Simple Method for Selecting Catalytic Monoclonal Antibodies That Exhibit Turnover and Specificity†

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ABSTRACT: Monoclonal antibodies were raised against a mono-*p*-nitrophenyl phosphonate ester to elicit catalytic antibodies capable of hydrolyzing the analogous *p*-nitrophenyl ester or carbonate. Potential catalytic antibody producing clones were selected, by use of a competitive inhibition assay, on the basis of their affinity for a "short" transition-state analogue, a truncated hapten which maximizes the relative contribution of the transition-state structural elements to binding. Of 30–40 clones that would have been examined on the basis of hapten binding alone, 7 were selected and 4 of these catalyzed the hydrolysis of the relevant *p*-nitrophenyl ester. This competitive inhibition technique represents a general approach for selecting potential catalytic antibodies and significantly increases the probability of obtaining efficient catalytic monoclonal antibodies. Further study of the catalytic antibodies revealed significant rate enhancement ($k_{cat}/k_{uncat} \sim 10^4$) and substrate specificity for the hydrolysis of the analogous ester and, for three of the antibodies, of the analogous carbonate. The antibodies displayed turnover, an essential feature of enzymes. Evidence that catalysis occurred at the antibody combining sites was provided by the identity of the binding and the catalysis-inhibition specificity patterns.

The idea that an antibody directed to a substance which closely mimics the transition state for a given chemical reaction should catalyze that reaction (Pauling, 1948; Jencks, 1969) has, together with hybridoma technology (Kohler & Milstein, 1975), proved fruitful in producing novel monoclonal antibodies (MABs)[1] which are enzymelike catalysts (Lerner & Benkovic, 1988; Green & Tawfik, 1989; Schultz, 1989). One of the most critical aspects in the overall process is the selection of those few MABs which are catalytic from among all the noncatalytic antibodies that simply bind the hapten (the transition-state analogue).

The long-range goal is to be able to directly screen the antibodies present in each of the hybridoma supernatant solutions for catalysis. However, an analysis that takes into account the amount of antibody present in such supernatants, the detection sensitivity, and the degree of nonspecific reaction (Tawfik, 1990) indicates that (a) a reaction that proceeds at any appreciable rate without antibody catalysis (as a result of noncatalyzed reaction, enzyme impurities in the medium, etc.) cannot be considered for direct screening of catMABs at the low antibody concentrations present in the hybridoma growth media and (b) if no background reaction exists, direct screening for catalysis is only possible for efficient catMABs and when a particularly sensitive as well as selective assay (able to detect small amounts of product in the presence of a 10^4–10^6 excess of unreacted substrate) is used. One must therefore seek other stratagems to screen for potential catalytic clones.

One approach we have taken is to test the secreted antibodies at an early stage in the screening procedure, for binding to substances that represent transition-state structures which are *different* from the immunizing haptens. Transition-state theory ascribes enzyme catalysis to the complementarity of the enzyme to the transition state (TS) and not to the substrate of the reaction to be catalyzed (Pauling, 1948). Thus, only those binding interactions that uniquely exist in the TS and not in the substrate will directly contribute to catalysis (Fersht, 1985); only antibodies that exhibit high affinity for these elements can be expected to display catalytic activity. Ideally, a "short" TSA is a molecule containing all the unique elements of the TS; its structure minimizes elements common to substrate, TS, and product. By use of competitive inhibition binding assays (CIEIA) those clones secreting MABs having sufficient affinity for the "short" TSA can conveniently be identified. Such MABs can be expected to bind the true TS more strongly than the reactant(s) and product(s) and thus to display turnover as well as high catalytic efficiency. One should therefore be able to screen for potential catalytic clones in a more rational way and expend less time and effort to obtain a larger number and variation of catalytic antibodies.

We chose to demonstrate this approach using the convenient hydrolysis of *p*-nitrophenyl ester as a model. MABs were raised against a protein conjugate (**2**) of the *p*-nitrophenyl phosphonate hapten **1** (Figure 1). These antibodies were expected to catalyze the hydrolysis of the corresponding *p*-nitrophenyl ester **8** and carbonate **9**. *p*-Nitrophenyl hydrogen methylphosphonate (**4**) was used as the short TSA in a competitive inhibition assay to select potential catalytic clones for further study. This method was found to be highly selective and readily allowed us to obtain catalytic antibodies that display not only rate enhancement and specificity (both critical enzymelike features) but also *turnover*, the ability of a single enzyme molecule to repetitively catalyze the reaction of many substrate molecules. This essential feature of enzyme-cata-

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[1] Abbreviations: MAB, monoclonal antibody; catMAB, catalytic monoclonal antibody; TS, transition state; TSA, transition-state analogue; CIEIA, competitive inhibition enzyme immunoassay; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; PLE, porcine liver esterase; TBS, tris(hydroxymethyl)aminomethane-buffered saline; PNP, *p*-nitrophenyl; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; MS, mass spectroscopy; RT, room temperature.

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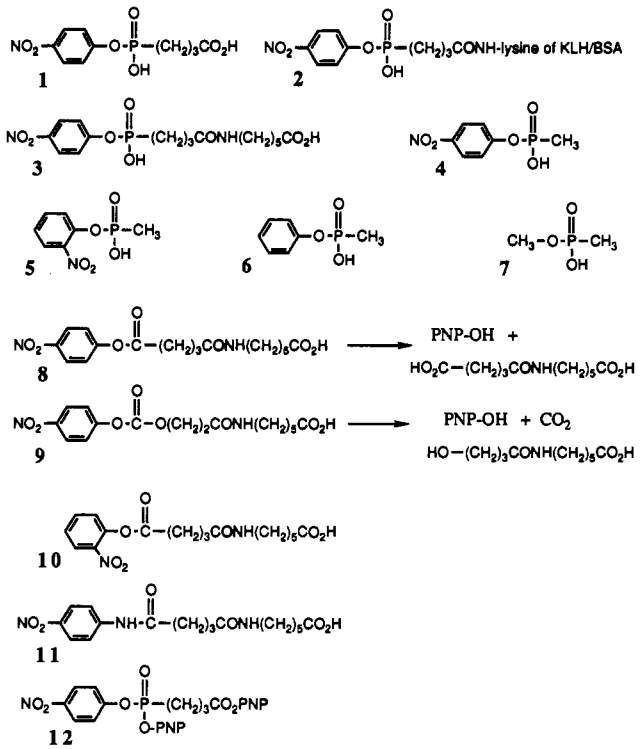

FIGURE 1: Chemical structures of hapten (**1**), conjugates (**2**), substrates (**8**–**11**), and inhibitors (**3**–**7**) used and the hydrolysis reactions (ester **8** and carbonate **9**) catalyzed.

lyzed reactions is rarely achieved by chemical or biochemical enzyme models.

Experimental Procedures

Synthesis of Haptens, Inhibitors, and Substrates. The structures of the haptens, conjugates, substrates, and inhibitors are given in Figure 1. All of the substances synthesized were purified to homogeneity (judged by TLC and NMR) by crystallization, distillation, or column chromatography. Structures were confirmed by NMR and MS data. Satisfactory elemental analyses were obtained for all crystalline substances. The phosphonate hapten and inhibitors and the substrates were prepared as shown in Figure 2. 4-Nitrophenyl (**4**), 2-nitrophenyl (**5**), phenyl (**6**), and methyl (**7**) hydrogen methylphosphonates were prepared by monodealkylation of the analogous methyl esters using lithium bromide. The 2-nitrophenyl ester **10** was prepared analogously to **8**.

Preparation of Conjugates, Immunization, Immunoassays, and Monoclonal Antibody Production. The tris(4-nitrophenyl) ester **12** (21.2 mg), dissolved in 1 mL of THF, was added to a solution of BSA or KLH (40 mg) in 2 mL of 0.1 M potassium borate. The mixture was stirred at 4 °C for 24 h and dialyzed against 2% Na_2CO_3 at 32 °C. The OD of the dialysate was monitored at 400 nm until it corresponded to the release of 2 equiv of 4-nitrophenol. Hapten density was determined by refluxing an aliquot of the protein conjugate in 2 N NaOH and measuring the OD at 400 nm. Determination of the hapten density using back titration with trinitrobenzenesulfonic acid (TNBS) (Habeeb, 1966) gave similar results.

Five BALB/c mice were each immunized (foot pad injection) with the KLH conjugate **2** (25 μg) emulsified in complete Freund's adjuvant. After 17 days, a boost (25 μg in incomplete Freund's adjuvant) was administered, and 3 days later the draining lymph node cells were fused with NSO myeloma cells according to procedures described before (Mirza et al., 1987; Eshhar, 1985). The hybridoma supernatants were screened

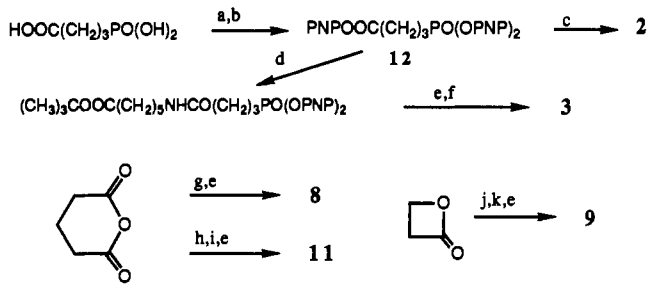

FIGURE 2: Reagents and conditions for the synthesis of haptens and substrates. (a) PCl_5, CCl_4, 45 °C/overnight; (b) NaOPNP, $CHCl_3$, RT/overnight; (c) THF, protein in 0.1 M $Na_2B_4O_7$, 4 °C/24 h; (d) $NH_2(CH_2)_5CO_2C(CH_3)_3$, THF, diisopropylethylamine; (e) TFA, CH_2Cl_2, RT/1 h; (f) Pyr:H_2O, 1:1, Et_3N, RT/24 h; (g) $NH_2(CH_2)_5CO_2C(CH_3)_3$; EtOAc, PNPOH, DCC; (h) $PNPNH_2$, dioxane, reflux 24 h; (i) isobutylchloroformate, Et_3N, −15 °C; $NH_2(CH_2)_5CO_2C(CH_3)_3$, THF, RT/30 min; (j) $NH_2(CH_2)_5CO_2C(CH_3)_3$,$H_2O$, RT/5 h; (k) PNP-chloroformate, THF, Et_3N, RT/overnight.

by an ELISA technique for binding to the hapten–BSA conjugate **2**, using peroxidase-linked, goat anti-mouse antibodies as previously described (Eshhar, 1985). In the competitive inhibition assays (CIEIA) the final inhibitor concentration was 1 mM.

Antibodies were obtained by propagating selected clones as ascites in BALB/c mice; they were purified by protein A (Pharmacia) affinity chromatography (Kronvall & Williams, 1969) and dialyzed against 30 mM TBS, pH 8. Protein concentration was determined by measuring optical density at 280 nm. Homogeneity of antibody was judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), which yielded only heavy and light chains under reducing conditions, using Coomassie blue staining.

Hydrolysis Assays. Reaction rates were determined by measuring the release of 4-nitrophenol (absorption at 405 nm) using a Titertek Twinreader (Flow). Substrates, in acetonitrile or DMSO, 40–100 mM stock solutions, were diluted with TBS and added to the antibody solutions; the final organic solvent concentrations were <1%. Kinetic parameters were determined by using a Lineweaver–Burk analysis; the observed rates were corrected for the uncatalyzed rate of hydrolysis in buffer. The uncatalyzed rate (k_{uncat}) was determined by using initial rates analysis and extrapolated to zero buffer concentration. As a positive control for catalytic hydrolysis of the substrates, porcine liver esterase (PLE; Sigma), diluted 1:500 in TBS, pH 8.0, was used.

Results

The conjugates **2**, using the protein carriers KLH and BSA, were prepared by an unambiguous route (Figure 2) and afforded hapten densities of 20 haptens per KLH (for 100000 MW) and 12 per BSA. A short immunization protocol using lymph node cells for fusion was adopted. The resulting hybridomas were first screened for binding to the BSA conjugate **2**, as well as to BSA alone, and then for inhibition of binding of the conjugate **2**–BSA using the hapten inhibitor **3** (**3** represents the hapten where 6-aminocaproate replaces lysine residue of the protein carrier) and the short TSA **4**. The results are presented in Figure 3.

The catalytic activity of 14 purified antibodies that demonstrated 60–100% inhibition by the short TSA **4** and that bound protein A was determined by measuring the hydrolysis of the *p*-nitrophenyl ester **8** (antibody concentration, 5.7 μM; substrate concentration, 0.4 mM). The rates were measured in the absence and in the presence of the hapten inhibitor **3** (10^{-4} M). Four of these 14 demonstrated hapten-inhibited, substrate-specific catalytic activity; net initial velocities

Table I: Kinetic Parameters of the Catalytic Monoclonal Antibodies

MAB	K_m[a] (mM)	K_i[b] (mM)	ester **8**			carbonate **9**	
			k_{cat} (min^{-1})	no. of turnovers	k_{cat}/k_{uncat}	no. of turnovers	k_{cat}/k_{uncat}
CNJ 157	0.11	3.4	2.39	50	0.97×10^4	6	1.60×10^4
CNJ 123	0.08	57.0	0.79	30	0.32×10^4		
CNJ 2	0.06	ND	1.1	38	0.46×10^4	4	0.80×10^4
CNJ 19	ca. 30[c]	0.99	0.63	28	0.26×10^4	2	0.63×10^4

[a] The K_m values were determined by using **8** as substrate. [b] The K_i values were determined by using **4** as inhibitor. [c] The K_m for CNJ19 was determined at substrate concentrations up to 1 mM; attempts to increase the substrate concentration to the K_m range resulted in inactivation of the MAB. The value given is therefore only an estimate.

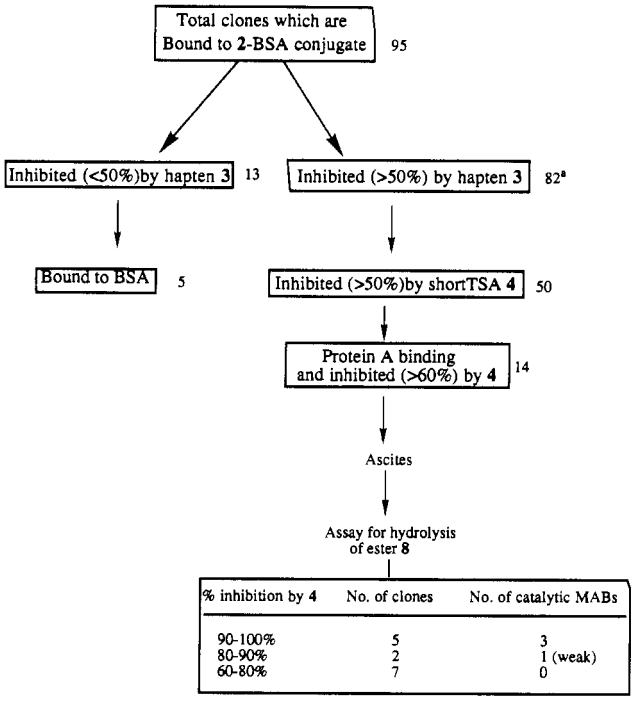

FIGURE 3: Scheme for the selection of catalytic monoclonal antibodies. Footnote *a*: Had all of these 82 clones been screened for protein A binding and propagated as ascites, a total of 30–40 would have been obtained as purified MABs to be assayed for catalysis.

(nonrelevant MABs were used as controls) of 1–13 μmol/(L·min) were measured. The antibody-catalyzed reaction was stoichiometrically inhibited by hapten **3**; 5.25 μM MAB and concentrations of **3** varying from 0.01 to 100 μM were used. A sharp break in catalytic activity is observed when the hapten concentration approaches the antibody combining site concentration: 0.1 μM, 3%; 1 μM, 19%; 5 μM, 63%; 10 μM, 98% ([**3**] and percent inhibition, respectively). Competitive inhibition assays (CIEIA) of the 14 purified antibodies using hapten analogues **6** and **7** gave low inhibition (3 MABs were inhibited 50–80% by **6**; none were inhibited >50% by **7**), and no correlation was found between the degree of inhibition and the catalytic activity.

Table I provides the kinetic parameters for the four catalytic antibodies. MAB CNJ 157 demonstrated the highest catlytic activity for the hydrolysis of both ester **8** (Figure 4a) and carbonate **9** (Figure 4b). The catalytic parameters for the MAB-catalyzed hydrolysis of **8** were obtained from initial velocities by using the substrate (**8**) in a concentration range 0.034–0.4 mM, the MAB concentration being 4.3 μM. No appreciable hydrolysis was detected with the corresponding 4-nitroanilide **11**. Substrate specificity was established by demonstrating the high rate of hydrolysis with the homologous 4-nitrophenyl ester substrate **8** and the absence of catalytic activity with the analogous 2-nitrophenyl ester **10** by using substrate concentrations up to 1 mM (Figure 6). When both the 2- and 4-nitrophenyl esters were exposed to the enzyme

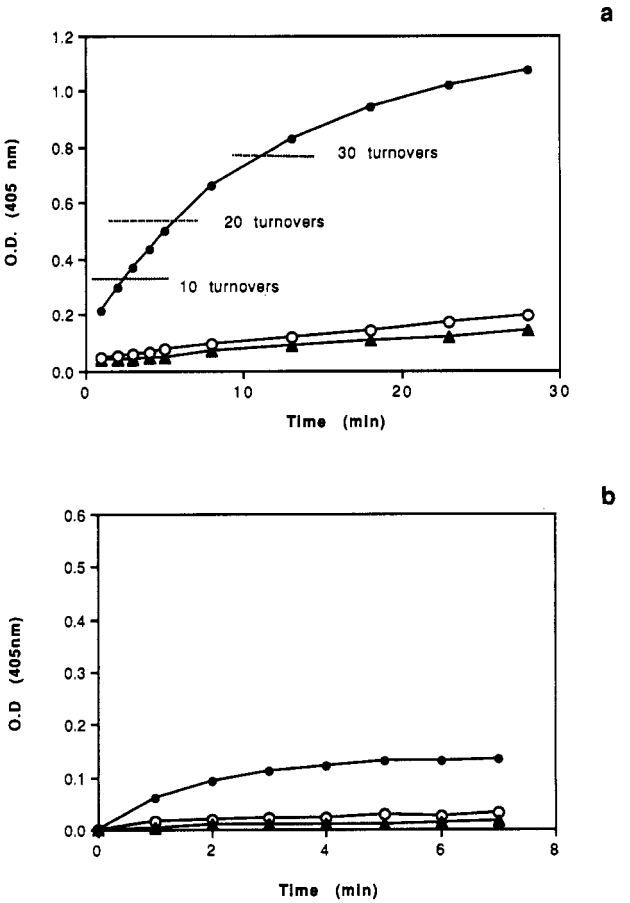

FIGURE 4: Hydrolysis of ester **8** (a) and carbonate **9** (b), each at 0.5 mM concentration, catalyzed by CNJ 157 at a final concentration of 5.7 μM in 30 mM TBS, pH 8.0 (●), in the presence of hapten inhibitor **3**, final concentration 10^{-5} M (○), and in 30 mM TBS buffer alone (▲).

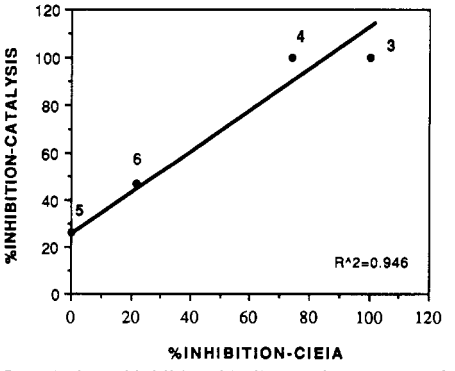

FIGURE 5: Correlation of inhibitor binding and inhibition of catalysis.

PLE, reaction selectivity was greatly decreased and the 2-nitrophenyl ester was hydrolyzed more rapidly (Figure 6). As shown in Table I, all four antibodies that catalyze hydrolysis of the ester **8** and two of the three that hydrolyze the carbonate **9** exhibit turnover. In each reaction there is a drop in catalytic

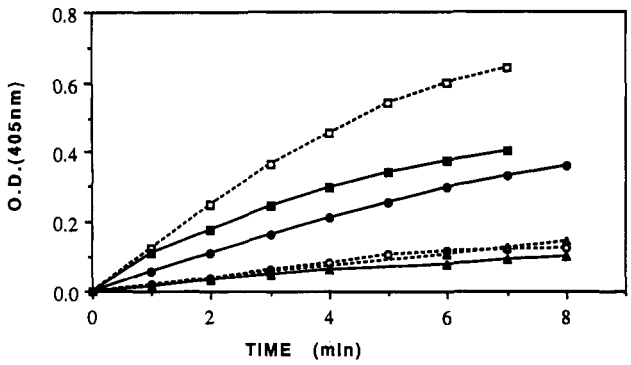

FIGURE 6: Hydrolysis of *o*-nitrophenyl ester **10** (open symbols) and *p*-nitrophenyl ester **8** (solid symbols), at 1 mM concentrations, in the presence of 5.7 μM CNJ 157 (○, ●), PLE (□, ■), and nonrelevant MAB (△, ▲). The OD values shown for *o*-nitrophenol were corrected (×5.3) to represent equimolar concentration values to *p*-nitrophenol.

activity after a given number of turnovers, depending upon the antibody and the substrate used (Table I).

The relative binding affinities of hapten **3**, the short TSA **4**, phenyl methylphosphonate **6**, and 2-nitrophenyl methylphosphonate **5** to the MAB CNJ 157 were determined by CIEIA (MAB concentration, 0.2 μg/mL; inhibitors, 0.9 and 0.09 mM). The relative inhibition of catalysis by each of these substances was determined independently by measuring inhibition of the hydrolysis catalyzed by MAB CNJ 157 (using 3.2 μM antibody concentrations, 1.0 mM concentrations of ester **8**, and 10 and 100 μM concentrations of the inhibitors). The correlation between the inhibition of antibody binding and the antibody-mediated catalysis is demonstrated in Figure 5.

Discussion

The crystalline, easily purified and characterized tris(*p*-nitrophenyl) ester **12** proved to be simple and reliable for the preparation of protein conjugates. The covalent linkage to the carrier protein amino groups proceeds solely through the carboxyl ester (coupling of the protein via the *p*-nitrophenyl phosphonate ester bonds does not occur under these conditions), and then one of the *p*-nitrophenyl phosphonate ester bonds can be easily hydrolyzed in situ, on the protein, releasing *p*-nitrophenolate, which is readily monitored spectroscopically; the phosphonate anion produced renders the remaining *p*-nitrophenyl ester far more resistant to hydrolysis. This approach avoids the free carboxyphosphonic acid (which would probably be prepared from the triester), a substance that is more difficult to isolate and, if conjugated to protein via carbodiimide reagents, would also be susceptible to covalent linkage through the phosphonate (Erlanger, 1980). We find the advantages of this approach to be generally useful and applicable to other phosphonate haptens of the form RP(=O)(OH)X using an analogous di-*p*-nitrophenyl ester [RP(=O)(OPNP)XCO_2PNP].

A short immunization protocol using lymph node cells for fusion was adopted. The affinity and repertoire of specific antibodies usually increase due to somatic hypermutations which start early (7–14 days) after immunization (French et al., 1989). Therefore, fusion of draining lymph node lymphocytes after a short immunization period, 17–20 days and boost, should afford a broad spectrum of antibody binding sites. The larger the repertoire, the greater the probability of obtaining combining sites that have the required features for efficient catalysis. Draining lymph node cells were found to yield a greater repertoire of clones than spleen cells when a short immunization protocol was used (Mirza et al., 1987).

Following the choice of hapten, the hybridoma selection procedure is the most crucial phase in the successful production of catalytic monoclonal antibodies. The selection of clones by inhibition assays with the eliciting hapten is widely used in the conventional selection of MABs to ensure that the selected clones are directed to the hapten and not to other, irrelevant epitopes on the conjugate, such as the linker group. For example, 5 of the 95 binding antibodies to conjugate **2**–BSA were strongly bound by the carrier, BSA, and were therefore not inhibited by the hapten **3**. This method has also been reported for the production of catalytic MABs (Schultz, 1989). However, hapten binding alone is not sufficiently selective (ca. 85% of the antibodies bound to **2**–BSA were >50% inhibited by hapten **3**; see Figure 3) and is not specific for catalytic activity. On the other hand, the selection of potential catalytic clones on the basis of their affinity for the short TSA **4**, using the CIEIA technique, was found to be more selective and specific: of the total number of binding antibodies, the number of MABs to be purified was reduced to seven (or even five, if a higher cutoff in inhibition by **4** is used). As seen from the boxed table of results in Figure 3, all of the catalytic clones exhibited highest affinity for the short TSA (i.e., >90% inhibition of binding at 1 mM inhibitor concentration, using hybridoma supernatants). The percent of inhibition was found to be independent of antibody concentration at the concentrations used: high inhibitor concentrations and very low MAB concentrations. The one catMAB (CNJ 19) found in the group inhibited 80–90% by the short TSA **4** was the weakest catalyst (Table I). Significantly, no catMABs were found among the clones having low to moderate affinity for the short TSA (<80% inhibition of binding under the same conditions).

The selection of potential catalytic clones on the basis of their affinity to a short TSA, i.e., a relatively small molecule which emphasized those structural elements of the hapten that are unique to the transition state and do not exist in the substrate, may therefore be used as a general procedure for the selection of catalytic antibodies. Successful results can be expected only if the short TSA has sufficient affinity to inhibit antibody binding to the hapten conjugate. The degree of inhibition exhibited by phenyl analogue **6** is too low, and therefore it cannot be used to indicate potentially active antibodies. In this case the contribution of the nitro group with its relatively high binding energy (2–5 kcal/mol) (Pressman & Grossberg, 1968) was essential to ensure the minimum required affinity. The design of the short TSA may also include elements of the substrate so that selection is for substrate specificity as well as for potential reactivity. For example, in this case the antibodies were chosen to be selective for the 4-nitrophenyl ester and do not therefore catalyze the hydrolysis of the 2-nitrophenyl ester.

The catMABs displayed in Table I illustrate the varying kinetic parameters present in a group of catalytic antibodies successfully selected by using the short TSA approach. If transition-state stabilization alone were determining the catalytic rate enhancement, and if the short TSA **4** is indeed closely related to the true transition state for the reaction, the observed rate enhancement, k_{cat}/k_{uncat}, would correlate well with K_m/K_i; i.e., catalysis is equivalent to strong binding of transition state and weak binding of the substrate (Kraut, 1988). On this basis CNJ 19 would have been expected to be the most powerful catalyst, rather than the weakest of the four. It therefore appears that other factors in the individual MAB–substrate interactions influence catalysis. The group of catMABs provides material for further detailed study to elucidate the amino acids present in the antibody combining site, the functional groups responsible for catalysis, and the

causes of enhancing or hindered catalysis. This study should also explain the mechanism of subsequent irreversible inhibition of reaction (Table I; see below) and suggest ways of overcoming this.

It is critical to have relatively large amounts of highly purified MABs to detect catalytic activity. In addition, to study the hydrolysis of substances such as the ester **8** or carbonate **9**, which can be catalyzed by many hydrolytic enzymes, it is essential to minimize enzymic impurities in the antibody preparation. Use of protein A or protein G affinity columns is very effective for purifying larger quantities of MABs from ascites, although it is limited to certain antibody subclasses (Kronvall & Williams, 1969). Fourteen of the 50 clones had affinity for the short TSA and bound protein A, and therefore only these MABs were studied further at this stage. (Additional catMABs are probably present in the nonprotein A group. Other methods such as affinity chromatography and HPLC are being investigated to obtain pure MABs from this group.)

As emphasized above, it is always possible that the catalytic activity of an antibody preparation is due to minor quantities of highly active enzyme impurities which are difficult to detect. Methods such as SDS–PAGE, preparation of Fab fragments, evaluation of Michaelis–Menten kinetic parameters, and even affinity chromatography (Schultz, 1989) cannot provide absolute assurance that the catalytic activity is exclusively due to the antibody. Two lines of evidence have been used to ensure that a catalytic reaction is occurring within the antibody combining site: (1) substrate specificity, i.e., the structures of the reactive substrates are analogous to the hapten used to elicit the antibody; (2) effective inhibition of catalytic activity by the hapten used to elicit the antibody. MAB CNJ 157 demonstrates specific reactivity for the 4-nitrophenyl ester **6** and does not show reactivity for the analogous 2-nitrophenyl ester **10** (Figure 6). MAB CNJ 157 activity is also inhibited by the hapten **3** (Figure 4a,b); titration of combining sites with a hapten, as demonstrated in this case (see Results), should be more indicative of antibody catalysis than simply showing inhibition at high hapten concentrations. One might argue, however, that enzyme impurities could also show selectivity for the 4-nitrophenyl substrates and that phosphonates are also known to effectively inhibit many hydrolytic enzymes.

We suggest a more convincing probe: the direct correlation between the binding specificities of the antibody combining site and the, independently determined, binding specificities of the catalytic site. This is readily performed by comparing the relative inhibition of conjugate binding by a panel of inhibitors (presented as percent of inhibition determined by CIEIA) to the relative inhibition of catalytic activity by these same derivatives of the hapten. As shown in Figure 5, the specificity pattern of the antibody (i.e., the relative affinities) toward the hapten **3**, the short TSA **4**, the phenyl analogue of the short TSA **6**, and the 2-nitrophenyl short TSA **5** is exactly as one would expect for antibodies raised against **2** (**3** > **4** ≫ **6** > **5**) and identical for inhibition of binding in ELISA and for inhibition of catalytic activity. This correlation of the specificity pattern, used by us previously (Kohen et al., 1980), provides convincing evidence that catalysis is indeed taking place within the antibody combining site and cannot be due to extraneous, catalytic impurities in the antibody preparation.

It is noteworthy that CNJ 157 (and, to a lesser extent, CNJ 2) catalyzes the hydrolysis of both the analogous ester and carbonate (Table I). With CNJ 19 an enhanced initial rate is observed, but the reaction is stoichiometric—one molecule of carbonate reacting per MAB combining site. On the other hand, CNJ 123 catalyzes only hydrolysis of the ester; no reaction (stoichiometric or otherwise) takes place with the carbonate. By use of a nearly identical phosphonate hapten (Jacobs et al., 1987), an antibody that hydrolyzed the corresponding carbonate (k_{cat}/k_{uncat} = 810) was obtained, while a different phosphonate hapten (Tramontano et al., 1988) gave rise to an antibody that hydrolyzed an analogous ester with an exceptionally high rate enhancement ($k_{cat}/k_{uncat} = 6.25 \times 10^6$) but did not display activity with the corresponding carbonate. The factors that give rise to these seemingly inconsistent results remain to be elucidated, but the possibility of having either selective, molecular-specific, or more general, shape-specific, catalytic activity may find useful applications.

A hallmark of an enzyme-catalyzed reaction is *turnover*, the ability of a single enzyme molecule to catalyze the reaction of many substrate molecules and, in principle, to be reused; this is rarely observed in simple organic models of enzymic reactions. The terms "catalytic" and "catalysis" are often incorrectly used in cases where rate enhancements are observed but turnover is not. Even though kinetic parameters such as EM (effective molarity), k_{cat}/k_{uncat}, K_m, ν_m, and even turnover number ($=k_{cat}$) can be presented, they do *not* indicate that turnover is occurring. Several of the reported antibody-promoted reactions cannot be expected to display turnover due to their design (Kohen et al., 1980; Balan et al., 1988; Pollack & Schultz, 1989), even though they may display high stereoselectivity and/or enhanced initial rates. Some hydrolyses promoted by selectively raised MABs were stoichiometric (Kohen et al., 1980; Tramontano et al., 1986a) or were followed to only a limited number of turnovers (Baldwin & Schultz, 1989). The nature of the phenolic or alcoholic leaving group appears to be an important factor in determining turnover; using a coumarin ester substrate, which incorporates a good leaving group, a catMAB studied by Tramonatano et al. (1986b) displayed a purely stoichiometric reaction. The same MAB displayed repetitive turnovers with the analogous 4-acetamidophenyl ester, which has a poorer leaving group.

CNJ 157 was found to display ~50 turnovers; the other three catMABs also display significant numbers of turnover with substrate **8**. This is especially noteworthy since *p*-nitrophenyl esters are activated substrates and readily acylate proteins and may therefore have been expected to react stoichiometrically with complementarity determining residues of the antibody [as observed with similar active esters (Kohen et al., 1980; Tramontano et al., 1986a)]. Similar turnover was recently reported for an antibody-catalyzed Diels–Alder reaction (Hilvert et al., 1989) which involves substrates that would not be expected to react with the protein. After the number of turnovers for the ester **8** and carbonate **9** indicated in Table I, rate enhancement by the catalytic antibodies becomes negligible. This decrease, which is much higher than expected from the decrease in concentration of substrate, is due to irreversible loss of activity and not due to product inhibition (catalytic as well as hapten binding activity of the antibodies was not recovered even after extensive dialysis at pH 3.2). Most likely, this inactivation results from nucleophilic substitution by amino acid residues in or near the combining site upon the substrate; such reactions occur readily with 4-nitrophenyl esters and carbonates.

Future applications of catMABs will exploit the exciting potential of this technique to produce new catalysts with tailor-made specificity. Clearly, efficient catalysis, i.e., rate enhancement as well as turnover, will be required; yet, the principles that determine the eliciting of efficient catalytic MABs remain to be identified. Selective screening for po-

tential catalytic clones such as that proposed here as well as novel approaches involving recombinant DNA technologies (Ward et al., 1989; Huse et al., 1989) will all contribute to achieving this goal.

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Effects of Cyclic GMP on the Secondary Structure of Cyclic GMP Dependent Protein Kinase and Analysis of the Enzyme's Amino-Terminal Domain by Far-Ultraviolet Circular Dichroism†

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ABSTRACT: Far-UV circular dichroism spectra of bovine lung cyclic GMP dependent protein kinase (G-kinase) show that the enzyme contains α-helical and β-pleated sheet elements. Binding of cyclic GMP changes the spectra in a way consistent with the induction of β-sheet from random coil. Examination of the amino-terminal sequence of G-kinase indicates the presence of a strongly α-helical segment with several features in common with the leucine zipper motif. We propose that this sequence may be the important part of the dimerization domain of the enzyme. A synthetic peptide corresponding to amino acids 1–39 of G-kinase has a strongly α-helical CD spectrum, supporting the predicted secondary structure of this amino-terminal sequence. In contrast to the native enzyme, a structure reduced in α-helix was found when a constitutively active form of G-kinase, which lacks amino acids 1–77, was studied.

It has been shown that cyclic GMP dependent protein kinase (G-kinase)[1] is one of the major receptor proteins for cyclic GMP [Lincoln and Corbin (1983) and Edelman et al. (1987), reviews]. In most tissues, the levels of G-kinase are relatively low compared with those of cyclic AMP dependent protein

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[1] Abbreviations: G-kinase, cyclic GMP dependent protein kinase; A-kinase, cyclic AMP dependent protein kinase; CD, circular dichroism; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; CAP, catabolite activator protein.

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