Subject: Biochemistry

Production of Courseware

Content for Post Graduate Courses

Paper : 14 Protein Biochemistry and Enzymology
Module : 20 Ribozyme

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Biochemistry

Protein Biochemistry and enzymology

Ribozyme
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1. Objectives
   a. To understand what are ‘Ribozymes’, their characteristics and role in biological systems
   b. To explore the brief history of Ribozymes
   c. To understand in detail on various types of naturally occurring ribozymes
   d. Various applications

2. Description
   2.1. Ribozymes:

   Since from time immemorial it was believed that enzymes are always proteins, but later several catalytic RNAs were discovered, known as ‘Ribozyme’ or ‘RNAzyme’, which were capable of catalyzing biochemical reactions. The discovery of ribozymes revealed that RNA contributed to the ‘RNA world hypothesis’ showing its role in replication as well as in biological catalysis. Hence suggeststhat RNA might be the ancestor of the very first living form. The RNA due to its catalytic nature can cleave / ligate RNA & performs other biochemical reactions like peptide bond synthesis.

   Their major role in biological systems includes:
   a. Hydrolysis of phosphodiester bonds
   b. Formation of bonds
   c. Catalyze amino transferase activity of the ribosome
      eg: RNA Polymerase
          Amino acyl Transferase
          RNAse P
2.2. **Characteristics of Ribozymes:**

- **Ribozymes** are contrary to central dogma and are rare in the living entities, but with vital roles in life. Like the molecular machinery which converts messenger RNA into proteins is fundamentally the ribozyme; coordinates with Mg$^{2+}$ (cofactor).

- The original discovery of ribozymes was in viruses which use ribonucleic acid (RNA) for storage of genetic information. These small RNA's were able to cut themselves out of larger RNA molecules without the involvement of any enzyme. This unique property led to them being studied in detail. Later it was shown that these RNAs were also able to cleave other RNA molecules which contained the NUX pattern of nucleotides (where N stands for any nucleotide, U for uracil, and X for adenine, uracil, or cytosine). This therefore made them act like true enzymes.

- Ribozymes are present in the organelles of eukaryotes (nucleus, mitochondria and chloroplasts), amphibians, prokaryotes, bacteriophages, viroids and in satellite viruses that infect plants.

- The grouping of these RNA catalysts is based on their chemical type. However, regardless of the type, all RNA are associated with metal ions such as potassium (K$^+$) or magnesium (Mg$^{2+}$). These metal ions are known to play essential roles in catalyzing reactions.

- All ribozymes specifically catalyze reactions that modify themselves. It is because of this nature, that ribozymes cannot be considered as true enzymes or biocatalysts. Howvere there is one exception. RNase P is associated with processesing of the 5’P end of tRNA precursors.
2.3. Structure and mechanism of Ribozyme

Ribozymes have diverse structures and mechanisms using the four nitrogen base choices other than the diverse amino acids for protein structure. The tertiary structure of the ribozyme–substrate complex has been examined using a variety of biochemical, biophysical, genetic, and computational methods. By combining techniques like NMR spectroscopy, X-ray crystallography, time-dependent (FRET) fluorescence resonance energy transfer, hydroxyl radical foot printing, and photo-crosslinking revealed the structure of the active site. It was found to be comprised of multiple conserved functional groups, located at and around the interface of the two domains in the docked, active tertiary complex.

![Image showing diversity of ribozyme structure](Image)

Figure 2: Image showing diversity of ribozyme structure
(Leadzyme, hammer head, twister ribozymes)

Ribozymes follow Michael-Menten kinetics like enzymes. The reaction rate can increase up to $10^{11}$ times while reaction efficiencies ($k_{cat}/K_m$), can go up to $10^8$ M$^{-1}$ min$^{-1}$. The enhancement in the reaction rate as provided by ribozymes are indeed impressive, but still it is of the order $\approx 10^3$-fold times less than the rate enhancement which can be provided by enzymes which may catalyzing similar reactions. Additionally, ribozymes also cannot be compared with proteins in erms of turnover number In other words, they cannot be considered as multiple-turnover enzymes. This is mostly because the release of the product is so slow that it leads to easy saturation of the ribozyme’s catalytic site. This appears to be a limitation of ribozymes. However, since ribozymes by nature generally catalyze intramolecular, single-turnover reactions, it also reflects evolutionary constraints of the RNA enzyme.
An exhaustive comparison of the enzymatic mechanistic of protein and RNA enzymes has recently been made.

Ribozymes, known so far have been shown to possess an absolute requirement for a divalent cation, generally Mg\textsuperscript{2+}. The divalent cations are required not only for catalysis, but also for proper assembly of the tertiary structures, especially in case of large catalytic RNAs. Therefore catalytic RNAs are therefore considered to be metalloenzymes in nature. Based on analogy with the properties of protein metalloenzymes, a two-metal-ion reaction mechanism has been proposed in case of large catalytic RNAs. However, in case of small catalytic RNAs, the role of divalent cations is not much clear, but they are generally considered essential for catalytic purpose. But in model systems, it was found that divalent ions are not much required for trans-phenylanation.

Most of the ribozymes are involved in RNA processing. They either serve to cleave chains of precursor RNA, known as “molecular scissors” or ligate two molecules of RNA together, known as “staplers”. The various types of reactions catalyze by ribozymes include splicing, RNA ligation, oligo nucleotide chain extension, endonuclease action, phosphatase action etc. Ribozyme may also assist in the folding of the proteinaceous infectious particles (prions), similar to chaperonins and may be involved in the viral cleavage and packaging.
2.4. History of Ribozyme discovery

- In 1967, Carl Woese, Francis Crick, and Leslie Orgel suggested for the first time that RNA could act as a catalyst. This idea was based upon the discovery that RNA can form complex secondary structures.
- T.R. Cech discovered catalytic RNAs in the early 1980s.
- In 1982, Kelly Kruger coined the term ‘Ribozyme’ for these catalytic RNA and published in “Cell”.
- During mid-1980s Sidney Altman unraveled RNase P and its catalytic role at University of Yale.
- In 1989, T.R. Cech and S. Altman shared the Nobel Prize in chemistry for their contributions in unraveling the catalytic properties of RNA.
- Artificial ribozymes are RNA enzymes which are able to catalyze their own synthesis reactions under very specific conditions. An example of this is an RNA polymerase. Improved variants of the "Round-18" polymerase ribozyme were developed by mutagenesis from 2001. "B6.61" could add up to 20
bases to a primer template within 24 hours. The newer one, "tC19Z" ribozyme could add up to 95 nucleotides with fidelity of 0.0083 mutations/nucleotide.

2.5. Types of Ribozymes

Ribozymes may be classified into natural ribozymes and artificial ribozymes. Natural ribozymes are discovered from different domains of life, while artificial ribozymes are synthesized in the laboratory based on the dual nature of RNAs as a catalyst and an informational polymer.
2.5.1. Natural Ribozymes:

- **Peptidyl Transferase 23S rRNA**: Peptidyl transferase is an aminoacyl transferase (EC2.3.2.12) which is associated with the formation of peptide bonds between adjacent amino acids during the translation process. It is also the primary enzymatic function of the ribosome. Peptidyl transferase activity is only mediated by ribosomal RNA (rRNA) has been well proven by site directed mutational studies. In prokaryotes, the 23S component of 50S ribosome and in eukaryotes, the 28S component of 60S ribosome performs the peptidyl transferase activity.
b. **RNAse P**: It’s ubiquitous endo ribonuclease and present in Archaea, Eubacteria, Eukaryotes and organelles. It is well known as true RNAzyme as it processes 5′ Leader sequences of t-RNA. Human RNAse P has also recently discovered with the role in transcription of small non-coding RNAs like U6snRNA, 5SrRNA, tRNA, SRP RNA etc. RNase P is known as a natural multiple turnovers RNAzyme also.

- **Bacterial RNAse P** has two components: Both components necessary for ribozyme function are M1RNA and C5 protein.
- **In archaea, bacteria ribonucleoproteins** comprise of protein subunits (4-5) with RNA component. *In vitro* reconstitution studies revealed that protein subunits are individually dispensable for tRNA processing which is very well mediated by the RNA counterpart.
- **Eukaryotic** RNAse P is more complex in terms of associated proteins. 9-10 proteins is associated with the RNA chain, among which five exhibit homology to archae bacteria. The protein subunits play a catalytic role in rRNA processing with in nucleolus. These protein subunits of RNase P are shared with RNase MRP, a catalytic ribonucleoprotein which is involved in the processing of ribosomal RNA within the nucleolus.
c. **Group I & II introns:**

Group I & II introns belong to large self-catalytic RNAs, involved in the processing of mRNA, tRNA & rRNA. The secondary structure comprised of 9 paired regions (p1-p9); with p4-p6 domains and p3-p9 domains. Group I introns normally have long ORFs. The mode of GTP dependent catalysis includes 2 sequential trans-esterification.

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**Figure 7. Structure of RNAase P**

**Figure 8. Group I Introns**
Group II introns are another type of autocatalytic RNAs present in all domains of life. The catalytic activity even was found at hyper saline conditions *in vitro*; however protein counter parts are associated for *invivo*-splicing. Group II performs GTP independent intron excision involving lariat intermediate. Spliceosome has also evolved from group II introns with a structural similarity of Domain V substructure V6/V2 extended SnRNA. Site-specific mobilization to new DNA regions explored can be used as a tool for biotechnology.

d. **GIR – 1 Branching Ribozyme:**

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**Protein Biochemistry and enzymology**

**Ribozyme**
The discovery of GIR-1 ribozyme resulted from the functional characterization of introns from extrachromosomal recombinant DNA of *Didymium iridis*. GIR-1 branching ribozyme, now also known as lariat capping ribozyme and has about 179 nucleotides. Another complex type of group I introns also known as twin-ribozyme introns catalyze.

e. **Leadzyme:** It is a small ribozyme, also known as ‘metallo-ribozyme’ due to its obligatory requirement of lead for catalysis. Leadzyme comprise of an asymmetric internal loop with six nucleotides and a helical region on each side of the internal loop; which acts as the site for cleavage. It specifically cleaves the phosphodiester bond. Even though it was discovered in artificial system the natural counterparts are in 5SrRNA.
f. **Hairpin Ribozyme:**

Hairpin ribozymes are small ribozymes found in RNA satellites of plant viruses. It assists in the cleavage & ligation of products of rolling circle replication to form circular and linear satellite RNAs. Even though these are similar to hammer head ribozyme, these do not require a metal cofactor for the reaction.

- satellite RNA of - tobacco ringspot virus(sTRSV), chicory yellow mottle virus (sCYMV), arabis mosaic virus (sARMV)


g. **Hammerhead Ribozyme:** The hammerhead ribozyme has been well studied as a model system for unraveling the structure and function of RNAs for therapeutic purposes. This is also a metallo-ribozyme. It plays a role in reversible cleavage and joining reactions at very specific site within RNA. It was named for its resemblance of the early secondary structure diagrams to a hammerhead shark. Hammerhead ribozymes RNAs were discovered initially in satellite RNAs and viroids (plant viruses). The self-cleavage reactions, first reported in 1986, mediated via rolling circle model of replication. The hammerhead sequence which forms a conserved three-dimensional tertiary structure (helices I, II &III) is sufficient for self-cleavage.
Figure 13. Hammerhead Ribozyme

h. **HDV Ribozyme:** The **hepatitis delta virus (HDV) ribozyme** found in the hepatitis delta virus plays role in viral replication. It is the only known ribozyme required for the viability of a human pathogen. It is the quickest known naturally occurring ribozyme. It possesses five segments which are helical connected by a double pseudoknot and found to be active in vivo even in absence of any protein factors. Studies also revealed the presence of an active-site cytosine and a divalent metal facilitating the cleavage reaction. The HDV ribozyme is very much similar to another group known as Mammalian CPEB3 ribozyme.

i. **Mammalian CPEB3 Ribozyme:** A genome-wide search for ribozymes revealed an HDV-like RNA homology in the human CPEB3 gene, known as mammalian CPEB3 ribozyme. In humans the homology region has been associated with mRNA polyadenylation. This catalytic RNA is highly conserved and found only among mammals. While other HDV-like ribozymes have been identified to be active in vitro in other eukaryotes.

j. **VS Ribozyme:** VS or Varkud satellite ribozyme is the largest known natural nucleolytic RNA without any available structural data and found as embedded in VS RNA. This satellite RNA is found in mitochondria of Varkud-IC and some fungal strains like Neurospora. The catalytic action includes a reversible cleavage and ligation by trans-esterification involving 2’ and 5’ oxygen and 3’ phosphorous atoms. VS ribozyme is unique in its primary, secondary and tertiary structure was unraveled by probing and mutagenesis studies. The active site appeared to be within helix VI and A 730 loop was found to have an intimate contact with the substrate. Another A756 was also revealed to have direct nucleobase participation in the phosphoryl transfer chemistry.
k. **Glms Ribozyme (the metabolite-responsive self-polymerizing ribozyme):** The Glucosamine-6-phosphate activated ribozyme act as a riboswitch, which is involved in the regulation of genes in response to the active concentrations of a metabolite i.e. Glucose-6-phosphate. Bioinformatic screening of 5' untranslated regions of glmS genes revealed its role and have evolved as the first ribozyme which require binding of an exogenous small molecule for activity. The GlmS enzyme is dependent on GlcN6P to achieve its self-catalysis which leads to the degradation of the mRNA, and thus reduction in GlmS enzyme, encoded by the glmS gene. It possess a double pseudo-knotted structure suggests that the amine group of GlcN6P is involved in the catalytic action. Binding of the ligands to conventional riboswitches that couple folding to binding is often enthalpically driven. The thermodynamics of the natural and unnatural ligand binding by much more rigid and pre-organized glmS ribozyme remain to be studied.

l. **CoTC Ribozyme:** The transcriptional termination of human β globin gene analysis revealed a new phenomenon-CoTCie.Co-transcriptional cleavage. This consists of an initial pre-termination cleavage(PTC) co-TC mainly involves the self-cleaving activity of 3 flanking region of β globin gene.

2.5.2. **Artificial Ribozymes:** After the discovery of natural ribozymes, the interest in the study of synthetic or engineered ribozymes has considerably risen. Tang and Breaker isolated self-splicing RNAs for the very first time by in vitro selection. Even though most of them were structurally similar to naturally occurring hammerhead ribozymes, few had novel structures as well. In 2015, Michael Jewett and Alexander Mankin using directed evolution approach have engineered aribosome and are named as ‘Ribosome-T, or Ribo-T.' Lincoln and Joyce developed a novel Ribozyme system, fastest till date, which are capable of self-replication within sixty minutes. A pair of ribozymes emerged by utilizing molecular competition of a candidate RNAmixture. Each ribozyme in this pair synthesizes the other by joining together the synthetic oligonucleotides, in the absence of any protein. Along with this, the development of ‘aptozymes’, which are engineered riboswitches, has also been an area of high interest and active research.

Riboswitches are regulatory RNA motifs capable of changing their structure in response to ligand for regulation of protein synthesis. A large number of riboswitches, which occur naturally, are known to bind a vast array of metabolites. An exception to this is one ribozyme based on a riboswitch (glmS). Early
work in characterizing riboswitches was performed using theophylline as the ligand. It was found that an RNA hairpin structure is formed to block the ribosome binding site. In the presence of ligand theophylline, the regulatory RNA region is cleaved off, allowing the ribosome to bind and proceed for translation. Thymine pyrophosphate is a recently found ligand which acts on riboswitches. (FACS) has been commonly used to engineer aptozymes. Flexizyme, a ribozyme which can catalyze the amino acylation of tRNA with a wide range of natural and unnatural amino acids.

2.6. **Applications:** The various applications of natural and artificial Ribozymes include:

- **Therapeutic:** Catalytic RNAs have simple structure, site-specific cleavage activity, and catalytic action and therefore they are effective modulators of gene expression because of their. Under the broader domain of gene therapy, such catalytic RNAs have been studied, proposed, engineered and developed for treating various diseases. The most important challenge which one comes across during the use of RNA based enzymes for therapeutic applications is its short half-life. To circumvent this, the 2’ position on the ribose moiety is modified. The inhibition of RNA-based viruses (both plant & animal) has been one major thrust area of ribozyme gene therapy. A synthetic ribozyme which is directed against HIV RNA called “gene shears” has entered the phase of clinical testing. Similarly, another ribozyme for targeting the hepatitis C virus RNA has been recently designed.

**Conclusion**

Thus ribozymes are contrary to central dogma, a proof for pre-RNA life. They exist in natural and artificial forms in wide variants with immense therapeutic applications.