into 10 m*M* PB, pH 7.4, for 16 h at 4°C. The methylation process was 73% efficient, and binding constants of the methylated and the unmethylated antibodies did not show significant differences, consistent with maintenance of the native conformation of the antibody. The similar behavior in the assays of the methylated and unmethylated antibodies serves to eliminate the possibility that any of the observed behavior is caused by NDA/CN reaction with the antibody itself.

Determination of the Number of Amino Groups in the Methylated 6D1 02E8

The number of amino groups remaining after methylation was calculated using the method of Habeeb *(21)* with *N*-α-acetyl-L-lysine as the standard. Standard solutions from 0.0125–0.2 m*M* were prepared in 4% $NaHCO_3$, pH 8.5, solution. The concentration of the MAb was measured at 280 nm (ε 1.48 mg^{-1} cm^{-1} mL). It was assumed that the absorptivity of the antibody did not change upon methylation. A solution of 0.1 mg/mL of the antibody 6D1 02E8 was used in this experiment. To 1 mL of the standards, and of unmethylated and methylated 6D1 02E8 samples, 0.25 mL of 0.1% 2,4,6-trinitrobenzenesulfonic acid (TNBS, Sigma, St. Louis, MO) was added. After incubation of this solution for 2 h at 40°C, a 0.25-mL solution of 10% sodium dodecyl sulfate (SDS, Fisher Scientific) was added, followed by 0.125 mL of 1 *N* HCl. The absorbance was read at 335 nm with a Shimadzu UV 160 spectrophotometer.

Reverse-phase HPLC Analysis of the NDA/CN Reaction Kinetics

This technique—which allows separation of the fluorescent products and their direct determination—was employed to confirm the results of the spectrophotometric and fluorimetric kinetic studies. The formation kinetics of CBI–amino acids was determined under isocratic elution conditions

using RP-HPLC (Phase Il-ODS, 3-mm column [100 × 3.2 mm] {Bioanalytical Systems, West Lafayette, IN}). The mobile phase consisted of 30% acetonitrile in 50 m*M* PB, pH 6.8. The flow rate was regulated at 1 mL/min. The formation of the CBI–amino acid was monitored using a xenon light source in the Shimadzu RF-535 HPLC fluorescence monitor at an excitation wavelength of 420 nm and an emission wavelength of 490 nm. Preliminary gradient elution experiments were performed to obtain the isocratic conditions necessary to prevent any MAb coelution. Aliquots of 60 μL of amino acid, 60 μL of MAb, 100 μL of CN (1 m*M*), 100 mL NDA (1 m*M*), and 80 μL of 10 m*M* PB, pH 7.4 were mixed, and the autoinjector was started immediately after the addition of NDA. The reaction was monitored for 40 min at 3-min intervals. Two amino-acid concentrations (0.25 m*M* and 0.5 m*M*) and two MAb concentrations (4.7 riM and 9.4 riM) were investigated. After each kinetic run, the column was flushed with 50% acetonitrile in 10 m*M* PB, pH 6.8, to remove the MAb. The average fluorescence of four trials was plotted against time to obtain the rate measurements.

Results

Preparation of MAb

The PPD–**2** conjugate proved to be a better immunogen than the KLH–**2** conjugate for the production of **2**-specific antibodies. Only 7.5% of the 2-specific hybridomas secreted antibodies that cross-reacted with PPD, compared to 95% of cross-reactivity with KLH. Therefore, subsequent experiments were carried out using hybridomas derived from PPD–**2**-immunized mice. The use of PPD as a superior carrier with respect to cross-reactivity with the antibodies to the hapten has been described by us elsewhere *(23)*.

Screening Results: Hybridomas

The hybridomas were screened for binding to the BSA–**2** conjugate, to BSA, to **2,** and to NDA. They were also screened for catalytic activity in the NDA/CN reaction of the five preselected amino acids listed here. The results are summarized in Table 1. Clones are included in the table if they 1) bound BSA–**2** only or 2) bound BSA–**2, 2,** and NDA. Table 1 excludes clones that had binding activity toward BSA even if they bound BSA–**2,** since it could not be established in such cases whether the affinity toward BSA–**2** arose from interactions with the TSA-portion or the BSA-portion of the conjugate.

Of the 77 clones examined, 46 (or 60%) showed some binding of the BSA–**2** conjugate. However, 75 (or 97%) exhibited catalytic activity in the NDA/CN reaction of at least one of the amino acids tested. Among the hybridomas that specifically did not show appreciable binding of BSA–**2, 2,** or NDA, 36% showed catalytic activity.

Table 1
Binding and Catalysis in Screening of Hybridoma Clones at the 96-Well Stage

Plate	Number of clones	Number of clones that showed binding toward			Number of clones that catalyzed the NDA/CN reaction of:		
		BSA–**2**, **2**, and NDA	BSA–**2** only	No ligand	All five amino acids	One to four acids	No amino acids
1	15	2	5	7	2	13	0
2	13	3	6	4	3	10	0
3	12	1	5	3	2	9	1
4	8	1	1	3	0	8	0
5	11	2	2	7	1	9	1
6	18	6	12	3	1	17	0

Screening Results: Purified MAbs

Purified MAbs were screened again for binding of BSA–**2**,**2**, and NDA and for catalysis of the NDA/CN reaction of the five preselected amino acids. The results are summarized in Table 2.

Of the seven purified antibodies, only two showed binding to the BSA–**2** TSA conjugate. Two others showed weak binding of **2** or NDA, and three showed no measurable binding of any of these ligands. Nevertheless, all of these antibodies exhibited catalytic activity in the NDA/CN reaction of two or more of the amino acids tested. Two of the antibodies that had no binding activity toward any of the ligands tested were catalytic in the reactions of all five amino acids.

Discussion

Hapten Design

Generally speaking, the design of the hapten appears to be successful, considering that antibodies were obtained that accelerated the fluorogenic NDA/CN reaction of the amino acids tested over the nonfluorogenic reaction. However, the detailed kinetic situation is more complicated, and the dependence of rate on antibody concentration is complex, passing through a maximum. The data supporting this assertion are not included in this article. The phenomenon and possible models to explain it will be documented and presented in a later publication.

Screening for Binding and Catalysis

Of the antibodies that showed binding toward the TSA conjugate (BSA–**2**) but not to BSA (and thus exhibiting affinity for the hapten portion of the conjugate), 97% were catalytically active in the NDA/CN reac-

Table 2
Binding and Catalytic Properties of Monoclonal Antibodies

	Monoclonal Antibodies						
Properties	2A111F2	2D91B11	2E81B8	1D21A8	6D102E8	2E101E11	6D53C12
Binding							
Ligand, binding constant, M^{-1}	no binding	NDA, too weak to measure	no binding	no binding	BSA–**2,** 10^8	NDA, **2,** BSA–**2,** 10^8 for all	**2,** too weak to measure
Catalysis							
N-α-Lys	+	+	+	+	+	–	+
Leu	+	+	+	+	–	+	+
Glu	–	+	+	+	+	–	–
Asp	–	–	+	+	–	+	+
Ser	+	–	+	+	–	+	+

tion of at least some of the amino acids tested. Thus, antibodies that bind the conjugate appear very likely to be catalysts, and in this system, binding can be considered to be a positive indicator for catalytic activity. On the other hand, of antibodies that failed to exhibit affinity for the TSA conjugate, nearly 40% were catalytically active. Lack of binding is therefore quite faulty as an indicator of the absence of catalytic activity. For the NDA/CN reaction at least, a screen that tests for binding affinity alone would probably overlook a substantial number of catalysts.

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Discussion

Paul: These are fascinating results. Al Tramontano and I published a paper in 1992 describing a similar example of biphasic antibody catalysis. In a subsequent work we published in *Molecular Immunology*, we hypothesized that the antibody catalyst can exist in alternate states dependent on the antibody concentration. The alternate forms of the catalysts are differentiated by their disulfide-bonding state. I wonder if you are willing to consider alternate states of the catalyst as an explanation for the biphasic concentration dependence you observed.

Schowen: It is entirely possible. We have not done experiments to exclude changes in disulfide-bond status.

Kirby: Your data illustrate the general point very well—that is, catalytic antibodies to a transition-state analog may not be terribly effective, but they are certainly very educational. I wondered whether there would be an interaction of the dialdehyde in the substrate with the amino groups on the surface of the antibody, which may present a problem in catalysis. If so, we are back to your point that when you have more than one serious chemical step in a reaction, the catalytic properties and pH dependencies of the antibody will not be the same at the different steps. The question is: Have you looked at the pH dependence?

Schowen: We've carried out the experiments at two pHs, and similar results are obtained at both. However, the number of variables involved is extraordinary. That is, there is the pH dependence of possible catalytic groups in the antibody and of the substrate. Changes in the overall structure can certainly occur as a function of pH, but the results appear to be independent of such changes because they are generally similar as using native antibody.

Kirby: Can I ask if you got so far as to identify product inhibition?

Schowen: No.

Kohler: Sudhir, I have exactly the same thought as yours. I think the data can be interrupted in terms of an allosteric second site having a coopera-

tive effect on the catalytic activity. We observed a similar cooperative effect with our affinity-labeling technique. By increasing the concentration of an inhibitor, we saw a biphasic labeling curve and increased affinity-labeling. I think the antibody molecule is highly flexible and cooperative.

Schowen: The allosteric effect—in this case—would have to be between antibodies. The variable here was antibody concentration. All other concentrations were constant throughout the experiment.

Paul: Perhaps as you saturate one valency with decreasing concentration of the antibody, the other valency is activated by a cooperation mechanism. In the classical antibody literature, there are data alluding to positive and negative cooperativity because of the two arms of the antibody, in which binding of the antigen to one arm influences the activity of the other arm.

Schowen: Yes.

Tramontano: You may remember, Dick, that a number of years ago we worked on antifluorescein antibodies as catalysts, and we had interesting observations in the realm of concentrations that you're working in. Your reaction system contains not only a substrate-binding site for an extended aromatic substrate, but as in the antifluorescein, there is also an anion-binding site which might enhance the addition of the anionic reagents to the substrates.

Schowen: Yes, that is a nice suggestion.

Green: We have also observed at least one case where an antibody seems to exist in two states. There seems to be some conformational change that we could detect from X-ray structures. But I would exercise caution in invoking conformational states to explain your data. As pointed out by Tony Kirby, the inhibition of your reaction may essentially be due to product inhibition. Secondly, you've linked the hapten to a carrier, and the resultant antibodies might lose the ability to recognize the primary reacting group. We've seen that you might get much better catalysis by linking small transition-state analogs to the carrier. On the other hand, maybe you would get much higher recognition of the nonreacting side-chains.

Schowen: I think it is absolutely right that we've designed a hapten that presents a structure most similar to the desired reaction partner.

Paul: Marian Schiffer has published several papers defining alternate states of light-chain dimers in the crystal form. So the antibody molecule is a flexible molecule, even when you crystallize it out of a supersaturated solution.

Green: In chemically complicated system, it is reasonable to first look at possible differences in the small chemicals, not in the antibody.

Paul: I think that's a bias we bring to the table—chemistry vs biology. I think both factors need to be kept in mind.

Green: I agree completely.

Thomas: In deciding this alternative between transition-state trapping and an allosteric mechanism, wouldn't it be crucial to detect the nonfluorescent product?

Schowen: That is a nice point. Of course, if we can do it properly, it should be examined.