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Antibody catalysis

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Abstract - It has recently become possible to exploit the diversity and specificity of the humeral immune system to create immunoglobulins capable of catalysis. Generation and characterization of catalytic antibodies for three concerted chemical reactions in which carbon-carbon bonds are made or broken are briefly reviewed.

The production of monoclonal antibodies with tailored catalytic properties is a promising new strategy for creating novel enzyme-like molecules able to speed chemical reactions (ref. 1). In this approach, first suggested by Jencks in 1969 (ref. 2), a stable analog of a reaction's rate limiting transition state is used as a hapten to elicit an immune response. If the design of the transition state analog is a good one, some fraction of the induced antibodies will possess the desired catalytic activity and can be identified through screening. Early attempts to generate efficient catalytic antibodies were largely unsuccessful (ref. 3), but progress in this field has been rapid since 1986 due to the availability of improved transition state mimics and monoclonal antibodies. A wide range of chemical reactions — from hydrolytic transformations to pericyclic processes — has now proved amenable to antibody catalysis (ref. 1).

Catalytic antibodies, like enzymes, exhibit rate accelerations, substrate specificity, and regio- and stereoselectivity (ref. 1). Significantly, the specificity and selectivity of these systems correlates precisely with the structure of the antigen used to elicit the immune response. These properties can therefore be set by the researcher through synthesis of an appropriate transition-state analog. The ability to design at will highly efficient catalytic antibodies for any given reaction is likely to have far reaching consequences in both medicine and industry. Tailored antibody catalysts are potentially important as tools for studying how natural enzymes work, and as practical agents for accelerating reactions for which natural enzymes are unsuitable or unavailable. Because to their specificity and biocompatibility, they are also attractive candidates for applications *in vivo*.

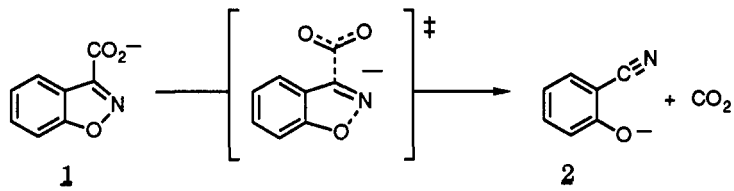
Concerted reactions in which carbon-carbon bonds are formed or broken are especially attractive targets for antibody catalysis, because they generally do not require the participation of catalytic general acids, general bases and nucleophiles which are difficult to introduce into the antibody combining site during immunization. For this reason, and as outlined below, we have selected several reactions for investigation that do not require chemical catalysis: a decarboxylation (ref. 4), a bimolecular Diels-Alder cycloaddition (ref. 5), and a Claisen rearrangement (ref. 6). These three transformations are of enormous importance in both biology and chemistry (including organic synthesis). In addition to their intrinsic chemical interest, concerted transformations of this sort have the potential to illuminate some of the elementary mechanisms by which proteins accelerate reactions. Specifically, they allow us to assess the roles of desolvation, proximity and strain in catalysis. A detailed understanding of these fundamental principles is essential if we hope to optimize the design of individual transition state analogs, improve the properties of our first-generation catalysts through mutagenesis, or expand the repertoire of reactions accelerated by antibodies.

DECARBOXYLATIONS

The rates of many reactions, including S_N2 nucleophilic substitutions and E2 eliminations, are highly sensitive to the choice of solvent. Enzymic reactions are similarly controlled by their protein microenvironment, although the precise contribution of solvation effects to the overall rate acceleration has been difficult to estimate. To study the role of medium effects in protein catalysis, we have investigated the ability of antibodies to accelerate the decarboxylation of 3-carboxybenzisoxazoles (ref. 4).

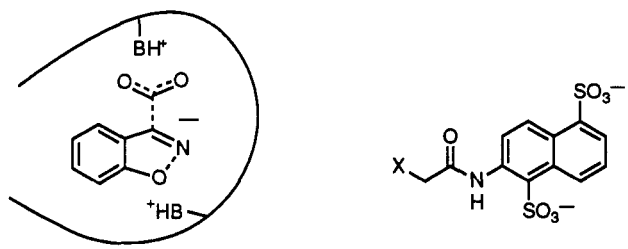
Conversion of 3-carboxybenzisoxazoles 1 into cyanophenols 2 (Scheme 1) is a concerted, intermediateless process that is particularly sensitive to microenvironment. As the reaction solvent is changed from polar protic to dipolar aprotic, the rate of decarboxylation increases up to 10^8-fold (ref. 7). This dramatic effect has been ascribed to substrate destabilization through desolvation and concomitant stabilization of the charge-delocalized transition state through dispersive interactions (ref. 7). The low dielectric environment of an immunoglobulin combining site is more like an aprotic dipolar solvent than it is like water. Given this, decomposition of antibody-bound 3-carboxybenzisoxazole might be significantly accelerated over the simple aqueous reaction. We anticipated that specific binding interactions could be utilized to extract the charged substrate from aqueous buffer and force it into the destabilizing environment of an immunoglobulin binding pocket.

Scheme 1



1 2

We employed the highly immunogenic napthalene disulfonate derivative 3 to elicit the desired antibody catalysts. Model studies by Kemp and coworkers had previously shown that hydrogen bonding to the substrate carboxylate strongly inhibits decarboxylation, whereas an adjacent positive charge is not deleterious (ref. 7). To elicit a hydrophobic environment able to exclude water, the apolar naphthalene framework was therefore utilized. Sulfonate groups were included to induce complementary cationic residues within the combining site to promote binding of the anionic carboxylate of the substrate and to stabilize the incipient phenolate of the product.



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Standard methods were employed in the preparation of monoclonal antibodies that recognize 3. By screening hybridoma supernatants directly for decarboxylase activity with 5-nitro-3-carboxybenzisoxazole on a kinetic microplate reader at 340 nm, however, we were able to evaluate many more antibodies than is typically possible. From a pool of ca. 1200 hybridomas, we identified 25 antibodies with significant decarboxylase activity. These were propagated in mouse ascites and purified to homogeneity. The best catalyst, 21D8, had a rate acceleration of 19,000-fold at 20 °C in aqueous buffer at pH 8.0 (10 mM Tris-HCl). It exhibited hyperbolic kinetics, indicating formation of a Michaelis complex between the antibody and substrate prior to carbon-carbon bond cleavage. As expected, the napthalene disulfonate hapten 3 was a potent competitive inhibitor of catalysis (K_i = 6.4 nM), demonstrating that decarboxylation occurs within the induced binding pocket of the antibody.

21D8 is a significantly better catalyst for the decarboxylation of 3-carboxybenzisoxazoles than a variety of micelles, cationic polymers and crown ethers (ref. 8). The causes of this dramatic rate acceleration are interesting. Using environmentally sensitive fluorescent probes, we have shown that the antibody combining site is very hydrophobic and virtually inaccessible to solvent molecules in the presence of bound ligand. Further, investigation of the temperature dependence of k_{cat} revealed that the observed rate enhancement is entirely due to a lower enthalpy of activation for the catalyzed reaction. Although enthalpy changes also account for the large rate accelerations seen in dipolar aprotic solvents, the antibody-catalyzed decarboxylation is mechanistically distinct from the solvent-accelerated processes in that its activation entropy is considerably less favorable. One plausible explanation for this observation is that, unlike solvent, the rigid protein matrix that must resolvate the substrate is unable to relax as the reaction approaches the transition state. The data thus support the notion that the antibody

promotes catalysis by destabilizing the substrate through desolvation (stripping away its hydration shell) and by stabilizing the charge delocalized transition state through dispersion interactions.

Because many synthetically useful transformations can be promoted by partitioning of the reaction into a less polar medium, extension and generalization of this strategy may lead to the development of antibody catalysts of increasing sophistication and practical utility. Nucleophilic substitutions and aldol condensations are only two of many exciting targets likely to be facilitated through antibody-mediated medium effects.

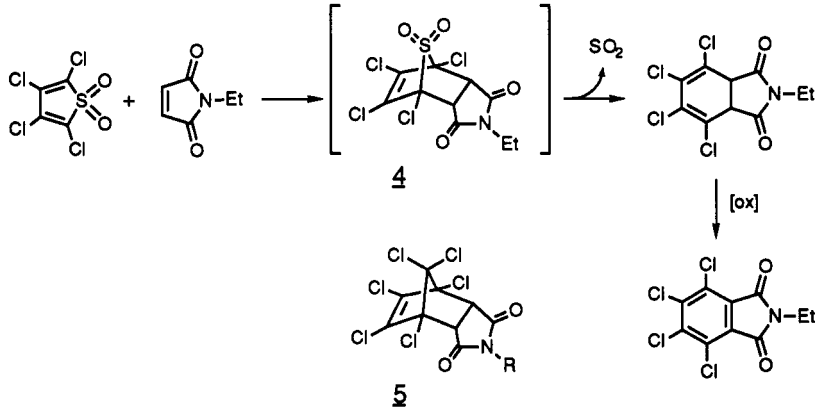
CYCLOADDITIONS

The Diels-Alder reaction between conjugated dienes and olefins to give cyclohexene derivatives is one of the most powerful and versatile methods available to synthetic chemists for assembling carbon-carbon bonds (ref. 9). Not only are two carbon-carbon bonds formed during the cycloaddition, but the reaction often occurs with high selectivity as well. While important in the laboratory, Diels-Alder reactions are rare in nature, and attempts to isolate enzymes that catalyze such processes have been entirely unsuccessful. The development of tailored "Diels-Alderase" antibodies could therefore fill an important niche, particularly as such catalysts would likely exhibit high regio-, enantio- and diastereoselectivity. Another intriguing possibility would be the use of antibodies to alter the normal endo:exo selectivity of a cycloaddition reaction in a predictable way.

Absence of natural "Diels-Alderases" in biology suggests that nature has discovered alternate efficient routes to cyclohexenes, not that proteins are intrinsically unable to catalyze cycloadditions. In fact, the use of binding interactions to bring two reactants together within an antibody active site is expected to greatly increase the probability of reaction. In this scenario, binding energy would be used to "pay" for the large loss in rotational and translational entropy incurred as the reaction coordinate is traversed. Exploitation of proximity effects may be thought of as catalysis by approximation, and theoretical calculations suggest that rate accelerations as high as 10^8 M (for 1 M standard states) can be achieved by this mechanism alone (ref. 10).

To test the feasibility of exploiting proximity effects to catalyze Diels-Alder reactions with antibodies, we targeted the two-step cycloaddition-cycloreversion sequence illustrated in Scheme 2 (ref. 5). Tetrachlorothiophene dioxide and *N*-ethylmaleimide undergo cycloaddition to give adduct 4, which rapidly eliminates sulfur dioxide (ref. 11). Dihydro-(*N*-ethyl)-tetrachlorophthalimide is subsequently oxidized to the phthalimide under the reaction conditions. The transition states for both the cycloaddition and chelotropic elimination resemble the high energy intermediate 4. Hence, the stable hexachloronorbornene derivative 5, which mimics this species, was chosen as our hapten. We anticipated that antibodies generated against 5 would have the correct topology for bringing the two substrate molecules together in the proper orientation for reaction. Because 5 has a very different shape than the final products of the reaction (phthalimide and SO_2), complications due to product inhibition could also be avoided.

Scheme 2



Several monoclonal antibodies recognizing 5 (R = $(CH_2)_5COOH$) substantially catalyze the addition of tetrachlorothiophene dioxide to *N*-ethylmaleimide with multiple turnovers (ref. 5). After reductively methylating the surface lysines of the antibody to avoid an undesired side-

reaction with the diene, the reaction was assayed by monitoring the disappearance of tetrachlorothiophene dioxide by HPLC or the appearance of SO_2 by the rapid bleaching of starch-iodine solutions. Both assay methods yielded equivalent kinetic parameters, indicating that the cycloaddition step is rate determining, while the usual control experiments established that the reaction occurs within the induced binding pocket. The effective molarity for the best catalyst, 1E9, was found to be > 10^2 M. The true chemical efficiency of the system must be much higher than this value implies, however, because we were unable to dissolve enough tetrachlorothiophene dioxide in aqueous buffer to saturate the antibody. The ability of 1E9 to undergo multiple turnovers is a consequence of the relatively high K_d value for *N*-ethyl-tetrachlorophthalimide, estimated by competition ELISA to be greater than 3 mM. Product inhibition is often a serious complication of enzyme-catalyzed bimolecular reactions, so the latter result is significant and validates the original choice of hapten. In the future, we hope to exploit other chelotropic eliminations, as well as acid/base and redox chemistry, to promote product release and catalyst turnover.

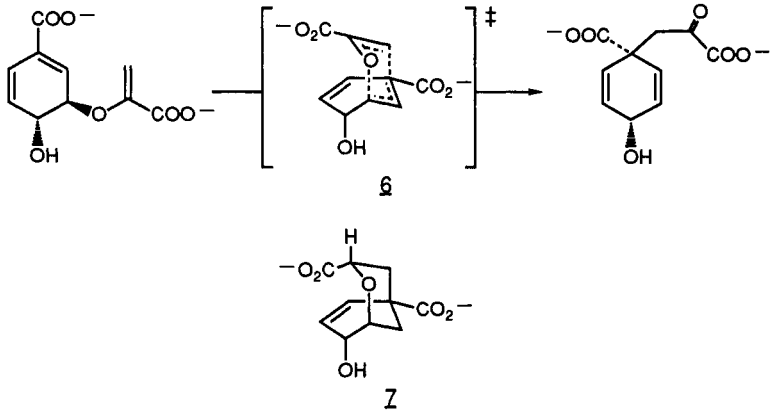
A second strategy for creating "Diels-Alderase" antibodies was recently reported by Braisted and Schultz (ref. 12), who utilized a [2.2.2]-bicyclooctene derivative to mimic the boat-like transition structure for the [4 + 2]-cycloaddition of an acyclic diene and an *N*-arylmaleimide. Lacking the ethano bridge of the hapten, the cyclohexene product is not locked in the relatively high energy boat conformation, but can undergo a conformational change that facilitates its release from the binding pocket. While this approach is promising in principle, the reported catalyst bound product nearly two orders of magnitude more tightly than either the substrate diene or dienophile, limiting its effectiveness in practice. In addition, the effective molarity for the system was only 0.35 M. Ultimately, however, more extensive screening of the immune response to the bicyclooctene hapten may yield more effective catalysts.

Preparation of "Diels-Alderase" antibodies is significant for several reasons. These molecules are the first reported protein catalysts for cycloaddition reactions. Thus, our studies show that it is feasible to exploit proximity effects in a rational way, and accelerate important non-physiological reactions. Because the Diels-Alder reaction is a prototype for a broad and important class of pericyclic processes, it should now be possible to extend this approach to many other transformations.

SIGMATROPIC REARRANGEMENTS

The Claisen rearrangement of allyl enol ethers is another pericyclic reaction of great importance in organic synthesis. Rearrangement of chorismate into prephenate (Scheme 3) is a biologically relevant example of this type of sigmatropic process, constituting a key step in the metabolic production of aromatic amino acids in plants and lower organisms (ref. 13). The enzyme chorismate mutase [EC 5.4.99.5] accelerates this rearrangement more than 10^6-fold. Although the mechanism of action of chorismate mutase is still debated, we anticipated that the sigmatropic rearrangement, like the Diels-Alder reaction, would be susceptible to strain and proximity effects, rather than to general acid/base catalysis, and hence particularly susceptible to catalysis by an antibody.

Scheme 3



6

7

Compound 7 is the best inhibitor known for chorismate mutase (ref. 14), binding to the enzyme ca. 10^2-times more tightly than chorismate itself. It was designed by Bartlett and coworkers to mimic the diaxial chair-like geometry of the putative transition state 6. Immunoglobulins

raised against protein conjugates of 7 also effectively catalyzed the conversion of chorismate into prephenate with rate accelerations in the range of 10^2- to 10^4-fold (refs. 6 &15). It is notable that the most effective of the antibody catalysts possesses 1% of the activity of chorismate mutase itself. While most antibody catalysts do not compare as favorably with their naturally occurring counterparts, this result does indicate the potential of this approach to catalyst design. The fact that not every antibody is equally potent again underscores the importance of screening as large a fraction of a given immune response as possible. The probability of finding an effective catalyst is likely to increase with the size of the population sampled.

The most significant property of the chorismate mutase antibodies is not their specific activity, but rather their high enantioselectivity. Even the antibody exhibiting only a 200-fold rate acceleration over background was able to effect a kinetic resolution of racemic chorismate and give optically pure (+)-chorismate (ref. 16). That antibodies, like enzymes, exert considerable regio- and stereochemical control over the reactions they promote is a property most likely to be useful for practical applications. In fact, when racemic haptens are employed, it is even possible to obtain catalysts from a single fusion that process each substrate enantiomer (ref. 17).

In addition to its other attributes, the chorismate mutase system is ideal in permitting study of important protein structure-function relationships. Over and above the natural enzyme, there now exist two different antibody catalysts for this reaction and several immunoglobulins that bind the transition state analog tightly but do not promote the sigmatropic rearrangement. Detailed characterization of these molecules — and, in particular, comparison of their properties — is likely to result in a far better understanding of the mutase reaction, and, perhaps, protein catalysis in general. As a prelude to detailed structural studies, we have cloned and sequenced the genes encoding the chorismate mutase antibody 1F7 (ref. 18). Interestingly, the light chain of 1F7 is closely related to the light chain of the anti-(2-phenyl-oxazolone) antibody NQ10.12.5 for which an x-ray structure was recently reported (ref. 19).

We have also successfully expressed 1F7 as an Fab fragment in the yeast *Saccharomyces cerevisiae* (ref. 18). In our expression system, the mature light chain and the truncated Fd heavy chain proteins are synthesized simultaneously from the bidirectional *GAL1-GAL10* promoter. Because the secretion signal sequences were removed, the gene products are produced in the cytoplasm of the host, where they assemble spontaneously into a functional unit. Experiments *in vitro* established that 1F7 produced in yeast is identical to the originally isolated, full-length IgG molecule in terms of its affinity toward hapten 7 and its catalytic potency.

The rearrangement of chorismate into prephenate is an essential metabolic reaction, and yeast cells that lack chorismate mutase are unable to grow unless supplemented with phenylalanine and tyrosine. We wondered whether intracellularly expressed 1F7 might replace the missing enzyme functionally and complement the metabolic disorder. We did not see complementation of an engineered *S. cerevisiae* strain containing an insertion mutation in the structural gene for chorismate mutase, presumably because 1F7 is 10^4-fold less effective than the natural enzyme and was overexpressed only by a factor of 10. However, we identified a permissive chorismate mutase-deficient cell line following extensive random mutagenesis of the antibody-harboring cells with ethyl methanesulfonate and classical genetic selection (ref. 20). Intracellular expression of 1F7 conferred a substantial growth advantage to this host under auxotrophic conditions. In contrast, complementation of the metabolic disorder was not observed when an esterolytic antibody was used in place of 1F7. Our experiments thus establish the feasibility of using catalytic antibodies to carry out vital biochemical transformations *in vivo*. They also suggest exciting opportunities for regulating cellular function, altering cellular metabolism, or destroying carcinogens and other toxins with these tailored catalysts. Perhaps most significantly, though, the availability of a growth selection assay that depends on antibody activity provides us with a powerful tool for identifying genetic changes in the antibody itself that augment catalytic potency. We are currently attempting to direct the evolution of the chorismate mutase antibody toward greater chemical efficiency through direct random mutagenesis of the antibody-encoding genes and subsequent genetic selection. Achieving this goal is important, given that most first-generation antibodies are orders of magnitude less effective than their naturally occurring counterpart enzymes. Genetic selection has the potential to be a general and generalizable solution to this problem.

CONCLUSIONS

Because catalytic antibody technology combines the intuition and synthetic skill of the chemist with the virtually inexhaustible combinatorial diversity of the immune system, it represents an unusually powerful and versatile strategy for creating tailored biocatalysts. In fact, this

technology complements and extends other current approaches to artificial enzymes, including the re-engineering of existing protein binding sites by site-directed mutagenesis (ref. 21), the *de novo* design of protein binding sites from their constituent amino acids (ref. 22), the development of ribozymes (ref. 23), and the creation of low molecular weight synthetic catalysts (ref. 24). In principle, any chemical transformation can be speeded by antibodies. As long as the reaction itself is compatible with an aqueous milieu and a protein microenvironment, and as long as a suitable transition state analog can be devised, catalysis can be achieved. As shown here, even non-physiological reactions such as the [4 + 2]-Diels-Alder cycloaddition and the decarboxylation of 3-carboxybenzisoxazoles, can be promoted efficiently by antibodies. In practice, of course, many issues must be addressed before the practical promise of this technology is fully realized. Development of general rules that relate hapten design to successful generation of efficient catalysts, implementation of strategies to introduce catalytic functionality and chemical cofactors into the antibody combining site, and increased access to the full diversity of the immune system will all increase the power of this approach. By also bringing the tools of molecular biology and genetics to bear on the study and improvement of first-generation antibody catalysts, in a few years it may even be possible to generate immunoglobulins that rival the efficiency of naturally occurring enzymes.

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