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Aspects of Antibody-Catalyzed Primary Amide Hydrolysis

RICHARD C. TITMAS,* THELMA S. ANGELES, RENEE SUGASAWARA, NURREDIN AMAN, MICHAEL J. DARSLEY, GARY BLACKBURN, AND MARK T. MARTIN*

IGEN, Inc., 1530 E. Jefferson Street, Rockville, MD 20852

ABSTRACT

Because there are many known C-terminally amidated peptides of biological importance, there is great potential in medicine and organic synthesis for antibodies that catalyze primary amide bond hydrolysis or formation. We characterized a catalytic antibody, 13D11, raised to a phosphinate hapten, that hydrolyzed the primary amide of a dansyl-alkylated derivative of (R)-phenylalaninamide (DNS-(R)F-NH_2). At pH 9.0, 13D11 hydrolyzed DNS-(R)F-NH_2 with a k_{cat} of 1.65×10^{-7} s^{-1} (k_{cat}/k_{uncat} = 132) and a K_m of 432 μM, and was stereospecifically hapten-inhibited (K_i = 14.0 μM). Control experiments indicated that the catalytic activity was not the result of a contaminating protease. In accordance with the hapten being a transition-state analog of base hydrolysis, the rate of DNS-(R)F-NH_2 hydrolysis increased with hydroxide concentration to an optimum pH of 9.5. Above pH 9.5, activity declined rapidly suggesting the antibody was inactivated during the long incubation period. This work demonstrates the feasibility of generating catalytic antibodies to hydrolyze unactivated amide bonds without cofactor assistance.

Index Entries: Amide hydrolysis; catalytic antibody; primary amide; small cell lung cancer.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

An often-cited goal of catalytic antibody research is the generation of antibodies capable of efficient, specific hydrolysis of unactivated amide bonds (*1–4*). Few amide-hydrolyzing antibodies have been produced and, with the exception of the work described here, no hapten-elicited antibodies have been shown to hydrolyze unactivated amides without cofactor assistance. The reasons why amide-hydrolyzing antibodies are generally more difficult to generate than either ester- or carbonate-hydrolyzing antibodies has partly to do with mechanistic complexity (in amide hydrolysis, the antibody must protonate the amino leaving group) and partly with the difficulties associated with screening antibodies for slow turnover rates. The uncatalyzed rate (k_{uncat}) of typical peptide bond hydrolysis (*5*) is much slower than that of typical esters (*6*), carbonates (*7*), or activated amides (*8,9*). Consequently, it is much more difficult to detect antibodies that catalyze amide hydrolysis. If two separate reactions of different uncatalyzed rates (k_{uncat}) are catalyzed by two antibodies with identical rate enhancements (k_{cat}/k_{uncat}), it will be harder to detect the antibody that is slower in absolute terms (k_{cat}). There are two main reasons for this. One is that in the slower reaction, there will be fewer turnovers per unit of time and the assay system must be able to detect extremely low concentrations of product. The other reason is that reactions with slower k_{uncat} values typically require longer periods of antibody-substrate incubation (days or weeks) than do faster reactions (minutes or hours), which may lead to false results because of the possible appearance of byproducts, antibody denaturation (especially at nonneutral pH values), and traces of adventitious enzymes may catalyze the reaction under consideration.

In nature, the most commonly encountered amide bonds are found in peptides and proteins, where the three main types of amides are: in peptide bonds linking individual amino acids, in asparagine and glutamine amino acid side chains, and in the carboxy termini of some peptides. Hydrolysis of peptide bonds and hydrolysis of primary amide bonds (asparagine, glutamine, and C-terminal amides) have similar Gibbs free energies of activation (*10–12*) and, hence, require similar amounts of transition-state stabilization to be catalyzed with the same rate enhancement.

Primary amides are attractive targets for catalytic antibody hydrolysis, and we have chosen to design a hapten that will hydrolyze C-terminal primary amide bonds. Antibodies with such activities have much therapeutic and industrial potential. A number of peptide hormones terminate in biologically active carboxamide groups whose hydrolysis could conceivably be valuable in treatments of various diseases (Table 1) (*13–15*). In addition, as described below, catalytic antibodies could be useful in the industrial synthesis of therapeutically valuable amidated peptides.

Some amidated peptide hormones that are important in disease states are calcitonin (*16,17*), calcitonin gene-related peptide (*17,18*), big gastrin (*19,20*), and bombesin-like peptides (BLP, otherwise known as bombesin-

Table 1
Selected Naturally Occurring Bioactive α-Amidated Peptides
(Adapted from refs. *13* and *14*)[a]

α-Amidated, C-terminal, residue	Peptide
Alanine	b,o CRH; μ-Conotoxin
Cysteine	Crustacean cardioactive peptide; conotoxins G1, M1, S1
Aspartic acid	Deltorphin
Glutamic acid	Joining peptide; melittin (bee)
Phenylalanine	FMRF-NH_2; gastrin; cholecystokinin; CGRP; γ_1MSH
Glycine	Oxytocin; vasopressin; GnRH; pancreastatin; leucokinin I, II; *Manduca* adipokinetic hormone; lutropin releasing hormone
Histidine	Apamin (bee); scorpion toxin II
Isoleucine	h, r CRH; PHI; *Manduca* diuretic hormone; rat neuropeptide El (melanin concentrating hormone)
Lysine	Cecropin A; PACAP38 (pituitary adenylate cyclase activating peptide 38); conotoxin G1A; egg laying hormone (snail); tertiapin (bee)
Leucine	b, h GHRH; β-amidorphin; mastoparan; cecropin B; buccalin; myomodulin; PACAP27; proglucagon (111-123)
Methionine	Substance P; Substance K; PHM; gastrin-releasing peptide; neurokinin A, B; neuromedin B, C; bombesin (frog)
Asparagine	VIP (mammalian); neuromedin U; corazonin; mast cell degranulating peptide
Proline	Calcitonin; Thyrotropin-releasing hormone
Glutamine	Melittin; levitide
Arginine	Preproglucagon (111–123); short insectotoxin (scorpion)
Serine	Frog granuliberin-R; cecropin B (moth); dermorphin (frog)
Threonine	Rat galanin; avian VIP; locust adipokinetic hormone
Valine	αMSH; r, p, h secretin; metorphamide/adrenorphin; urotensin I (fish)
Tryptophan	Cockroach myoactive peptide; sea anemone peptide; crustacean erythrophore concentrating peptide
Tyrosine	Neuropeptide Y; PYY; PP; ω-conotoxin; amylin

[a]Most of the peptides listed are commercially available and can be found in the appropriate company catalogs.

related peptides [BRP] or gastrin-releasing peptides [GRP] (*21,22*). Elevated calcitonin levels have been associated with several disease states, including medullary thyroid carcinoma, C-cell hyperplasia, chronic renal failure, pancreatitis, mineral and bone disorders, and hyperthyroidism (*16*). Elevated gastrin is associated with gastrinomas, and BLP with small-

Table 2
Hormones Produced and Receptors Expressed by Human SCLC (*21*)

Hormone name	Produced by SCLC	Receptor expressed by SCLC
GRP	+ (Met-NH_2 terminus)	+
IGF-1	+ (Pro-NH_2 terminus)	+
Transferin	+	+
Calcitonin	+	+
AVP	+ (Gly-NH_2 terminus)	+
Opioid Peptides	+	+
Neurotensin	+	+
Glucagon	+	?
Substance P	+ (Met-NH_2 terminus)	?
Somatostatin	+	?
CCK	− (Phe-NH_2 terminus)	+
TGFα	+	+
VIP	− (Asn-NH_2 terminus)	+
ACTH	+	?
GGAP	+	+
ANF	+	?
Galanin	? (Ala-NH_2 terminus [porcine], Thr-NH_2 terminus [rat], unknown for human)	+
Bradykinin	?	+
Interferon-γ	?	+
GM-CSF	?	+
Neurokinin A	+ (Met-NH_2 terminus)	+
Neurokinin B	+ (Met-NH_2 terminus)	+

cell lung cancer (SCLC) (*20*). Experimental treatments are under way to neutralize the effects of these hormones in disease states using monoclonal antibodies (anti-BLP) (*23*), neutral endopeptidase 24.11 (BLP hydrolysis) (*24*), and synthetic antagonists (BLP) (*25–28*). Another therapeutic target could be antibodies that hydrolyze the asparagine side chain. Administration of bacterial asparaginase, which hydrolyzes the side chain carboxamide of free asparagine, has been shown to be an effective antileukemia agent (*29*).

SCLC may be a particularly attractive therapeutic target for primary amide-hydrolyzing antibodies. A great majority of patients with SCLC die despite current conventional chemotherapeutic regimens (*21*). SCLC has been shown to have "neuroendocrine" properties associated with it (*21,30,31*). As shown in Table 2, SCLC cells not only produce the peptide hormones, but also express receptors for these hormones (*21*). Thus, SCLC tumors probably accelerate their own growth by an autocrine

mechanism (*30*). A catalytic antibody capable of deamidating one or more of these peptides, or preferably a battery of different catalytic antibodies each capable of deamidating a different peptide hormone expressed by SCLC cells, might be therapeutically effective. Such catalytic antibodies might be especially effective if directed to a tumor site by being conjugated to a tumor antigen-binding monoclonal antibody (*32*).

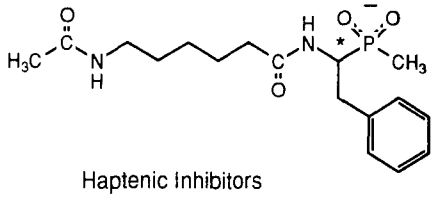
In industrial synthesis, there is a need for new methods for producing C-terminally amidated peptides (*33–37*). Peptides such as growth-hormone-releasing factor (*35*), calcitonin (*35,37*), calcitonin gene-related peptide (*17*), and neurokinin A (*38,39*), may have therapeutic value, and tailor-made catalytic synthetic methods would be extremely useful. Methods currently being explored include the use of recombinant rat α-amidating enzyme (*35,36*) and yeast carboxypeptidase Y (*37*). Catalytic antibodies capable of amide hydrolysis could be used for preparation of C-terminal peptide amides. Peptide hydrolysis is thermodynamically a reversible reaction and could be made to go in the opposite (synthetic) direction by using anhydrous organic or organic/aqueous solvents (*40–42*). Indeed, a catalytic antibody has been shown to function catalytically in organic solvents (*43*). Amide synthesis using catalytic antibodies in a transesterification reaction has also been reported (*44,45*).

In this article, we describe the characterization of the first monoclonal antibody capable of hydrolyzing an unactivated amide bond without cofactor assistance. Extensive screening of 68 antiphosphinate (Fig. 1) monoclonal antibodies against four primary amide (Fig. 1) and four methyl ester (Fig. 1) substrates resulted in the identification of one antibody that catalyzed the hydrolysis of the dansyl-alkylated derivative of (R)-phenylalaninamide (DNS-(R)F-NH_2) (Fig. 1).

MATERIALS AND METHODS

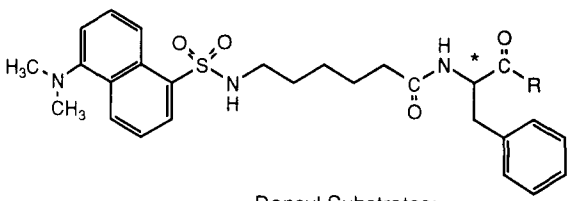
Hapten **1** was synthesized as an enantiomeric mixture as outlined in Scheme 1. Preparation of [1(R/S)-1-amino-2-phenylethyl] phosphonous acid **2** was carried out as previously described (*46*). [1(R/S)-N-CBZ-1-amino-2-phenylethyl] phosphonous acid **3** was prepared from **2** by reaction with benzyl chloroformate. *O*-methylation of **3** with trimethylsilyldiazomethane and methanol followed by P-methylation using sodium hydride and methyl iodide gave compound **4.** Lithium hydroxide hydrolysis of **4** afforded **5.** Finally, catalytic hydrogenation of **5** followed by acylation with the symmetrical anhydride of 6-maleimidocaproic acid **6** gave **1.**

Acetylated versions of the hapten were prepared as individual enantiomers for inhibition studies (Fig. 1). Compound **3** was resolved by recrystallization of its (−)(*S*)-α-methylbenzylamine salt (*46*). Following acidification, the pure enantiomer was converted to the acetylated haptenic



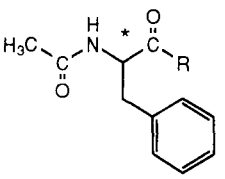
Haptenic Inhibitors

* = (R) and (S) prepared separately



Dansyl Substrates:

R = -NH_2 or -OCH_3
* = D- or L-



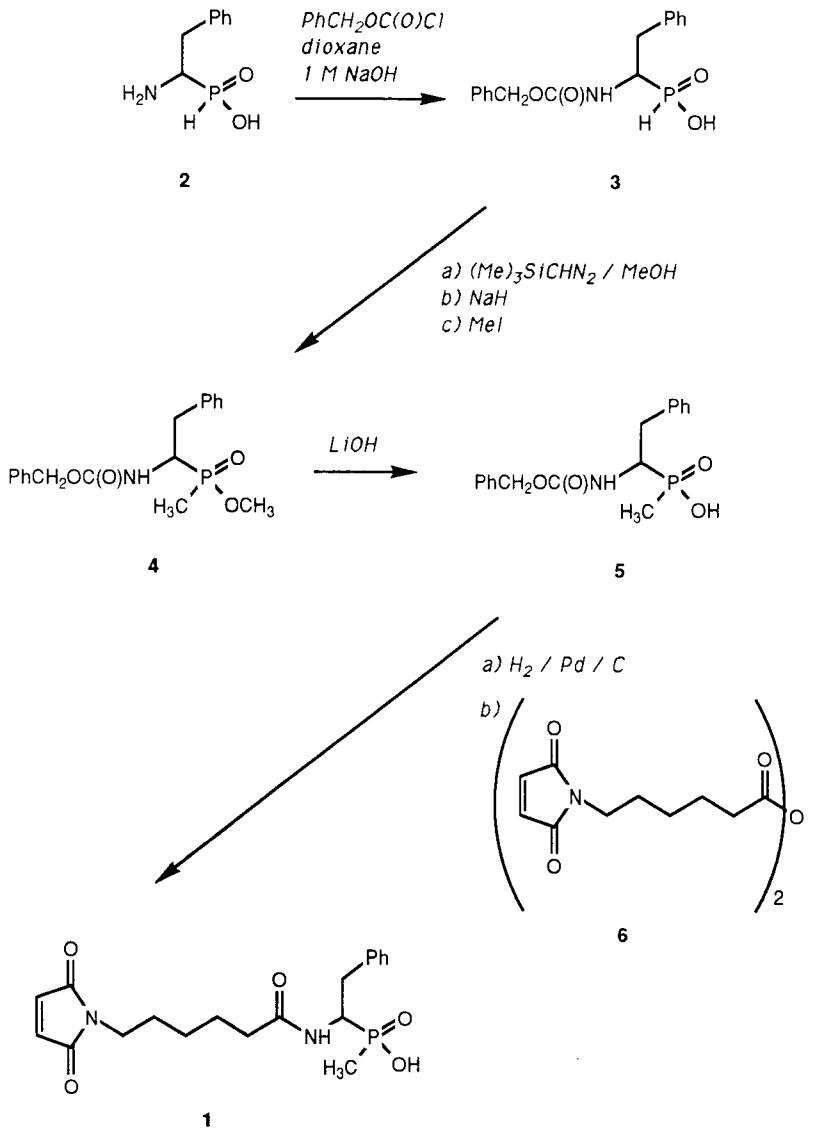
Acetyl Substrates:

R = -NH_2 or -OCH_3
* = D- or L-

Fig. 1. Structures of the acetylated hapten, dansyl-alkylated substrates, and acetylated substrates.

compound as in Scheme 1, except the symmetrical anhydride of 6-*N*-acetylamino-*n*-caproic acid was used instead of 6-maleimidocaproic acid. The other enantiomer was prepared in the same way except that the recrystallation was done with (+)(R)-α-methylbenzylamine. Because of atomic number differences of the chiral center substituents, the (S)-hapten structurally corresponds to the (R)-substrate.

BALB/c mice were immunized with hapten 1-keyhole limpet hemocyanin (KLH) conjugates. The hapten was coupled via the maleimide group to iminothiolane-modified KLH and bovine serum albumin (BSA). Splenocytes of mice immunized with the KLH conjugate were fused with SP2/0 myelomas to produce hydridoma cell lines. Sixty-eight monoclonal



Scheme 1. Preparation of hapten 1.

antibodies were selected, on the basis of binding affinity to hapten-BSA, for further screening for catalytic activity. Quantitation of antibody binding to the hapten-BSA conjugate was performed by standard ELISA protocols.

Antibodies were produced in large scale in ascites fluids and were purified by standard procedures. These involved lipid extraction, ammonium sulfate precipitation, and column chromatography on protein A, DEAE, Mono Q, and finally Alkyl Superose (Pharmacia LKB, Piscataway, NJ). Purified antibody was >99% homogeneous as determined by SDS-polyacrylamide electrophoresis.

The Fab' fragment of purified 13D11 was produced by insoluble pepsin-agarose digestion and stepwise reduction and alkylation (47). Fab' was purified by gel filtration on a Superose 12 HR10/30 column (Pharmacia LKB).

Antibodies in hybridoma culture supernatants (typically 1–10 μg/mL) were screened for catalytic activity. Antibodies were immobilized on anti-mouse immunoglobulin affinity gel (Calbiochem, LaJolla, CA), transferred to Millititer GV 96-well filtration plates (Millipore, Bedford, MA), and washed thoroughly by aspiration. Appropriately buffered substrate solutions were added, and reactions were allowed to proceed in the wells. The potential substrates (four esters and four amides; Fig. 1) were incubated, not individually, but as (R)/(S) pairs, in the plates at pH 5.0, 7.0, and 9.0 at room temperature for various times (3–24 h) depending on the conditions that affected background hydrolysis (pH and type of scissile bond). Assay buffers contained 140 m*M* NaCl, 0.01% NaN_3, and either 25 m*M* Mes (pH 5.0), 25 m*M* HEPES (pH 7.0), or 25 m*M* Tris (pH 9.0). After incubation, the solutions were withdrawn from the wells, and product was detected by fluorescence using TLC (dansyl substrates) or absorbance using HPLC (acetylated substrates). Because the immobilized antibodies remained in the wells, they could be washed and reassayed several times with different substrate and buffer combinations. Antibodies that were identified as possibly catalytic in the hybridoma supernatant screen were purified from ascites fluids and rescreened for catalysis.

Substrate turnover was generally determined at pH 9.0 and 37.0C in MTEN buffer (50 m*M* Mes, 25 m*M* Tris, 25 m*M* ethanolamine, 100 m*M* NaCl) (*48*) containing 0.01% NaN_3. Product formation was quantitated by reversed-phase HPLC. Reported kinetic parameters have been corrected for buffer effects. Substrate concentrations and velocities (typically after 6 d) were fitted to the Michaelis-Menten equation (*49*):

$$V/[E_t] = (k_{cat}[S])/([S] + [E_t] + K_m) \quad (1)$$

In most assays, the antibody-combining site concentration $[E_t]$ was 13 μ*M*.

The inhibition constant (K_i) for hapten was determined fitting the effect of hapten concentration on substrate turnover velocity:

$$V_i/V_o = (2[Ab] - [I] - K_i' + \{([I] + K_i' - 2[Ab])^2 + 4K_i'2[Ab]\}^{0.5})/4[Ab] \quad (2)$$

where V_i and V_o are the inhibited and uninhibited velocities, respectively, 2[Ab] is the antibody-combining site concentration, [I] is hapten concentration, and $K_i' = K_i/(I + [S]/K_m)$ (*50*).

RESULTS AND DISCUSSION

Dialkylphosphinate hapten **1,** designed as a transition-state analog (TSA) of phenylalaninamide hydrolysis, was prepared as an enantiomeric mixture. Murine immunization of the KLH-conjugated hapten resulted in a large number of monoclonal antibody-producing hybridoma cell lines.

To enable the screening of a large number of monoclonal antibodies for catalysis with various ester and amide substrates at three pH values, we used a hybridoma culture supernatant screening protocol in which the low concentrations of antibody present in supernatants are concentrated and purified on affinity gel for screening for catalysis. Because the antibodies remained immobilized, the gel could be washed and incubated with multiple substrates and pH values. In the screen, 68 antibodies were tested at pH 5.0, 7.0, and 9.0 with four primary amide and four methyl ester substrates (Fig. 1).

The hybridoma supernatant screening procedure yielded a few potentially catalytic antibodies that were produced in larger quantities in ascites fluids. Rescreening of the highly purified antibodies resulted in the identification of one antibody, 13D11, that hydrolyzed the dansylated (R)-amide (DNS-(R)F-NH_2) at pH 9.0. The corresponding methyl ester substrate (DNS-(R)F-OMe) was not hydrolyzed by 13D11 at a rate above (the significantly faster) background. The effect of substrate concentration on the rate of hydrolysis showed 13D11 to have a $k_{cat} = 1.65\ (\pm 0.24) \times 10^{-7}$ s^{-1} and a $K_m = 432\ (\pm 132)\ \mu M$.

A number of observations and control experiments showed that the hydrolytic activity associated with 13D11 was not the result of a contaminating amidolytic enzyme:

1. Activity was observed only toward the "unnatural" (R) isomer, not toward the "natural" (S) phenylalaninamide substrate;
2. Only one of the acetylated hapten enantiomers (Fig. 1), presumably the (S)- isomer, which spatially corresponds to the (R)-amide substrate, inhibited 13D11 ($K_i = 14.0\ (\pm 1.6)\ \mu M$);
3. The 13D11 Fab' fragment was found to have the same specific activity as the whole IgG molecule;
4. Monoclonal antibody purified from two different batches of ascites was found to be equally active;
5. Nine common inhibitors of known proteases were tested for their effect on the antibody-catalyzed reaction. Amide-hydrolyzing activity was virtually unaffected by PMSF, EDTA, aprotinin, antipain, leupeptin, and phosphoramidon, and only partial inhibition occurred with the zinc aminopeptidase inhibitor bestatin, the aspartic protease inhibitor pepstatin, and the irreversible inhibitor of cysteine proteases E-64. Since concentrations of these inhibitors used were adequate to inhibit completely their known target enzymes, the partial inhibition is presumably the result of nonspecific antibody binding; and
6. Finally, the acetylated (R)- amide was not accepted as a substrate, suggesting that the five-carbon alkyl linker of the substrate, which was present during immunization, recognized by the antibody.

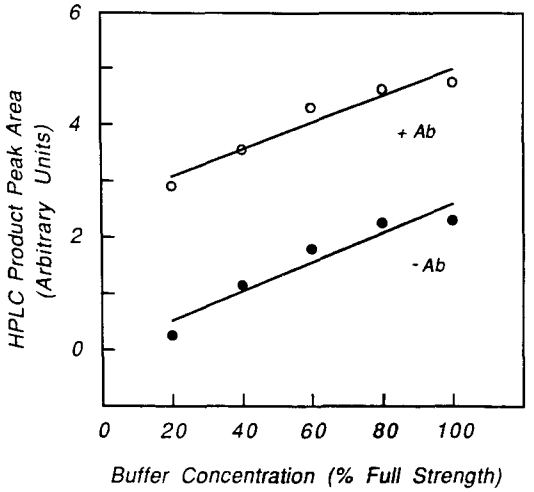


Fig. 2. Hydrolysis of DNS-(R)F-NH_2 (100 μM) in the absence (closed circles) and presence (open circles) of 13 μM 13D11. The reactions were carried out in various dilutions of MTEN buffer (50 mM Mes, 25 mM Tris, 25 mM ethanolamine, 100 mM NaCl) containing 0.01% NaN_3 for 7.1 d at pH 9.0 and 37°C.

Slow, buffer-catalyzed hydrolysis of DNS-(R)F-NH_2 was observed under the usual assay conditions in the absence of antibody. Incubation of 100 μM DNS-(R)F-NH_2 in a range of assay buffer dilutions showed that background hydrolysis linearly depends on MTEN buffer concentration and nearly disappeared at zero buffer concentration (Fig. 2, closed circles), showing that uncatalyzed hydrolysis is primarily buffer-catalyzed. When 13 μM 13D11 and 100 μM DNS-(R)F-NH_2 were allowed to react in a range of buffer dilutions, the rate of hydrolysis vs buffer concentration gave a line parallel to that in the absence of antibody (Fig. 2, open circles). This shows that buffer is not involved in the mechanism of 13D11 hydrolysis of DNS-(R)F-NH_2 and has no effect on the antibody-catalyzed reaction.

An experiment similar to that shown in Fig. 2 (closed circles, buffer dependence in the absence of antibody) was performed to determine the uncatalyzed rate of DNS-(R)F-NH_2 hydrolysis at zero buffer concentration. The experiment differed in that more points were measured (7) and a longer incubation time (9.0 d) was used to get a more accurate ordinate intercept. The resultant plot (not shown) gave an uncatalyzed rate of primary amide hydrolysis of 1.25 ($\pm$0.23) $\times$ 10^{-9} s^{-1} at pH 9.0 and 37.0°C. Thus, 13D11 catalyzes amide hydrolysis with a rate enhancement (k_{cat}/k_{uncat}) of 132 at pH 9.0.

The effect of pH on catalysis was studied. The pH rate profile increases with hydroxide concentration to an optimum of pH 9.5, above which the rate declines sharply (data not shown). We suspect that the drop-off of activity at pH values above the optimum pH of 9.5 was because

of inactivation of the antibody over the long incubation time used (13.0 d). The hapten was designed as a transition-state analog of hydroxide hydrolysis of phenylalaninamide, so it is not surprising that the rate has an alkaline pH maximum. The rate of catalysis increased 7.7-fold between the normal assay pH of 9.0 to the optimum pH of 9.5, whereas the hydroxide concentration increased 3.2-fold over the same pH range. This indicates that in catalysis there is a somewhat greater than first-order dependence on hydroxide concentration. Reported studies of the pH dependence of uncatalyzed base hydrolysis of amides show a similar dependence on hydroxide concentration (*51* and from data presented in *5*).

Primary and secondary amides have similar stability to hydrolysis, although secondary amides may be somewhat more stable because of steric hindrance around the scissile bond (*12*). Although the uncatalyzed rates of primary and secondary amide bond hydrolysis are similar, it may be more difficult to raise antibodies to catalyze one or the other. Because transition-state analogs of primary amide hydrolysis are presented on the tip of the hapten, they are more exposed than analogs of peptide bond analogs. As a result, elicited antibodies may tend to bind to the former with higher affinity. Consequently, such antibodies may generally have higher affinity toward the hydrolytic transition state. A contrary possibility is that since primary amides have a smaller leaving group (ammonia) than do substituted amides (e.g., peptides), less ground state destabilization (*52*) of the amino leaving group can be used to accelerate catalysis. Other features of antibody-catalyzed amide hydrolysis will result from pecularities of individual haptens unrelated to the number of substituents on the amide nitrogen.

Finally, it should be pointed out that it requires no more transition-state binding energy for an antibody to hydrolyze a peptide bond than, for example, an ester. It is a common misconception that it is always easier to generate a catalytic antibody to catalyze a reaction that has a fast uncatalyzed rate (a kinetically labile reaction) than a reaction that has a slow uncatalyzed rate (a kinetically inert reaction). In fact, the likelihood of generating an antibody to catalyze a reaction with a given rate enhancement (k_{cat}/k_{uncat}) is independent of the magnitude of the uncatalyzed rate of the reaction. For example, an antibody that binds the transition state of a stable peptide bond with a given binding energy will accelerate hydrolysis of that bond by the same degree as an antibody that binds an ester transition state with the same binding energy. This can be theoretically demonstrated as follows. The relationship between a first-order uncatalyzed rate (k_{uncat}) and the Gibbs free energy of activation ($\Delta G‡$) is;

$$k_{uncat} = (kT / h) \exp^{-\Delta G‡/RT} \quad (3)$$

where T is temperature (Kelvin), k is the Boltzmann constant, h is the Planck constant, and R is the gas constant. The catalyzed rate of the same reaction (k_{cat}) can be similarly expressed as:

$$k_{cat} = (kT / h) \exp^{-(\Delta G‡ - \Delta \Delta G‡)/RT} \quad (4)$$

where $\Delta\Delta G‡$ is the change in the Gibbs free energy of the transition state brought about by antibody binding. Thus, the rate acceleration (k_{cat}/k_{uncat}) brought about by a catalytic antibody is:

$$(k_{cat} / k_{uncat}) = [(kT / h) \exp^{-(\Delta G‡ - \Delta \Delta G‡)/RT} / (kT / h) \exp^{-\Delta G‡/RT}] \quad (5)$$

which can be reduced to:

$$(k_{cat} / k_{uncat}) = \exp^{\Delta\Delta G‡/RT} \quad (6)$$

From Eq. (6) it can be seen that the rate acceleration brought about by a catalytic antibody is independent of the magnitude of the transition-state energy barrier ($\Delta G‡$) and, hence, the rate of the uncatalyzed reaction. Why are there many more esterolytic antibodies reported in the literature than amide-hydrolyzing antibodies? Some of the reasons relate to ease of monitoring a rapid rate (monitored over minutes or hours rather than days) and the potential problems associated with traces of adventitious enzymes that may become apparent in long incubations. Another reason is that the mechanism of ester hydrolysis is simpler than that of amide hydrolysis, and hence, transition-state analogs are more easily designed.

SUMMARY

Many peptide hormones, especially neuropeptides, have C-terminal primary amide moieties that are required for biological activity. There are many potential applications in therapy and synthesis for catalytic antibodies that hydrolyze and/or form primary amides. A dialkylphosphinate transition-state analog of phenylalaninamide hydrolysis has been prepared and used as a hapten in murine immunization. Screening of elicited antihapten monoclonal antibodies resulted in the identification of a catalytic antibody, designated 13D11, that stereospecifically catalyzed hydrolysis of a derivative of phenylalaninamide. Kinetic characterization of 13D11 showed that 13D11 has a $k_{cat} = 1.65 \times 10^{-7}\ s^{-1}$ ($k_{cat}/k_{uncat} = 132$) and a $K_m = 432\ \mu M$, and is hapten-inhibited with a K_i of $14.0\ \mu M$. This work demonstrates the feasibility of antibody-catalyzed unactivated amide hydrolysis without using cofactors.

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DISCUSSION

This paper was presented by Richard Williams.

Zouali: You have shown us that the hydrolytic activity is present in the Fab fragments. We have heard that the isolated light chains of some antibodies have some catalytic activity. Have you looked at the light or heavy chains of your antibody for activity?

Williams: No, but this will be a reasonable extension of the work that I have described. We are now cloning the antibody to permit these and other types of experiments.

Hansen: You may do yourself a disservice by saying that the terminal carboxyamide hydrolysis is as difficult a reaction as peptide bond hydrolysis. I think the amide hydrolysis will be even more difficult than peptide bond hydrolysis because of the higher pK_a of the ammonia vs the amino acid.

Williams: If you use the 7-yr half-life, we have a rate acceleration of approx 110-fold. However, if you accept Dave Hansen's estimate of 500 yr, it is in the range of 10,000-fold.

Paul: Did you directly screen for catalysis by the antibodies without going through the binding step?

Williams: No, the binding step was done first.

Paul: How many catalyzers emerged from how many binders?

Williams: There were over 4000 binders. Sixty-eight were tested in detail, and out of these, one catalyzer was identified.

Tramontano: Your work shows antibodies to phosphonates or phosphonamidates can bind and hydrolyze amides. The general assumption has been that an antiphosphonate or antiphosphonamidate antibody will not, in fact, bind an amide. Why is a phosphonate not a good analog of a strained configuration of an amide bond?

Hansen: I think that amides will, in fact, bind to these antibodies, but this type of antibodies has not previously shown an amidase activity. If I were to speculate, a lot of these antibodies probably do catalyze amide- and peptide-bond hydrolysis. The problem may be that it may take special assays to detect small levels of catalysis. A half-life of 500 yr for the uncatalyzed reaction corresponds to about 0.2% hydrolysis in 90 d. Even if you have 100-fold rate acceleration, there would only be 0.2% hydrolysis in a day. You have to be very careful to find that.

Green: Your results of antibody-catalyzed hydrolysis of a stable bond give a lot of encouragement for future work. Al Tramontano's comment raises a problem in understanding the mechanism. In the case of ester hydrolysis, you would expect that you simply have to achieve the transient state from which the alkoxy group can fly out as a stable

group. However, in the transient state of a hydroxyl attacking an amide bond, protonation of the amine is required, because H^- or an H_2^- cannot serve as a leaving group. A subsequent protonation step is needed for catalysis. Your hapten design does not allow this. Quite the opposite—you have a stable hydrophobic methyl group that is not expected to mimic a protonated amino group. For this reason, one is not generally surprised that immunization with phosphonates does not generate amide-hydrolyzing antibodies. In a sense, however, this is a numbers game, and if you look hard enough, you might find antibodies with unusual reactivities.

Tramontano: I am not concerned with the catalysis as much as the binding step alone. Do you, for instance, see binding of amides to the antibodies raised against phosphonates?

Green: Well, yes. We certainly find amide binding, but from IGEN's work, maybe we will learn that the methyl can somehow mimic the leaving ammonium group, in the same way that tertiary butyl groups have been found in the case of acetylcholinesterase to mimic the quaternary ammonium group.