Ribozymes, recognition and evolution

Precision in the recognition and orientation of substrate is important in the selectivity of catalysis by natural enzymes. Several new ribozyme species have been evolved using *in vitro* selection/mutagenesis which make use of precise substrate recognition to catalyze a variety of reactions.

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You must turn a key switch before starting a car, you must know the password before operating a computer system, and you must know the combination before opening a safe. All of these prerequisite recognition events are used to exert control, that is, to allow desirable events, while preventing unwanted ones. Researchers have been applying this principle to biological catalysis carried out by antibodies or ribozymes. In these systems, prerequisite recognition is programmed into macromolecules in order to exert control over the accelerated reactions. As is the case for natural enzymes, a substrate must bind to the macromolecule in a precise fashion before any reaction takes place.

A recent report describes a new chapter in this story. Dai *et al.* [1] have produced a ribozyme with hydrolytic activity sufficient to accelerate the cleavage of an unactivated amide bond. The ribozyme uses base-pairing to position the substrate precisely before reaction, so that only the amide bond is cleaved. This system is not the result of successful *de novo* design or even a simple selection procedure, but rather the directed *in vitro* evolution of the RNA ribozyme.

Enzymes use recognition to exert control

In general, synthetic organic chemistry methodology is based on inherent reactivity. That is, the product distribution of a given reaction will be determined by the inherent reactivities of the functional groups present on the reacting molecules, regardless of the product desired by the chemist. The key to synthesizing complex molecules is accurately predicting these reactivities, and adjusting the synthetic strategy accordingly. Chemists also use a variety of approaches to increase control over reactions. For example, the reaction conditions are adjusted to provide for equilibrium rather than kinetic product distributions, protecting groups are employed to block otherwise reactive groups, metal ions are used to chelate functional groups, thereby controlling molecular conformation, and specifically-designed metal ligands are used to modulate the reactions of a catalytic metal species, among other methods.

In contrast, Nature uses a prerequisite recognition event to exert control over chemical reactions. That is, a substrate must fit in the active site of an enzyme catalyst before the reaction takes place. This specific binding to the macromolecular catalyst achieves at least three levels of control over the catalyzed reaction. First, only desired substrates bind to the enzyme, so other molecules with similar inherent reactivities can be present in the solution, but do not react because they do not bind. This substrate selectivity allows many reactions to take place simultaneously inside living cells without appreciable interference from unwanted side reactions on other molecules. Second, once the correct substrate is bound, only the desired portion of the molecule is positioned near the catalytic machinery of the enzyme. Thus, the desired functional group takes part in a reaction, even if other functional groups of equal or greater inherent reactivity are present on the same substrate molecule. Finally, since enzymes are themselves chiral, the precise binding allows exquisite stereoselectivity in the catalyzed reaction.

Catalytic antibodies and ribozymes also control reactions using recognition

Researchers have been exploring ways of combining prerequisite binding to biological macromolecules with novel catalytic activity. The goal is to create highly selective and efficient catalysts not previously known in nature using molecules that are already known to have the capacity to recognize targets specifically. For example, catalytic antibodies are produced by immunizing an animal with a molecule that is designed to elicit catalytic activity in the resultant antibodies [2]. The classical approach to producing catalytic antibodies involves immunizing an animal with a stable molecule that resembles, in terms of geometry and/or charge distribution, the transition state of a desired reaction rather than the starting materials or products. The antibodies elicited by this so-called transition state analog are thus more complementary to the reaction's transition state than to the starting materials or products. Because of this complementarity, binding forces within the binding pocket of the antibody can lower the energy of the transition state, thereby reducing the overall energy barrier leading to catalysis of the reaction. As expected, catalytic antibodies exhibit Michaelis–Menten or saturation kinetics that are consistent with a mechanism in which the substrate must first bind in the antibody binding pocket, then undergo reaction. This prerequisite substrate binding, reminiscent of natural enzymes, means that catalytic antibodies can exert the same kinds of control over the catalyzed reactions as do natural enzymes. Best of all, this control can be programmed into the antibody catalysts by using an appropriately

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designed immunizing molecule to stimulate production of the antibodies. As a result, catalytic antibodies have now been produced that exhibit impressive levels of preprogrammed substrate selectivity, regioselectivity, and/or stereoselectivity while catalyzing a wide variety of different chemical transformations with values of k_{cat}/k_{uncat} that routinely exceed 10^3–10^4 [2–4].

The immune system has proven to be a fertile source of interesting new antibody catalysts, but proteins are not the only biological macromolecules capable of accelerating chemical processes. Certain RNA molecules, or ribozymes, can accelerate chemical transformations as well. Since the original discovery and characterization of the *Tetrahymena* group I intron [5], a large number of different RNA sequences have been shown to accelerate phosphoester transfer reactions [6,7]. In these cases, detailed kinetic analyses have indicated that the substrate oligonucleotide must bind via specific base-pairing interactions to the so-called internal guide sequence (IGS) of the ribozyme before the chemical reaction occurs. Thus, like natural enzymes and catalytic antibodies, ribozymes control the chemical reaction within their active site by requiring specific recognition of substrate to occur first. This specificity results in cleavage of only one of the several phosphoesters in an oligonucleotide substrate.

The recognition-dependent reactivity of all the biological catalysts is worth emphasizing, because it is one of the key features of natural enzymes that makes them so powerful. On the other hand, catalytic antibodies and ribozymes are not similar in very many other respects. Catalytic antibodies are proteins whose production is based upon mechanistic consideration of the reaction to be catalyzed, while most of the ribozymes produced *in vitro* result from a selection based on function. Of the two, catalytic antibodies have thus far been shown to bind a broader spectrum of substrates, and the reactions that they catalyze are more diverse.

RNA molecules accelerate reactions and also possess their own genetic information

As well as being chemically functional as a catalyst, RNA carries a genetic description of itself that can be replicated. This has led to the concept that an 'RNA world' may have evolved during the early history of the Earth [8–10]. If the notion of an RNA world has merit, then it is reasonable to expect RNA molecules to be able to catalyze a wide variety of chemical transformations, not just phosphoester transfer. The known repertoire of reactions catalyzed by RNA was expanded when Cech and coworkers [11] showed that the *Tetrahymena* group I intron could accelerate the hydrolysis of a carboxyester, appropriately placed within an oligonucleotide substrate.

The repertoire of ribozyme-promoted reactions was further increased when Schultz and coworkers [12] demonstrated that the transition state analog idea, successful with catalytic antibodies, could also be extended to RNA molecules. In this case, the transition state analog was a molecule that resembled the predicted transition state for a bond isomerization. Candidate RNA molecules were selected from an artificial library of RNA sequences based on their ability to bind the transition state analog species with high affinity. The selected molecules were amplified using the polymerase chain reaction (PCR) and reselected for their ability to bind the transition state analog covalently bound to a matrix. Seven rounds of selection/amplification yielded an RNA sequence that exhibited a k_{cat}/k_{uncat} value of 88 for the isomerization of a diastereomeric biphenyl compound. It remains to be seen whether this transition state analog approach can produce RNA species that can efficiently catalyze reactions involving bond cleavage and/or bond formation like those observed with catalytic antibodies.

New ribozymes have also been directly selected from a large (>10^{12} molecules) randomized pool of RNA molecules on the basis of their ability to accelerate a chosen reaction [13–19]. Generally, the ribozyme is self-modified during the course of the reaction, and acceleration of the self-modification reaction enables the preferential PCR amplification of the active ribozyme. Using this type of selection scheme, ribozymes have been selected that function as improved templates in a self-copying reaction [13] and tRNA-derived molecules have also been selected with an increased ability to undergo Pb^{2+}-dependent cleavage [19]. Using a similar procedure, Joyce and coworkers [20] have just reported the isolation of DNA sequences that accelerate the Pb^{2+}-dependent cleavage of a ribophosphoester embedded in a DNA molecule. It will be interesting to see if DNA molecules, presumed to be generally less capable of forming higher order structures, can be produced that show the same level of catalytic activity as RNA ribozymes.

Ribozymes can be improved by directed molecular evolution *in vitro*

Researchers have taken the selection of ribozymes an important step further by using directed Darwinian evolution *in vitro* on a molecular level [14–18, 21]. In these studies, acceleration of a chosen reaction is also used in a selection scheme for the isolation and subsequent amplification of desired RNA sequences. The key feature of the *in vitro* evolution approach is that the selected sequences are themselves subjected to limited random mutagenesis before undergoing the next round of reaction-based selection. As a result, catalytically active RNA sequences are continually evolved in a Darwinian sense, not simply isolated. Using this approach, ribozymes have been evolved *in vitro* that can accelerate phosphoester transfer reactions with altered metal dependence [15], accelerate an RNA ligation reaction [16], and catalyze a polynucleotide kinase reaction [17]. In related studies, novel guide sequences have been evolved that direct the efficient cleavage of mRNA by human ribonuclease P [21].

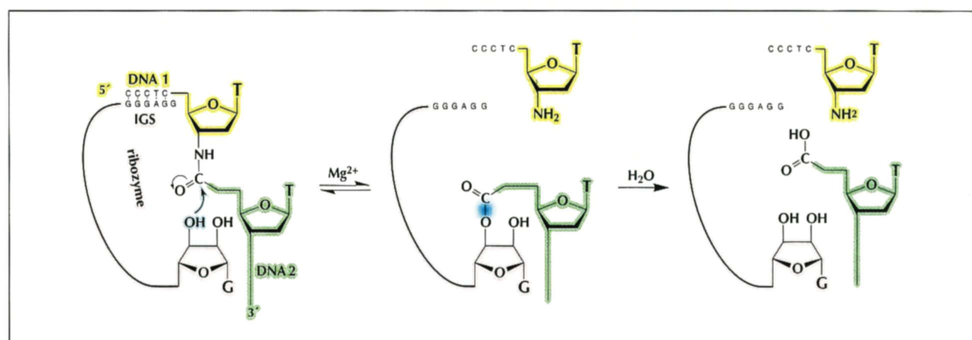

Fig. 1. Proposed mechanism for Mg^{2+} dependent ribozyme cleavage of an unactivated amide bond embedded in a DNA molecule. A short sequence at the 5' end of the DNA substrate hybridizes to the internal guide sequence (IGS) at the 5' end of the ribozyme (pink) positioning the single amide bond in the DNA sequence near the 3' terminal guanosine of the ribozyme. In the presence of Mg^{2+} the amide bond is cleaved resulting in release of DNA1 (yellow) and formation of a ribozyme-bound acyl intermediate with DNA2 (green). This intermediate undergoes spontaneous hydrolysis, releasing DNA2 and renewing the ribozyme for another round of catalysis.

A selection/mutagenesis scheme has also been used to evolve a ribozyme that uses a DNA substrate [14,18]. Following 27 rounds of mutagenesis/selection, RNA molecules evolved with 10^5-fold improved ability to carry out the Mg^{2+}-dependent cleavage of a DNA substrate compared with the wild-type group I ribozyme. Wild-type group I ribozymes prefer RNA substrates and possess a limited ability to react with DNA substrates. During the multiple rounds of mutagenesis/selection, selection conditions were intentionally altered, causing the population of ribozymes to shift in response. In all, an average of 17 mutations compared to the wild type were responsible for the improved DNA cleavage ability. As expected for ribozymes, kinetic analysis showed that the DNA substrate was specifically bound via base-pairing to the IGS of the evolved ribozymes before cleavage, explaining the observed reaction selectivity. Attempts are also being made to evolve the activity of recombinant catalytic antibodies by using the reaction catalyzed by the antibody to impart a selective advantage to the microorganism expressing it (see, for example, [22–24]).

An evolved ribozyme can accelerate cleavage of an amide bond

The DNA-cleaving ribozymes were next challenged with an unactivated amide group [1]. A modified DNA substrate was synthesized in which the reacting phosphoester function was replaced with an amide bond. These ribozymes were able to accelerate the cleavage of the amide with an estimated value of $k_{cat}/k_{uncat} = 10^3$–10^4 depending on the substrate used. The reaction was Mg^{2+}-dependent and probably involved a ribozyme-bound acyl intermediate (Fig. 1). This remarkable result indicates that directed evolution *in vitro* can produce ribozymes with the ability to accelerate difficult chemical transformations other than phosphoester transfer.

Results of ribozyme evolution studies have profound implications for theories about the evolution of an RNA world [14–18, 21]. Of particular interest are the RNA ligation activity reported by Szostak and coworkers [16] and the novel reactivities evolved by Joyce and coworkers for DNA substrates [14,18] as well as substrates with amide bonds [1]; the evolution of these three activities would be important steps in the conversion of an early RNA world into our present one. Analysis of the mutations that evolved to accelerate the reactions also provides an extremely comprehensive set of data on structure–activity relationships that can be used to delineate the details of ribozyme catalysis [16,18]. It will be particularly interesting to see if repetition of these evolution studies generally produces similar or different evolved ribozymes, and whether the same or different evolutionary paths are taken.

Reason for optimism

Evolved ribozyme species generally exhibit exquisite specificity while accelerating even difficult chemical transformations, due to the prerequisite binding of substrate. The possibility of exploiting this specificity of ribozymes *in vivo*, perhaps as highly specific therapeutic agents, has been discussed [25]. Of course, specificity is a double-edged sword, as anybody knows who has lost a car key, forgotten a computer password, or misplaced the combination of a safe. A major challenge will be to see if more general selection/mutagenesis schemes can be devised to use substrates of differing types, not simply oligonucleotides and to carry out reactions that do not involve self-modification. Szostak and coworkers [17] have recently described the selection of specific RNA 'aptamers' from a random-sequence RNA population that can bind a non-oligonucleotide target ligand (ATP) with high affinity and the evolution of catalytic activity from these aptamers. Despite this question of generality and other practical considerations [26], there is reason for optimism concerning the development of important new recognition-based catalysts using the *in vitro* evolution of ribozymes. After all, if the concept of an RNA world is correct, then all of the complexity of modern-day life, including us, must have eventually evolved from it.

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