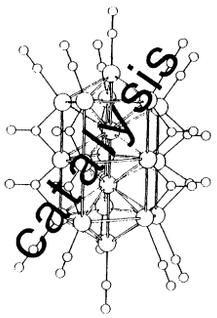
Semisynthetic Enzymes Are New Catalysts

Enzymes may be the ultimate catalysts, especially if their specificities can be changed by chemical modification



A continuing theme in the story of catalysis is the search for selectivity. Investigators are continually looking for catalysts that will carry out only one reaction, ideally at only one reactive site, and preferably with only one stereochemical outcome.* That type of catalyst has, of course, been available all along. It is called an enzyme.

Enzymes have been used relatively little in organic synthesis, says George Whitesides of Harvard University, primarily because of lack of motivation and lack of familiarity by synthetic chemists. Now, however, investigators are working with the complicated compounds that appear in, for example, medicinal chemistry, immunology, neurochemistry, and endocrinology, he says, "and a new class of techniques is needed to make them. It is plausible to think of going to enzymes to do so."

A major problem associated with the use of enzymes, however, is that many of the reactions investigators want to carry out with enzymes involve "unnatural" substrates or products. This problem can be attacked with new approaches that seek to bend enzymes to the investigator's will. The simplest of these may involve nothing more complicated than testing known enzymes with unusual substrates or changing the solvent or reaction conditions to change the activity of the enzyme.

More complicated procedures may involve combining the enzyme with an external reagent to produce a semisynthetic enzyme with different catalytic potential. The most complicated may involve a complete redesign of the enzyme itself, a topic that will be the subject of a second article. Research has only just begun on such applications, but the results so far suggest that there is great potential for success and that it might be possible one day to find or make an enzyme to carry out nearly any reaction desired.

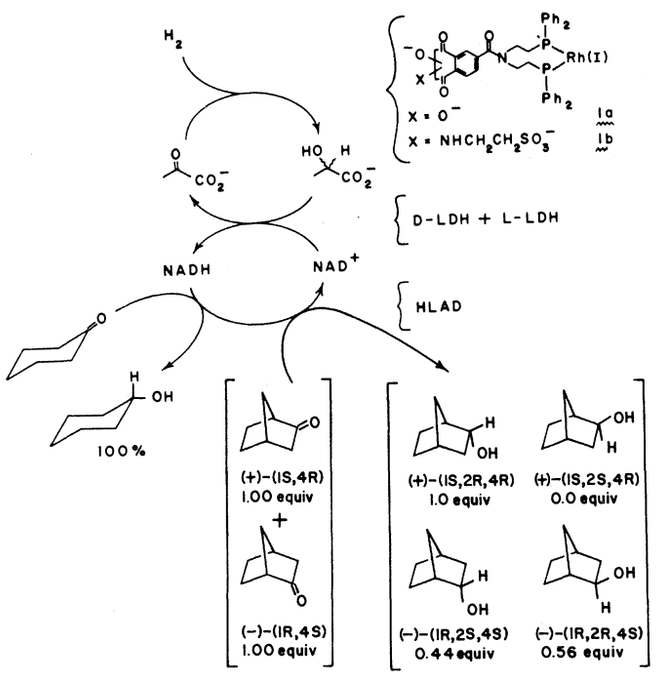
More than 2000 different enzymes have been described, but only about 200 of these are commercially available in small quantities and only 16 in industrial amounts. Ideally, says Alexander Klibanov of the Massachusetts Institute of Technology, a proposed chemical synthesis would involve one or more of the available enzymes because that would be simplest. The most important thing to keep in mind is that the reaction for which the enzyme is named is almost certainly not the only one that can be effected by the enzyme. Many other possible reactions might be found with only a little knowledge and a little experimentation.

Consider the enzyme glucose oxidase, which catalyzes the oxidation of β-D-glucose with molecular oxygen to produce D-gluconolactone and hydrogen peroxide. "Neither of the products of the glucose oxidase-catalyzed reaction is a coveted target," Klibanov says, "and hence it would seem that glucose oxidase has a very limited potential for the chemical industry. Textbooks, furthermore, assert that it is an extremely specific enzyme. . . . These statements, although correct, are somewhat misleading."

Glucose oxidase is, in fact, very specific with respect to the electron donor (D-glucose), but it is not specific at all with respect to the electron acceptor (O_2). It was known that oxygen could be replaced by artificial dyes, such as indophenol. Klibanov therefore reasoned that the enzyme might be useful as a reducing agent for the production of commercially important products.

The enzyme can be used, for example, to reduce benzoquinone to hydroquinone, which is used as a photographic developer and as an inhibitor of autooxidation and polymerization. In this case, the reaction is actually three times as fast as when oxygen is used as the acceptor. Glucose is consumed in the reaction, but it is an inexpensive material. The enzyme will work on a number of substituted quinones, many of which, such as vitamin K derivatives, have biomedical applications. It can also be used on aromatic nitro compounds; it will, for example, reduce nitrobenzene to nitrosobenzene.

Klibanov cites some other examples that have potential use. Galactose oxidase can oxidize a number of nonsugar aliphatic and aromatic alcohols selectively to the corresponding aldehydes. This reaction can be very stereospecific. Glycerol is oxidized exclusively to *S*-(−)-glyceraldehyde; with enantiomeric mixtures of 3-halo-1,2-propanediols, only the *R*-alcohol is oxidized. Xanthine



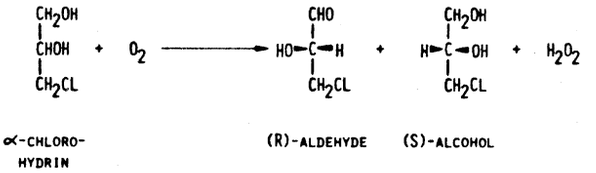
Linked catalysis

Reductions of carbonyl compounds using a hybrid organometallic-enzymatic catalyst system. LDH, *lactate dehydrogenase;* HLAD, *horse liver alcohol dehydrogenase.* [*Source: George Whitesides, Harvard University*]

*Preceding articles in this series have appeared in the 1983 issues of 4 February, p. 474; 25 February, p. 944; 25 March, p. 1413; 6 May, p. 592; 3 June, p. 1032; 17 June, p. 1261; 22 July, p. 351; 30 September, p. 1358; and 14 October, p. 151.

oxidase can be used for the separation of *cis*- and *trans*-isomers of β-arylacroleins, such as cinnamaldehyde, that could be precursors of amino acids. In most cases, the *trans*-isomer is oxidized about 100 times faster than the *cis*-analog.

Alteration of reaction conditions can also change enzyme specificity. Horseradish peroxidase, for example, effects hydroxylation of aromatic compounds, but yields have been low and several different products are formed in each reaction. By decreasing the temperature from 25° to 0°C, Klibanov found that side reactions could be eliminated and specificity improved. Under these conditions, *para*-substituted aromatic compounds are hydroxylated exclusively at the *meta* position and vice versa.



Selective oxidation

Galactose oxidizes only the R*-isomer of 1-halopropanediols, leaving behind the* S*-alcohol. [Source: Alexander Klibanov]*

Hog liver carboxyl esterase can catalyze transesterifications, but it has not been used for this purpose synthetically because many of the poorly soluble alcohols on which it might be used cannot compete effectively with water molecules; ester hydrolysis therefore predominates. Klibanov and Bernard Cambou have found that this problem can be overcome by using a biphasic system in which the enzyme is dissolved in a minimal quantity of water. The ester and the alcohol constitute the organic phase. The substrates, says Klibanov, will freely diffuse into the aqueous phase, undergo conversion, and then diffuse back into the organic phase. The fraction of water in such a system can be made extremely low, and hence esterification will be greatly favored over hydrolysis. Conceptually, the system is an emulsion, but experimentally it is advantageous to use

A Step Toward Wholly Synthetic Enzymes

One step beyond the combination of a protein binding agent with a synthetic catalyst is the construction of a completely synthetic enzyme. That term could encompass a variety of species, but perhaps the best definition is that it is a synthetic molecule with a cavity for binding a substrate and one or more catalytic groups attached.

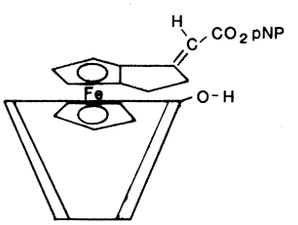
A great deal of work has been accomplished in the design of organic molecules with binding cavities. These materials, called cavitands by Donald J. Cram of the University of California, Los Angeles, have been discussed previously (*Science*, 11 March 1983, p. 1177). Unfortunately, there has been little reported success in attaching catalytic groups to the cavitands.

The one area where there has been success involves a family of materials called cyclodextrins. These are doughnut-shaped molecules typically containing six (α-cyclodextrin), seven (β-cyclodextrin), or eight (γ-cyclodextrin) glucose molecules. These contain a cavity that is 7 angstroms deep and 5 to 9 angstroms in diameter; the top is somewhat larger than the bottom so that the cavity is shaped like a cone rather than a cylinder. Much of the work on cyclodextrins has been performed by Myron L. Bender of Northwestern University and Ronald Breslow of Columbia University.

The cavity in α-cyclodextrin is large enough to accommodate a benzene ring comfortably. That cyclodextrins can be used in catalytic reactions was first shown by Breslow many years ago for the chlorination of anisole (methoxybenzene). In free solution, the chlorination yields a mixture of *ortho*- and *para*-chloroanisole. α-Cyclodextrin binds anisole with the methoxy group inside the cavity so that only *p*-chloroanisole can be formed. Furthermore, one of the sugar hydroxyls on the rim of the cavity catalyzes the reaction so that it proceeds more rapidly. In this case, then, both the selectivity and the rate are affected just as would be the case with an enzyme, but the increase in rate is rather modest.

Much of Breslow's subsequent work has involved an attempt to provide significant rate accelerations for simple catalytic reactions, with the sugar hydroxyls serving as catalytic species. β-Cyclodextrin, for example, can catalyze the hydrolysis of *m-tert*-butylphenyl acetate at a rate about 100 times greater than the uncatalyzed rate; strictly speaking, the reaction is not catalytic because the acyl group remains attached to the sugar, but this provides a good model for one step in most enzymatic esterolytic reactions.

The rate enhancement in the preceding example is small because binding of the ester in the cavity does not bring the ester linkage near the catalytic hydroxyl. This situation can be improved by use of the *p*-nitrophenyl ester of ferrocene-acrylic acid; in this case, the ferrocene fits comfortably into the cavity while the flexible side chain brings the ester near the catalytic hydroxyl; a rate enhancement of 10^5 is observed. If the acrylic side chain is incorporated into a fused ring system so that the ester is held near the hydroxyl more rigidly, a rate enhancement of 3.2×10^6 is obtained.



More recently [*J. Am. Chem. Soc.* **105**, 2739 (1983)], Breslow and his colleagues added one more degree of steric constraint to the system by attaching the ester to the fused ring system through a double bond. In this case, the catalytic reaction is 5.9×10^6 faster than the uncatalyzed reaction, the greatest increase so far observed. The reaction, furthermore, is carried out in aqueous dimethyl sulfoxide, which is necessary to solubilize the reactants; this solvent is more hydrophobic than water, and might be compared to the relatively hydrophobic environment at the active site of many enzymes. The uncatalyzed reaction in aqueous dimethyl sulfoxide proceeds about 24 times faster than the reaction in water, so the overall rate increase, compared to hydrolysis in water, is about 1.5×10^8, well within the range of accelerations produced by enzymes.

—T.H.M.

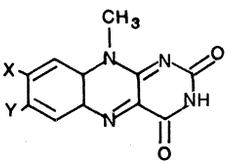
porous supports whose pores are filled with the aqueous phase. This system has the additional advantage that it will often result in the transesterification of only one isomer of a racemic mixture.

If an appropriate catalyst cannot be obtained in this manner, it may be possible to make one by combining the binding specificity of a protein with the catalytic activity of an organometallic. Whitesides and Michael Wilson, for example, attempted to use the biotin-binding protein, avidin, as a chiral template in hydrogenation reactions. They worked with a complex diphosphinerhodium complex that is a moderately active hydrogenation catalyst, but which does not exhibit any enantioselectivity; that is, in the hydrogenation of α-acetamidoacrylic acid, it produces equal quantities of (*R*)- and (*S*)-*N*-acetylalanine. When the rhodium catalyst is attached to the avidin through an iminolinkage, however, the yield of (*S*)-*N*-acetylalanine is 34 percent greater than that of its (*R*)-enantiomer. This result suggested that such procedures are viable, but other hydrogenation catalysts have produced better results.

Harry Gray and Ruth Margalit of the California Institute of Technology and Israel Pecht of the Weizmann Institute of Science in Israel have combined a ruthenium electron-transfer catalyst, $[Ru(NH_3)_5]^{3+}$, with sperm whale myoglobin to produce a "semisynthetic bioinorganic enzyme." Myoglobin binds oxygen and carries it through the circulatory system, but has no catalytic activity. When three ruthenium complexes are bound to it through surface histidine residues, however, the new material can reduce oxygen while oxidizing various organic substrates, such as ascorbate or durohydroquinone. For ascorbate oxidation, says Gray, the semisynthetic enzyme is about 200 times as effective as the ruthenium moiety (complexed with imidazole), and nearly as effective as naturally occurring ascorbate oxidase. This is a good example, he says, of how "a drastic modification of protein function can be achieved by a simple change in structure."

Equally good results have been achieved by Emil Thomas Kaiser and his colleagues at Rockefeller University with a group of semisynthetic enzymes that Kaiser calls flavopapains. Papain is a proteolytic enzyme (that is, one that hydrolyzes amide bonds in proteins) isolated, as the name suggests, from papaya; it is a thoroughly studied enzyme whose three-dimensional structure is well known. The flavins are a family of nitrogen-containing, aromatic, tricyclic compounds (isoalloxazines) that are known to catalyze many different reactions. Flavin-derived compounds are also cofactors in many enzymatic reactions.

The x-ray structures of papain, Kaiser says, show that it has an extended groove in the vicinity of cysteine-25, which is known to participate in the proteolytic reaction. It thus seemed likely that the sulfhydryl group of cysteine-25 could be alkylated with a flavin compound without destroying the accessibility of the binding region to potential substrates. Since the sulfhydryl participates in the enzymatic reaction, loss of hydrolytic activity would serve as a way to monitor the alkylation. Study of the x-ray structures also showed that the backbone nitrogen of cysteine-25 and the



Compound **1**: X = H ; Y = C–CH_2–S–Papain

Compound **2**: Y = H ; X = C–CH_2–S–Papain

side-chain amide of glutamine-19 should be able to hydrogen bond with an appropriately designed flavin.

Beginning in the late 1970's at the University of Chicago, Kaiser and Howard Levine, now at Genentech Corporation, constructed several flavoproteins, some with a carbonyl group near the alkylation site that can hydrogen bond to the interior of the protein, and some without the carbonyl. These were then used to oxidize N^1-alkyl-1,4-dihydronicotinamides, and their activities were compared to those of the flavins alone or of the naturally occurring enzyme NADH (reduced nicotinamide adenine dinucleotide) dehydrogenase, which also contains a flavin.

The flavopapains in which the flavin could not hydrogen bond to the protein showed only about a threefold rate enhancement compared to the reaction of the flavins alone; this increase, says Kaiser, is "practically insignificant." The flavopapain prepared from a 7-bromoacetyl substituted flavin (compound **1**), in contrast, gives rate increases (compared to the flavin alone) of one to two orders of magnitude, depending on the substrate. This material also shows the saturation kinetics characteristic of an enzymatic reaction.

The flavopapain produced from an 8-bromoacetyl substituted flavin (compound **2**) gives the best results of all. This material not only displays saturation kinetics, but gives a thousandfold increase in rate compared to the model reactions. Compound **2**, says Kaiser, "is the most efficient semisynthetic enzyme constructed to date" and it "approaches the activity displayed by all but the most efficient flavin-containing oxido-reductases known."

More recently, Kaiser, Czelaw Radziejewski, and Soumitra Ghosh have synthesized flavins with strong electron-withdrawing substituents (a chlorine atom or a cyano moiety) at the 7-position and have found that these are excellent catalysts for dihydronicotinamide reduction. Also, these undergo reaction with a variety of substrates containing chiral centers at their reactive functions, such as mandelic acid and benzoin. They are now attaching the flavins to papain and hope not only to get still better catalysts, but also catalysts where reaction occurs at a chiral center.

A new catalytic center does not have to be attached to the enzyme to achieve good results. Whitesides and Obsidiana Abril have developed a hybrid system that uses an organometallic complex in addition to an enzyme to regenerate NADH, a process that could be useful in the operation of enzymatic systems requiring NADH as a cofactor. The organometallic bis(phosphine)rhodium complex catalyzes the hydrogenation of pyruvic acid to lactic acid. The enzymes D- and L-lactate dehydrogenase then catalyze the oxidation of lactate back to pyruvate, in the process reducing NAD^+ to NADH. The net effect is that hydrogen is consumed to reduce NAD^+. Whitesides and Abril then coupled this system with the enzyme horse liver alcohol dehydrogenase, which requires NADH as a cofactor. The complete system could then carry out the reduction of (±)-2-norbornanone or cyclohexanone, using only catalytic amounts of NADH.

This system, unfortunately, has several shortcomings, says Whitesides. The rhodium complex has only modest catalytic activity toward pyruvate, and that step seems to be the rate-limiting factor in the system. The organometallic catalyst also seems to be poisoned relatively rapidly, presumably by sulfhydryl groups on the enzymes. Probably because of this deactivation, the investigators have not been able to scale up the reaction reproducibly. Nonetheless, the system does establish the possibility that biological and inorganic catalysts can coexist in one system. That type of linkage may eventually prove very useful to synthetic chemists.

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