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Nonspecific Catalysis By Protein Surfaces

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Abstract

Catalytic antibodies are the best available allaround enzyme mimics. They provide a unique experimental approach and some special insights into general questions about catalysis by enzymes. They offer enantiospecific reactions and levels of substrate binding that compare well with typical enzyme reactions, but not—so far—comparable catalytic efficiency. We and others have used the Kemp elimination as a probe of catalytic efficiency in antibodies. We compare these reactions with nonspecific catalysis by other proteins, and with catalysis by enzymes. Several simple reactions are catalyzed by the serum albumins with Michaelis–Menten kinetics, and can be shown to involve substrate binding and catalysis by local functional groups. Here, we report the details of one investigation, which implicate known binding sites on the protein surface and discuss implications for catalyst design and efficiency.

Index Entries: Antibodies; catalysis; transition-state analog; albumin; BSA.

Catalysis by Enzymes and Other Proteins

The underlying theme of this work is catalytic efficiency. Catalysis on protein surfaces—the regions of the main and side-chains in contact with the solvent—is possible at various levels. These range from ordinary unremarkable manifestations of normal functional group reactivity to the extraordinary levels of reactivity of the most efficient enzymes. Most interesting to the chemist are those enzymes that accelerate—to typical enzyme-like rates—the reactions of the least reactive substrates, like the phosphodiesters. The range of reactivity we are dealing with is enormous *(1)*. Yet we will demonstrate that a continuum of behavior is involved.

To simplify the analysis, I suggest the following categories, which represent increasingly efficient catalysis:

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- Nonspecific catalysis by protein surfaces.
- *Exceptionally efficient catalysis by protein surfaces.
- *Abzyme catalysis with broad (accidental) specificity.
- *Abzyme catalysis with good specificity.
- Enzyme catalysis with broad (including accidental) specificity.
- Enzyme catalysis with normal (i.e., high) specificity.

Of special interest for these proceedings are the three categories I have starred. The other three can be discussed very briefly. We know that the polar groups of proteins—carboxylate anions, ammonium cations, and others—typically cluster on the surface in contact with solvent water. Such groups are known to act as general acids, bases, and nucleophiles in reactions of activated substrates, and those on protein surfaces behave—as expected—in the same way: there is nothing special about such reactions. On the other hand, there is a great deal that is special about catalysis by enzymes, which can catalyze the slowest reactions of specific substrates with the highest efficiency. This involves binding, and thus specifically stabilizing the rate-determining transition-state for the reaction concerned. *Dynamic binding (2)* of the reacting center is reinforced by passive binding of the nonreacting parts of the substrate structure to provide optimal stabilization at this unique point on the reaction energy profile. (This is of course a considerable oversimplification, particularly because almost any enzyme-catalyzed reaction involved multiple steps and transition states.)

For enzymes like alkaline phosphatase, with broad specificities, transition-state binding of the reacting center seems to be relatively more important than passive binding. Also, what might be called accidental specificities are sometimes observed in the case of unnatural substrates: as in the efficient acylation of some serine enzymes by diisopropyl phosphorofluoridate. Binding of a reactive unnatural substrate in an enzyme-active site can trigger reactions with the catalytic groups that are present simply through proximity.

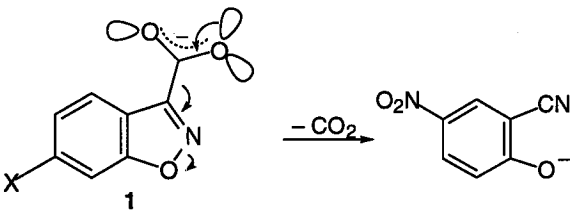
A similar type of reactivity, in principle, can be observed with any protein which can bind a potentially substrate molecule. A relevant example is the esterolytic reactivity of bovine serum albumin (BSA). BSA reacts with *p*-nitrophenyl acetate much faster than most other proteins: it reacts through a specific tyrosine, and the reaction shows burst kinetics, just like chymotrypsin—and for the same reason, the formation of an acyl enzyme intermediate *(3)*.

Catalytic Antibodies

We are interested in reactions of all types where reactivity is enhanced by functional-group proximity. Apart from enzyme catalysis, the most efficient examples are all intramolecular reactions, where effective molarities [EM *(4)*)] up to 10^{13} *M* are attainable in certain special situations. This level of reactivity makes it possible to study the mechanisms of reactions of some unactivated substrates at or close to enzyme rates, but the rigid systems

necessary seriously limit the geometries of approach of the reacting groups—a crucial factor when stereoelectronic preferences are strong. Advances in supramolecular chemistry allow the design of strong binding interactions between separate molecules, but not yet to interactions between attached functional groups efficient enough to elicit high reactivity *(2)*. So the possibility of raising antibodies with custom-made binding sites containing polar groups complementary to charged groups on a well-designed hapten is an exciting prospect.

Our first venture in the area was an attempt to produce an antibody with a controlled active-site microenvironment, capable of catalyzing the Kemp decarboxylation of benzisoxazole-3-carboxylates, **1**. This reaction is known from the physical organic work of the Kemp group *(5)* to be extraordinarily sensitive to the polarity of the medium, being up to 10^8 times faster in less polar solvents than in water.



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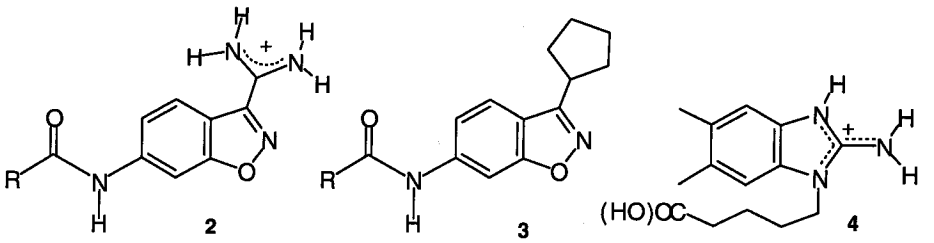
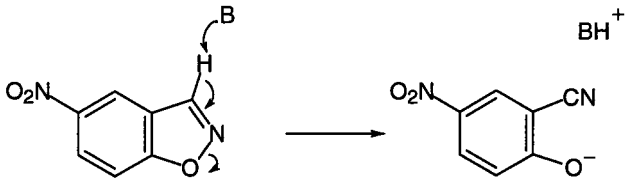
Kemp decarboxylation

Antibodies were raised against a range of 3-substituted benzisoxazole haptens, including the cationic amidine, **2** *(6)* and the cyclopentanyl derivative, **3** *(7)*, but none accelerated the Kemp decarboxylation to a measurable extent.



However, 5 of 11 antibodies raised against the amidine hapten **2** were found to catalyze the Kemp elimination, another closely related reaction showing extraordinary sensitivity to the polarity of the solvent when the general base is a carboxylate anion *(6)*.

Since the amidine group is known to form a stable double-hydrogen bond with carboxylate, the inference was clear that the catalytic antibodies had COO^- groups close to the hydrophobic site induced by the aromatic part of the hapten. Catalysis by these antibodies was not particularly efficient, but this was no surprise: intramolecular general-base catalysis is known to be intrinsically inefficient in model systems *(4,8)*. Efficient catal-



Kemp elimination

ysis of proton transfer requires the development of a strong hydrogen bond in the transition state, possible only with precise positioning of the catalytic group *(8)*. It would have been remarkable to have achieved this with the first attempt using our rather crude hapten-design principles.

For these reasons, we were most interested in a report *(9)* from the Hilvert group of the generation of "remarkably efficient" antibody catalysts for the Kemp elimination, using as the general-base carboxylate groups elicited by the same basic design strategy (hapten **4**). The rate constants concerned were one to two orders of magnitude greater than those we had found, and it was important to determine if this relatively efficient catalysis was a result of precise positioning of the general base. The obvious alternative was that the reaction took place in a hydrophobic microenvironment, thus taking advantage of the large medium effect observed by Kemp on the reaction in less polar media.

Thorn et al. had recognized this as a possible contributing factor, but there is no obvious way of quantifying the separate contributions of the two effects. So we adopted an entirely different approach. If efficient catalysis is caused primarily by a medium effect, with the participating general base present but not necessarily precisely positioned, the particular conditions—a hydrophobic binding site with a basic group in close proximity—are likely to occur frequently on the surfaces of proteins. So we screened a number of candidate proteins—including calmodulin, various serum albumins, and the enzymes barnase, lysozyme, chymotrypsin, chymotrypsinogen, and trypsin—as catalysts for the Kemp elimination of 5-nitrobenzisoxazole. The serum albumins turned out to be rather good catalysts *(10)*, showing Michaelis–Menten kinetics and k_{cat} values comparable to those observed by Thorn et al.

A detailed investigation of this catalysis showed slightly different pH dependences for albumins from different sources, signifying minor differences in active-site arrangements, but in the majority of cases reactivity depended on the basic form of at least one group with a pK_a close to 9. This could be identified as a lysine amino group by chemical inactivation experiments, confirmed by results from other workers *(11)*.

The identification of the catalytic general base as a lysine was a bonus; because the amine-catalyzed Kemp elimination is known *(12,13)* to be far less sensitive to the polarity of the medium than the reaction catalyzed by carboxylate. Thus, effective molarities calculated for the lysine amino groups in BSA and HSA differ by less than an order of magnitude when based on

second-order rate constants measured in water and acetonitrile, falling in the region of 10 *M* for BSA and HSA. The EM range for the carboxylate-catalyzed reaction calculated on the same basis lies between 10^{-4} and 10^{4} *(10)*. In the case of the serum albumins, the positioning of the general base evidently contributes no more than about 10 *M* to the effective molarity. In the case of the catalytic antibodies of Thorn et al. the much wider range combines contributions from both positioning and medium effects. Since 10 *M* is a respectable EM for positioning of a general base *(4)*, the BSA figure is interesting, but not exceptional: it is likely that positioning makes a similar contribution in the most efficient antibodies of Thorn et al.

The Catalytic Site of the Serum Albumins

It is important to stress that although many serum albumins catalyze the Kemp elimination—a relatively rapid single-transition-state reaction—this is far from being a common property of proteins. Aside from the proteins listed above, a large number of "relevant" antibodies have been screened for activity and found to be deficient. A catalytic site achieving an EM of 10 *M* matches all but a few intramolecular general-base catalyzed reactions in efficiency, so the arrangements involved are of general interest.

The serum albumins are the most abundant proteins in the circulatory system, and act as transport proteins for long-chain fatty acids. They have up to five binding sites, and these have been identified in some detail in recent crystal structures. The most recent *(14)* shows the structure of human serum albumin (HSA) with five myristates, plus two 2,3,5-triioidobenzoates bound. HSA is also of pharmaceutical interest as a carrier for heteroaromatic drug molecules, by virtue of the aromatic binding site or sites. The various types of molecules known to bind to HSA act, as expected, as competitive inhibitors for the HSA-catalyzed Kemp elimination *(10)*.

The alkyl chains of fatty acids are bound in hydrophobic binding regions, but the carboxyl groups are ion-paired with lysine or arginine cations. Thus, these hydrophobic binding sites have an adjacent general acid group, which under basic conditions—at least for lysine—is converted to a general base.

Inhibition studies with octanoate and various drug molecules known to bind to HSA suggest that two sites contribute to the observed catalysis of the Kemp elimination. The majority (about 70%) of the catalysis can be attributed to a single site [the Sudlow IIA site *(15)*], which can be identified in the Curry structure *(14)* by the presence of a bound triiodobenzoate anion. The carboxylate group is H-bonded to Lys-199, which is the candidate general base for the HSA-catalyzed Kemp elimination. (This position is occupied by an arginine in BSA, but in a complementary change Arg-222 in HSA becomes lysine in BSA, and a simple rotation of a bound aromatic system is all that is needed to set up the necessary geometry for H-bonding or catalysis.)

Catalytic Efficiency

In terms of the categories defined in the introduction, the serum-albumin-catalyzed Kemp elimination counts as exceptionally efficient catalysis by a protein surface. In terms of catalytic efficiency, as shown by the plot in Fig. 1, it compares with a middle-range catalytic antibody. The more general message of Fig. 1 is the relatively low catalytic efficiency of abzymes compared to enzymes *(16)*. It is important to understand the factors responsible in order to determine whether there is an intrinsic barrier to significantly higher activity by antibodies.

The most efficient enzymes are those that catalyze the reactions of the least reactive substrates, like phosphodiesters, glycosides and—somewhat less extreme—peptide bonds. Enzymes that catalyze the cleavage reactions of such substrates use nucleophilic catalysis, with general acids and often general bases also involved in vital proton-transfer steps. The involvement of functional groups is crucial. The majority of successful antibody catalysts—for example, esterases based on phosphonate and related transition-state analogue haptens—do not involve a functional group directly, but simply stabilize the transition state for hydroxide attack on C=O. It is significant that one class of reaction where enzyme and abzyme efficiencies are comparable (Fig. 1) is pericyclic. (The two least efficient enzyme reaction points are for carbonic anhydrase—where the uncatalyzed reaction is so rapid that a large acceleration is not required—and chorismate mutase.) If catalysis involves little more than binding the substrate in the right conformation, then abzymes can do relatively well.

It is reasonable to argue that the field of catalytic antibodies is new and still developing and that the evidence thus far is too thin to support far-reaching conclusions about the potential of abzymes as catalysts, and that a better understanding of the complex interaction of transition-state analogs, the immune system, and mechanisms will lead eventually to custom-made catalysts with efficiencies comparable to at least middle-range enzymes.

What we need is evidence to support this proposition. I have been encouraged at this meeting to hear of antibodies isolated from patients with autoimmune diseases that catalyze the cleavage of DNA. Data from the papers given by Roddkey, Gabibov, Gololobov, and Nevinsky particularly suggest significant DNase activity of certain autoantibodies. Intrinsically, phosphodiesters are extraordinarily unreactive substrates, and any detectable reaction catalyzed by an antibody is of interest. Apparently, the most efficient reaction is the cleavage of plasmid DNA catalyzed by an Fab fragment from a polyclonal preparation described by Gabibov and his colleagues *(17)*. An apparent k_{cat} of the order of 10 min^{-1} would put this system in the region depicted in Fig. 1, which is currently the exclusive province of enzymes. Questions remain to be answered: as the authors are well aware, it is always difficult to rule out contamination by enzymes, and it is important that the catalyst and the reaction should be fully character-

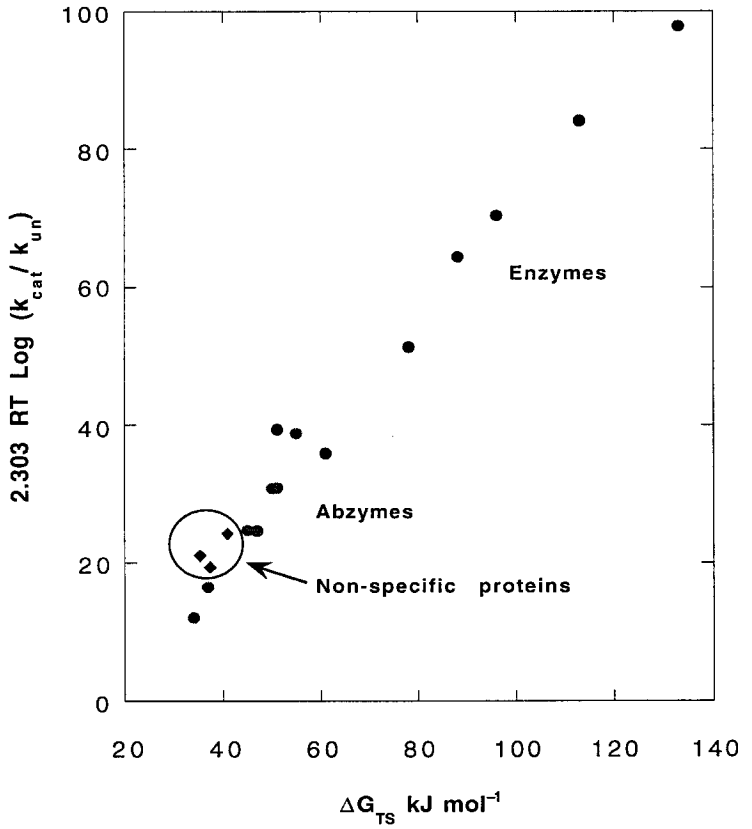


Fig. 1. Comparison of catalytic efficiencies of selected catalytic antibodies with a range of enzymes and with the nonspecific proteins discussed in this article. An earlier version of the plot appeared in reference 16.

ized and quantified. Nevertheless, if just one antibody can be conclusively shown to be capable of such high-efficiency catalysis—with or without the involvement of metal cations—this would provide the key to a significant advancement in the field.

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Discussion

Schowen: At first glance it seems counterintuitive that the immune reaction would be less sensitive to medium effects, in the sense that when the antibody removes the proton you generate a negative charge in the transition state, whereas in the case of the carboxylate base, you start with a negative charge and you end with the same charge. Does it have to do with the solvation of the negative ion and water?

Kirby: The big energetic stabilization effect is the desolvation of the carboxylate compared with water. In this particular reaction, it's fairly clear that desolvation of the lone pair that serves as the base is necessary. Because of the size of the aromatic system, it is probably also necessary to desolvate the other oxygen on the facing side. At any rate, that reaction shows the very large sensitivity to medium effects, much larger that the pK_a change. However, the pK_a does jump when you go into an organic medium because the conjugate acid is now different.

Paul: What is the concentration of the albumin in circulation?

Kirby: About 14 m*M*.

Paul: Indeed, and even weak reactivities might be biologically important at this enormous concentration. Is there thinking that the so-called nonspecific catalysis by albumin might be biologically important, although it is weak?

Kirby: That is a very interesting question. The philosophical answer is: because it's such a high concentration, nature has probably taken great care that albumin is not a random catalyst. But I don't know the answer to that question.

Paul: I didn't catch what you meant by the term "random catalyst."

Kirby: We should distinguish between two possibilities—biologically purposeful catalysis vs accidental catalysis.

Paul: I was actually thinking quite simplistically. Is albumin known to catalyze a biologically important reaction—low-level catalysis, as it might be? I am not referring to the model organic reactions that have been previously examined with antibodies to TSAs.

Kirby: I don't know of anybody who has identified such a reaction, but it's an interesting possibility. As you may know, albumin has been shown to

catalyze quite a lot of different reactions which need general acids or general bases and a hydrophobic binding site.

Zouali: This may be a disturbing or radical thought: we know we can antigenize antibodies, which is basically putting in epitopes by CDR replacements. Couldn't we use this approach to put in a catalytic site? We can take the catalytic site from an enzyme, copy it into a CDR, and retain the other CDRs to provide the substrate specificity.

Kirby: It would be a complicated operation because almost all enzyme active sites are noncontiguous pieces of polypeptides—there are several different peptide pieces located distant from each other in the linear sequence. The three-dimensional approach you are suggesting has been tried to some extent—not on antibodies, but in other protein systems—without any great success. It is long-term and a rather interesting proposition. The architecture looks quite complicated at the moment, but probably somewhere in the US someone is trying it.

Green: You used the term "surfaces" initially, but you meant pockets and cavities. Is that correct?

Kirby: I was using "surfaces" as in "surface of the moon." Or a dimpled face.

Green: Sudhir asked a question about possible reactions of BSA. I always thought this might be one possible application of a catalytic antibody. There are some terrible autoimmune reactions to drugs because of binding to albumin. The albumin becomes an autoantigen. Antiinflammatory agents can also trigger this hypersensitivity reaction. So there is some chemistry that in principle could be reversed by a catalytic antibody. If you we know the mechanism, we can imagine an antibody that would prevent the anbumin conjugate from forming.

Kirby: That is just really a binding question, not a catalysis issue.

Paul: Alcohol and certain anesthetics can indeed produce chemical modifications of albumin at basic residues, leading to autoimmune reactions. I'm not sure if the chemical reactivity of albumin is connected to its ability to act as a low-turnover catalyst.