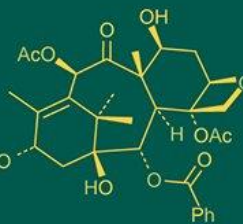
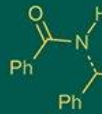


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; 10(3): 251-261
www.biochemjournal.com
Received: 15-11-2025
Accepted: 01-01-2026

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Enzymes mechanism and regulation

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i3d.7841>

Abstract

Enzymes are not mere molecular workhorses; they are dynamic, regulatable, and evolvable machines that shape every cellular process. This review weaves classical and contemporary perspectives to present an integrated view of enzyme mechanism and regulation. Beginning with foundational kinetic concepts and transition-state stabilization, we examine how modern structural and dynamic studies reframe catalysis as a product of transient conformational cycles and finely tuned energy landscapes. The essay then surveys powerful experimental approaches notably radioisotope exchange and positional isotope experiments that reveal microscopic steps and hidden intermediates inaccessible to bulk assays. Moving from mechanism to control, we consider multiple layers of regulation: fast, reversible allostery and feedback inhibition; durable covalent switches such as phosphorylation and acetylation; spatial control via compartmentalization; and emerging network-level metabolite-activation circuits. At the systems scale, enzyme filamentation and bifunctional proteins emerge as architectural strategies that tune flux and confer robustness. The review also highlights evolutionary themes that link catalytic promiscuity to the rise of pseudoenzymes, and describes extremozymes and catalytic nucleic acids (ribozymes, DNazymes) as examples of nature's catalytic repertoire beyond canonical proteins. Throughout, examples and recent modeling studies illustrate how single-enzyme behavior scales to pathway and cellular phenotypes. Finally, we outline methodological gaps and future directions from high-resolution time-resolved structural methods to genome-scale kinetic reconstructions emphasizing how deeper mechanistic insight will accelerate therapeutic design and metabolic engineering. By synthesizing mechanistic, structural, evolutionary, and systems viewpoints, this review aims to redefine enzymes as integrated nodes of chemistry and control.

Keywords: Enzyme mechanism, allostery, enzyme regulation, bifunctional enzymes, pseudoenzymes, enzyme promiscuity, extremozymes, catalytic nucleic acids

Introduction

Enzymes lie at the centre of life's chemistry without them, even the simplest metabolic reactions would crawl at rates incompatible with biological survival. As Robinson (2015) ^[41], emphasizes, enzymes are remarkable catalysts capable of accelerating reactions by factors of millions to trillions through an exquisite ability to lower activation energy. This catalytic power is not merely a biochemical curiosity; it is the essential foundation that drives cellular respiration, photosynthesis, DNA replication, signaling, and virtually every physiological process. Unlike inorganic catalysts, enzymes function with high specificity, frequently recognizing only a single substrate, and doing so with precision and regulation that allows life to remain organized and responsive. The study of catalytic mechanisms continues to unfold as structural and kinetic insights accumulate. Schramm (2006) ^[42] described enzymatic catalysis not as a simple lock-and-key interaction but as a dynamic process determined by stabilization of the transition state, an idea that transformed modern enzymology. Similarly, Agarwal (2018) ^[1] highlighted the role of protein conformational flexibility and dynamic motion, showing that enzymes are not rigid scaffolds but continuously shifting molecular machines whose internal movements drive catalysis. The work of Cárdenas and Bowden (2013) reinforced the historical evolution of enzyme kinetics, explaining how early models from Michaelis and Menten provided a simplified but foundational understanding of catalytic rate behavior. However, as Goldbeter (2013) ^[24] later stressed, real enzyme systems often display oscillatory behavior, cooperativity, and non-linear kinetics that cannot be captured by classical models.

Reiss *et al.*, (2020) ^[50] explored how allosteric regulation evolved as a central control strategy, where effector molecules bind at regulatory sites to alter enzyme conformation. The work of Holzer and Duntze (1971) ^[29] earlier paved the conceptual foundation by showing that allosteric enzymes operate as cooperative systems whose activity is fine-tuned through multi-subunit communication rather than independent catalytic units. Building on these ideas, Borkakoti (2025) ^[5] demonstrated that cooperativity and allostery allow enzymes to operate like biochemical switches, responding smoothly or abruptly to small changes in metabolite concentration. Enzyme regulation also extends beyond allostery. Post-translational modifications such as phosphorylation, acetylation, and ubiquitination rapidly reorganize metabolic priorities within cells. Although not the earliest voice in this field, recently pointed out how our interpretation of enzyme kinetics must be grounded in accurate parameter estimation, since biological regulation often modifies kinetic constants in real time. This perspective aligns with Scrutton (2023) ^[43], who noted that emerging experimental and computational tools are revealing complexities previously unappreciated such as transient catalytic intermediates, multi-step conformational cycles, and dynamic energy landscapes.

Enzyme evolution and diversity add yet another dimension. Ribeiro *et al.*, (2022) ^[51] examined catalytic mechanism similarity across enzyme lineages, illustrating that nature often repurposes structural frameworks to develop entirely new biochemical functions. This reinforces a growing view that enzyme structure, rather than sequence alone, determines mechanistic potential. Meanwhile, genomic advances continue to uncover new enzyme families whose activities challenge traditional categories of enzyme classification.

Together, these advances paint a picture of enzymes not as static catalysts but as dynamic, evolvable, and exquisitely regulated molecular systems. This review synthesizes current knowledge on enzyme mechanisms and regulatory strategies, integrating structural, kinetic, evolutionary, and systems-level perspectives to illuminate how these remarkable biomolecules sustain the chemistry of life. Understanding enzyme mechanism and regulation is therefore not an abstract academic pursuit, but a key pathway to designing novel therapeutics, engineering metabolic pathways for biotechnology, and interpreting disease processes at a molecular level.

Beyond classical catalytic mechanisms and kinetic regulation, modern enzymology explores deeper layers of mechanistic understanding involving radioisotope tracer approaches, multifunctional enzymes, pseudoenzymes that regulate without catalysis, enzyme promiscuity that drives metabolic innovation, extremozymes adapted to harsh environments, and catalytic nucleic acids such as ribozymes and DNAzymes. These emerging areas reshape our perception of how enzyme mechanisms and regulation operate in real biological systems.

Mechanism determination by radioisotope exchange

Understanding how an enzyme actually converts substrates into products requires more than measuring overall rates. It demands methods that can follow individual atoms through

the reaction pathway. Radioisotope exchange experiments provide exactly that. By introducing a radioactive or stable isotope label (for example, ^{14}C , ^{32}P , ^3H , or ^{18}O) at a defined position in a substrate or product, and then observing how that label redistributes during catalysis, one can infer the presence of intermediates, the order of binding and release, and even whether the reaction proceeds by a single-step or multi-step mechanism.

As Fromm (1975) ^[21] pointed out in an early methodological chapter, isotope exchange is “one of the single most useful procedures for studying the kinetic mechanism of enzyme action,” because it can reveal mechanistic features that are invisible in ordinary steady-state assays where only initial velocity is measured. In a typical experiment, an enzyme is allowed to reach a near-equilibrium mixture of substrate and product. A small amount of isotopically labeled substrate or product is then added, and the rate at which the label appears in the opposite pool is monitored. A measurable exchange of label, even in the absence of net product formation, is strong evidence that the enzyme catalyzes reversible microscopic steps within its catalytic cycle.

A classic conceptual framework for these experiments was laid out by Rose (1979) ^[52], whose review on *positional isotope exchange* showed how carefully placed labels can distinguish between different multi-step mechanisms. In positional isotope exchange, the label does not simply move between substrate and product; instead, it redistributes within a single molecule (for example, between different oxygen atoms of a phosphate group) as the enzyme transiently breaks and reforms specific bonds. Detecting such intramolecular scrambling indicates that the enzyme passes through a covalent or tightly bound intermediate in which the labeled group has time to reorient before the reaction completes.

Isotopes are also used at a more general level to study reaction mechanisms, not just in enzymes but in organic and metabolic chemistry. Cook (1988) ^[13] described how isotopic labels can act as “markers” for specific atoms, allowing chemists to track where those atoms end up after a reaction and thus discriminate between competing mechanistic proposals. When this logic is applied to enzymes, tracer studies can, for example, determine whether a carboxyl group in a citric acid cycle intermediate originates from CO_2 , from acetate, or from internal rearrangement within the molecule.

Role of enzymes in regulation of metabolism

Cells depend on more than just catalytic speed they require control. Metabolism must respond dynamically to changes in environment, nutrient availability, energy demand, stress, and developmental signals. Enzymes thus play a double role: they catalyze reactions, and they act as regulatory nodes that integrate biochemical fluxes, signaling, and adaptation.

Table 1: Examples of radioisotope-based approaches for probing enzyme mechanisms

Type of isotope experiment	What is labeled / measured	Mechanism	Reference
Equilibrium isotope exchange	Substrate or product pool labeled (<i>e.g.</i> , ATP or Pi) in a reaction at or near equilibrium	Demonstrates reversibility of individual steps; evidence for enzyme-bound intermediates even when there is no net product formation	Boyer, (1978) ^[6] .
Positional isotope exchange (PIE)	Label at a specific atom within a group (<i>e.g.</i> , one oxygen of phosphate) and redistribution within the same molecule is monitored	Indicates formation of covalent or tightly bound intermediates that allow intramolecular scrambling; distinguishes single-step vs multi-step pathways	Rose (1979) ^[52]
Steady-state isotope exchange kinetics	Time-dependent exchange of label between substrate and product under steady-state turnover conditions	Allows derivation of kinetic schemes, including binding order and presence of multiple binding sites or allosteric behavior	Darvey (1973) ^[16] .
General isotope tracer studies	Labeled atoms (<i>e.g.</i> , ¹⁴ C or ¹³ C) in metabolic intermediates	Map metabolic pathways, cyclic vs linear routes, and origin of specific atoms in products	Cook (1988) ^[13] .
Kinetic isotope effects (KIEs)	Substrate analogues differing only in isotopes at key bonds (<i>e.g.</i> , H vs D)	Identify which bond cleavage is rate-limiting; provide information on transition-state structure and tunneling	Fromm (1975) ^[21] .

Recent research over the last few years has expanded and refined our understanding of how enzymes regulate metabolism often via mechanisms far more elaborate than classical allosteric inhibition or feedback loops. Several major themes have emerged: enzyme-metabolite activation networks, structural reorganization (polymerization/filament formation), post-translational modifications (especially acetylation), metabolic reprogramming in specialized contexts (like immune cells), and integration of metabolism with gene regulation.

Allosteric regulation: rapid, reversible tuning of flux

One of the most elegant ways cells control metabolism is via allosteric regulation binding of small molecule effectors at sites other than the catalytic (active) site, which induces conformational changes that alter enzyme activity (increase or decrease) (Mingione *et al.*, 2023) ^[53].

- Allosteric enzymes often govern rate-limiting steps in pathways, acting as metabolic “valves” that open or close depending on cellular conditions. For example, recent structural work on human Phosphofructokinase-1 (PFK1) a key control point in glycolysis revealed how binding of allosteric effectors reshapes its structure, changing enzyme activity and thus influencing glycolytic flux (Lynch *et al.*, 2024) ^[54].
- The diversity of allosteric regulation is remarkable: binding of cofactors, substrates, products, or unrelated small molecules can shift the enzyme’s conformation ensemble effectively biasing it toward active or inactive conformations (McCullagh *et al.*, 2024) ^[55].

Allosteric regulation is powerful because it’s fast, reversible, and sensitive: small changes in effector concentration translate into large changes in enzyme flux enabling cells to rapidly adapt metabolism.

Feedback inhibition & metabolic homeostasis

Allosteric regulation often underlies feedback inhibition, a strategy whereby the end-product of a pathway binds to and inhibits the first committed enzyme of that pathway preventing overproduction and conserving resources (Malathi 2023).

- This is essential for biosynthetic pathways (amino acids, nucleotides, vitamins), where balance of intermediates is critical.
- Feedback inhibition, often combined with cooperativity (sigmoidal kinetics) and allostery, ensures smooth regulation rather than abrupt on/off switching - useful when metabolic demand fluctuates moderately (Naz *et al.*, 2023) ^[56].

Thus, feedback inhibition via allosteric binding helps maintain metabolic homeostasis in changing internal or external environments.

Covalent modification: “molecular switches” for longer-term control

Beyond fast, reversible control, cells often use covalent modification (*e.g.*, phosphorylation, acetylation, redox modifications) to regulate enzyme activity - allowing integration of hormonal signals, stress responses, or longer-term metabolic adjustments (Koshland 1970) ^[34].

- For instance, in mammalian metabolism, lysine acetylation has emerged as a key regulator: many metabolic enzymes are acetylated, which can alter their catalytic activity, substrate accessibility, or stability - thus affecting metabolic flux at multiple levels (Xiong and Guan 2012) ^[49].
- Post-translational modifications (PTMs) allow enzymes to act as molecular switches: turned on/off or modulated in efficiency in response to cellular signals such as hormones, nutrient levels, or stress - enabling metabolic reprogramming.

This mode of regulation is slower than allostery, but often more durable - bridging the gap between acute response and long-term adaptation.

Compartmentalization & substrate accessibility

Physical separation of metabolic pathways into subcellular compartments is another critical layer of control. By localizing enzymes and substrates in different organelles (*e.g.*, mitochondria, cytosol, and peroxisomes), cells can prevent unwanted cross-reactions and channel metabolites efficiently through pathways.

- Compartmentalization helps avoid futile cycles (*e.g.*, simultaneous synthesis and degradation), reduces diffusion losses, and preserves unstable intermediates.
- This spatial organization also allows regulation by substrate availability, local pH, cofactors, and redox environment all of which vary among compartments.

Thus, compartmentalization complements allosteric and covalent regulation to ensure metabolic efficiency and fidelity (Metallo and Vander Heiden 2013) ^[39].

Metabolite-enzyme activation networks & cross-pathway regulation

A recent landmark study by an enzyme activation network reveals extensive regulatory crosstalk between metabolic

pathways (Zubaidi *et al.*, 2025) ^[3] reconstructed a genome-scale metabolic network (in yeast) integrated with kinetic data, revealing that activator metabolites are widely distributed across virtually all metabolic pathways. They found that many enzymes not just those at the start of a pathway are subject to activation by small-molecule metabolites. This suggests that regulation is not limited to “committed steps” or classical feedback inhibition; instead, entire metabolic networks may be dynamically re-wired by changes in metabolite levels. Importantly, highly activated enzymes tended to be non-essential under standard conditions, indicating that metabolic plasticity and adaptability may be controlled through such activation networks (Donati *et al.*, 2018) ^[18].

Structural reorganization: enzyme filaments & polymerization

Beyond small-molecule regulation, enzymes are increasingly recognized as dynamic structural entities that can self-assemble into higher-order architectures filaments, polymers, or clusters in response to metabolic cues. In the review Mechanisms of metabolic regulation by enzyme filaments (Hvorecny & Kollman, 2023) ^[30], the authors describe how a variety of metabolic enzymes especially those in intermediary metabolism form filamentous structures when substrate or metabolite concentrations change.

Filament formation modulates enzyme activity often repressing under one condition and activating under another thereby offering a regulatory layer that is faster than gene expression changes and more flexible than classic allostery. This phenomenon is further exemplified by Metabolic regulation by enzyme polymerization (Kollman, 2024), which illustrates ongoing work in nucleotide-biosynthesis enzymes showing how self-assembly regulates flux in *de novo* pathway.

Post-translational modifications and metabolic enzyme regulation

Another layer of regulation comes from chemical modifications of enzymes especially acetylation which

modulate enzyme activity, stability, or localization. The review Mechanistic insights into the regulation of metabolic enzymes by acetylation (Xiong & Guan, 2012) ^[49] remains a foundational reference: almost all major metabolic enzymes in glycolysis, gluconeogenesis, TCA cycle, fatty-acid oxidation, amino acid metabolism, oxidative phosphorylation - are subject to lysine acetylation (Xiong and Guan 2012) ^[49].

Metabolism, signaling, and immunometabolism

Metabolism does not operate in isolation; it intersects markedly with signaling pathways, cell differentiation, immune response, and disease states. The review The Role of Metabolic Enzymes in the Regulation of Inflammation (Godfrey and Kornberg 2020) ^[22] has demonstrated how certain metabolic enzymes act beyond basic metabolism regulating inflammation by controlling flux through metabolic pathways or producing metabolites that act as signaling molecules.

Bifunctional enzymes

Enzymes that carry out two distinct catalytic activities in a single polypeptide chain known as bifunctional enzymes represent an intriguing and efficient biological design. Rather than relying on separate enzymes for successive or related reactions, a bifunctional enzyme merges both functionalities into one molecular unit. This fusion offers tight coordination, efficient substrate channeling, and effective regulation of metabolic flux (Moore 2004; Raben 2013) ^[40, 57].

What is a bifunctional enzyme?

A Bifunctional enzyme catalyzes two different biochemical reactions. These may be sequential steps in a pathway or two distinct functions that are physiologically linked. Often the two catalytic activities reside in distinct structural domains within the same polypeptide (Moore 2004) ^[40]. Because the two activities are encoded together, gene regulation, protein folding, and degradation become more streamlined an efficient arrangement for critical metabolic control points (Fig.1).

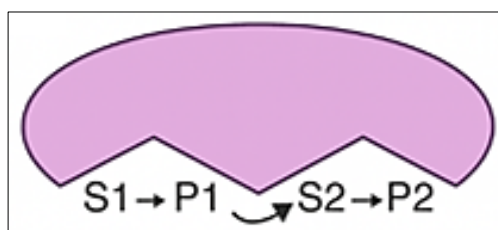


Fig 1: A bifunctional enzyme with two catalytic domains. (S1, substrate 1; S2, substrate 2; P1, product 1; P2, product 2.)

6-Phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (pfk-2/fbpase-2)

Perhaps the best-studied bifunctional enzyme is PFK-2/FBPase-2. In a single polypeptide chain (Fig.2):

- The PFK-2 domain phosphorylates fructose-6-phosphate (F6P) using ATP to produce fructose-2,6-bisphosphate (F2,6BP),
- The FBPase-2 domain hydrolyzes F2, 6BP back to F6P and inorganic phosphate (Raben 2013) ^[57].

Because F2, 6BP is a potent allosteric regulator of central metabolism - activating the committed step of glycolysis (Phosphofructokinase-1, PFK-1) while inhibiting

gluconeogenesis PFK-2/FBPase-2 functions as a metabolic “switch”: tipping the balance between glycolysis and gluconeogenesis depending on physiological needs.

Emerging Insights: bifunctional enzymes and network robustness

Beyond classical metabolic control, recent theoretical and computational studies reveal a deeper, systems-level advantage of bifunctional enzymes. According to a 2024 study, incorporation of “paradoxical” bifunctional enzymes - which both produce and degrade a substrate under different contexts can impart absolute concentration robustness in biochemical networks. In other words, certain

metabolite concentrations remain stable despite fluctuations in enzyme expression or substrate supply, conferring robustness to the system (Joshi and Nguyen 2025)

A 2025 follow-up work extended this concept using a hypergraph-based modeling approach to show that such robustness along with stable steady states can emerge even without detailed kinetic parameters, implying that

bifunctionality itself is a design principle for metabolic stability (Joshi and Nguyen 2024) ^[32]. Thus, bifunctional enzymes may not only optimize local reaction efficiency but also support global metabolic homeostasis by buffering against perturbations: a beneficial characteristic especially in fluctuating environments or varying cellular states.

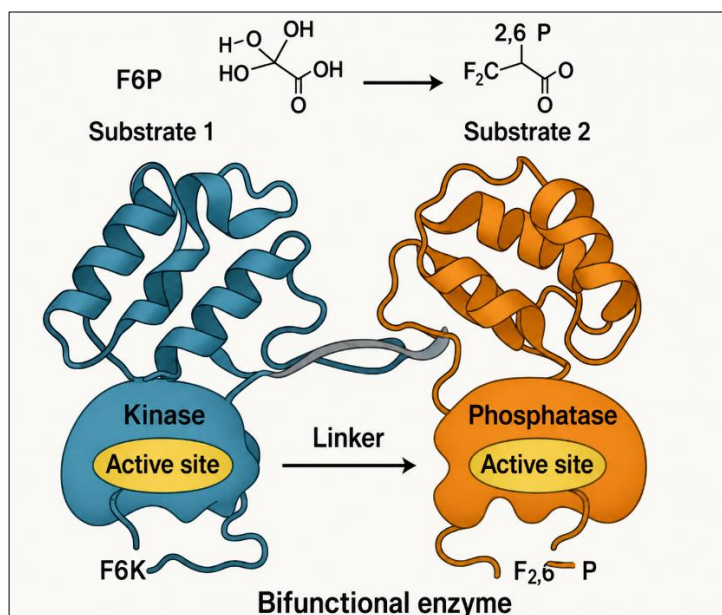


Fig 2: structural representation of a bifunctional enzyme showing two distinct catalytic domains and substrate binding sites.

Advantages & significance of bifunctionality

Integrating two enzymatic activities in one polypeptide gives several advantages:

- Tight regulation and coordinated control of sequential steps: ensures that production and consumption of an intermediate are closely balanced.
- Substrate channeling: reduces diffusion losses and protects labile intermediates.
- Robustness to perturbations: as shown by recent systems biology studies helps maintain steady metabolite levels despite environmental or cellular fluctuations.
- Reduced genetic and translational burden: a single gene and translational product instead of two separate ones; useful in compact genomes of microbes or efficient regulation in higher organisms.
- Flexibility in regulation: different domains can be regulated independently (*e.g.*, by post-translational modification, isoform expression), allowing dynamic control depending on tissue, developmental stage, or stress.

Pseudoenzymes

Pseudoenzymes are a unique class of protein relatives that structurally resemble active enzymes but lack detectable catalytic activity due to mutations in one or more residues essential for catalysis. Although originally considered evolutionary relics or non-functional paralogs, current evidence shows that pseudoenzymes play crucial regulatory and signaling roles in cells (Jeffery, 2020) ^[31]. They function

as molecular scaffolds, allosteric modulators, signaling switches, and binding partners that modulate the activity of their catalytically active counterparts. Pseudoenzymes thus expand the functional diversity of enzyme families by providing non-catalytic but biologically essential regulatory functions (Goldberg and Sreelatha, 2023) ^[23].

Structural basis of loss of catalysis

Most pseudoenzymes originated through gene duplication events followed by functional divergence. The key feature defining pseudoenzymes is the disruption of canonical catalytic motifs. For example:

- **Protein kinases** typically require the conserved HRD and DFG motifs for ATP binding and phosphotransfer. Many pseudokinases lack the catalytic Asp in the HRD motif or the Gly-rich loop required for ATP coordination (Hassan and Noor, 2022) ^[26].
- **Proteases** often lose the nucleophilic serine, cysteine, or the metal-coordinating histidine residues essential for peptide bond cleavage.
- **Phosphatase pseudoenzymes** lack the invariant cysteine in the HCX5R motif responsible for nucleophilic attack (Reiterer *et al.*, 2020) ^[58].

Despite these losses, pseudoenzymes often retain near-native 3D structures. This structural preservation enables them to interact with substrates, cofactors, or partner proteins even though they cannot catalyze reactions (Fig. 3).

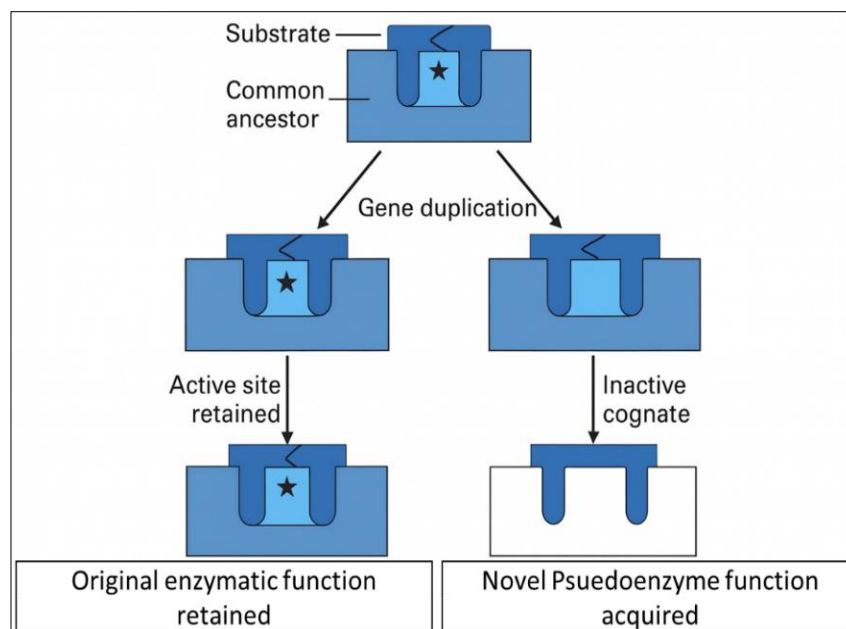


Fig 3: Structural Divergence after Gene Duplication Producing Enzymes and Pseudoenzymes

Functional roles of pseudoenzymes

1. Molecular scaffolds

Pseudoenzymes frequently act as scaffolds that bring together active enzymes and their substrates. By stabilizing multiprotein complexes, they control the spatial and temporal dynamics of signaling pathways (Ribeiro *et al.*, 2019) ^[59].

2. Allosteric modulators

Some pseudoenzymes bind ATP or other small molecules even without catalysis. This ligand binding induces conformational changes that regulate nearby catalytic domains. The pseudokinase MLKL, for instance, binds ATP to undergo conformational activation during necroptosis signaling (Tomoni *et al.*, 2019) ^[60].

3. Competitive inhibitors

Because of their structural similarity to active enzymes, pseudoenzymes can compete for substrate or binding partners, thereby acting as dominant-negative regulators. Pseudophosphatases in the DUSP family inhibit MAPK pathways by occupying substrate-binding surfaces without performing dephosphorylation (Hepworth and Hinton, 2021) ^[27].

4. Signaling switches

Pseudoenzymes function as reversible switches in pathways that require rapid “on/off” responses. The pseudokinase STRADA activates the tumor suppressor LKB1 by forming a heterotrimeric complex that mimics active kinase-substrate assembly, enabling LKB1 to phosphorylate AMPK family kinases (Reiterer *et al.*, 2020) ^[58].

As highlighted in Table 2, pseudoenzymes are distributed across multiple enzyme superfamilies, where they adopt

specialized non-catalytic functions such as scaffolding, allosteric regulation, substrate sequestration, and modulation of signaling complexes. Representative examples for each class and their experimentally validated roles are provided below.

Enzyme promiscuity

Enzyme promiscuity refers to the ability of an enzyme to catalyze reactions other than its primary, physiologically evolved function. Modern enzymology recognizes promiscuity as a common and essential property of enzymes, contributing to catalytic flexibility, metabolic resilience, and the evolution of new biochemical functions. Promiscuity is “*an inherent feature of many enzymes,*” enabling them to perform secondary reactions at low efficiencies (Tawfik, 2010) ^[47].

Types of enzyme promiscuity

1. Substrate promiscuity

Substrate promiscuity is the ability of an enzyme to accept and act on multiple different substrates, typically those that share similar structural or chemical features, instead of being restricted to a single specific substrate. This is common in enzymes with broad or flexible binding pockets, such as cytochrome P450 monooxygenases, which metabolize diverse xenobiotics and endogenous compounds (Guengerich, 2001) ^[25].

2. Catalytic promiscuity

A single active site can catalyze chemically different reactions, indicating deep mechanistic flexibility. A major example is the evolution of cyclohexadienyl dehydratase from

Table 2: Functional classification of pseudoenzymes across major enzyme families

.	Function	Examples	References
Pseudokinase	- Allosteric modulation of active protein kinases - Regulation of phospholipid kinase activity	- STRADα-LKB1, HER3-HER2, JAK JH2-JAK JH1, FAM20A-FAM20C, plant RKS1 - Vps15 controlling PI3K Vps34	(Kwon <i>et al.</i> , 2019; Klymiuk <i>et al.</i> , 2019) [35, 62]
Pseudo-histidine kinase	Acts as a protein-protein interaction module	DivL binding DivK in <i>Caulobacter</i> cell division	(Childers <i>et al.</i> , 2014) [11]
Pseudophosphatase	- Blocks substrate entry or prevents catalytic access - Controls spatial stability and localization of partner enzymes	- STYX binding ERK1/2 to restrict DUSP4 - MTMR13 stabilizing and positioning MTMR2	(Reiterer, & Farhan, 2017; Reiterer <i>et al.</i> , 2017) [63]
Pseudoprotease	- Non-catalytic inhibition of protease activation - Regulation of receptor trafficking	- cFLIP suppressing caspase-8 - iRhom proteins guiding receptor movement	(Chang <i>et al.</i> , 2002; Adrain <i>et al.</i> , 2012) [15, 8]
Pseudo-deubiquitinase	- Enhances/activates DUBs through complex formation - Provides allosteric support to ubiquitin-processing enzymes	- Abraxas1/2 activating BRCC36 - CSN6 supporting CSN5 in the COP9 signalosome	(Eddins <i>et al.</i> , 2006; Lingaraju <i>et al.</i> , 2014) [19, 64]
Pseudo-E2	- Allosteric tuning of E2 ubiquitin ligase activity - Regulation of localization in viral assembly	- Mms2 (UEV) modulating Ubc13 - Tsg101 UEV domain in viral processes	Strickland <i>et al.</i> , 2017; Walden <i>et al.</i> , 2019 [65, 66]
Pseudonuclease	- Non-catalytic modulation of nuclease function - Stabilization and assembly of protein-nucleic-acid complexes	- CPSF-100 regulating CPSF-73 - TEFM acting as mitochondrial elongation clamp	Mandel <i>et al.</i> , 2006; Hillen <i>et al.</i> , 2017 [67, 28]
PseudoATPase	Allosteric regulation of ATPase activity in linked domains	PseudoATPase regions of EccC	Rosenberg <i>et al.</i> , 2015 [68]
PseudoGTPase	Binding-mediated control of GTPase signaling	Rnd1/Rnd3 modulating p190RhoGAP $\rightarrow$ RhoA	Foster <i>et al.</i> , 1996 [20]

A non-catalytic solute-binding protein-an evolution experiment that revealed hidden catalytic activities in ancestral proteins (Clifton *et al.*, 2018) [12].

3. Condition-dependent promiscuity

Changes in pH, metal ions, or mutations can unmask secondary activities.

Such conditional promiscuity was shown in chalcone isomerase evolution, where ancestral proteins displayed multiple latent activities (Kaltenbach *et al.*, 2018) [33].

Promiscuity and pseudoenzymes

Although promiscuous enzymes and pseudoenzymes appear fundamentally different, they are deeply connected at the evolutionary and mechanistic levels. Promiscuous enzymes retain catalytic activity but possess secondary or low-efficiency side activities that broaden their functional range. In contrast, pseudoenzymes lack catalytic activity due to mutations in key residues but have evolved essential regulatory, scaffolding, or signaling roles. Importantly, enzyme promiscuity is now recognized as a major evolutionary intermediate in the transition from active enzymes to non-catalytic pseudoenzymes. Under the Innovation Amplification Divergence (IAD) model, weak promiscuous activities can be amplified and specialized, or alternatively, catalytic residues may deteriorate if selective pressure shifts toward regulatory rather than catalytic roles (Aharoni *et al.*, 2005) [2]. This framework explains why several pseudoenzyme families retain structural folds similar to their active counterparts but show loss-of-function mutations at conserved catalytic motifs.

Recent structural studies also highlight that pseudoenzymes often emerge from enzyme ancestors that once displayed broad substrate or catalytic flexibility. For example, the Tribbles pseudokinases, MLKL pseudokinase, and Fam20A arose through progressive loss of catalytic residues while

retaining regulatory binding interfaces, supporting the idea that an initially permissive (promiscuous) scaffold can drift toward a purely regulatory function (Aharoni *et al.*, 2005) [2]. Furthermore, studies show that structural plasticity, which underlies catalytic promiscuity, also predisposes certain enzyme families to evolve into pseudoenzymes. This is because flexible, promiscuous active sites can tolerate mutations that reduce catalysis without disrupting protein stability or binding capacity (Tawfik, 2010) [47]. As a result, such enzymes can shift from catalytic to non-catalytic but regulatory roles while maintaining functional relevance. Collectively, these findings position promiscuity as a bridge between enzymatic catalysis and pseudoenzymatic regulation, explaining how diverse pseudoenzyme families emerged and why many retain latent, vestigial features of their catalytic ancestors.

Extremozymes

Extremozymes are enzymes produced by extremophilic microorganisms that thrive in environmental conditions considered hostile to life, such as extreme temperatures, salinity, pH, pressure, or desiccation. These enzymes exhibit exceptional catalytic robustness and structural stability, enabling them to remain active under physicochemical conditions that denature typical mesophilic enzymes. Because of this inherent stability, extremozymes are increasingly viewed as “next-generation biocatalysts” for industrial and biotechnological processes requiring harsh reaction environments (Kumar *et al.*, 2022) [61].

At a molecular level, extremozymes display a refined repertoire of adaptations that support their function under extreme conditions. For example, thermophilic extremozymes possess enhanced hydrogen-bonding networks, increased core hydrophobicity, improved electrostatic packing, and reduced loop flexibility. These

structural features minimize thermal unfolding and confer exceptional thermostability. Littlechild (2015) [38]. Emphasized that such stabilizing strategies underpin the utility of thermophilic extremozymes in biofuel production, chemical synthesis, and polymer degradation, where elevated temperatures accelerate reaction kinetics and reduce contamination risks. Table 3 summarizes the major categories of extremozymes, highlighting their source organisms, key adaptive

mechanisms, and potential industrial applications. This classification provides a clear overview of how enzymes from extremophiles have evolved to function under environmental extremes. The table also compiles verified literature references that support each enzyme type. Such organization helps in understanding their biotechnological relevance.

Table 3: Types of extremozymes and their key characteristics (Kochhar *et al.*, 2022; Swaminaathan *et al.*, 2024; Wang *et al.*, 2024) [48].

Type of Extremozymes	Source Organisms / Environment	Key Structural or Functional Adaptations	Industrial / Biotech Applications
Thermostable extremozymes (Thermozymes)	Thermophiles & hyperthermophiles (<i>Thermotoga</i> , <i>Pyrococcus</i> , <i>Geobacillus</i>)	Highly packed hydrophobic core, strong ionic networks, shortened loops, increased thermostability	Biofuel production, high-temperature starch processing, polymer degradation
Cold-active extremozymes (Psychrozymes)	Psychrophiles (Antarctic bacteria, Arctic marine microbes)	Highly flexible active sites, low activation energy, reduced proline content	Cold-wash detergents, seafood processing, low-temperature bioremediation
Halophilic extremozymes	Halophiles (salt lakes, salterns, hypersaline lagoons)	Acidic surface residues, strong hydration shells, solvent stability	Biodiesel synthesis, peptide formation, saline food fermentation
Acid-stable extremozymes (Acidophilic enzymes)	Acidophiles (<i>Sulfolobus</i> , <i>Acidithiobacillus</i>) at pH 0.5-3	Stabilized acidic residues, strong electrostatic interactions at low pH	Starch hydrolysis, beverage clarification, leather industry
Alkaline extremozymes (Alkaliphilic enzymes)	Alkaliphiles (<i>Bacillus</i> species from soda lakes)	Stabilized active-site ionization at pH 10-12; strong electrostatic networks	Detergents, pulp & paper, textile bio-bleaching
Pressure-stable extremozymes (Barozymes)	Deep-sea piezophiles (trenches, hydrothermal vents)	Highly packed hydrophobic cores, pressure-resistant conformations	High-pressure food processing, pressure-assisted synthesis
Xerotolerant extremozymes	Desert microbes, fungi, endolithic bacteria	Stabilized by compatible solutes (trehalose, ectoine) and enhanced protein-water interactions	Dry enzyme formulations, powdered foods, enzyme stabilizers
Radiation-stable extremozymes	Radiation-resistant organisms (<i>Deinococcus radiodurans</i>)	Highly efficient DNA repair enzymes, antioxidant enzymes	Bioremediation of radioactive sites, radiation-tolerant biocatalysis
Polyextremophilic extremozymes	Organisms tolerating multiple extremes (hot & acidic; cold & salty)	Multi-layered structural adaptations enabling tolerance to compound stresses	Chemical synthesis under mixed extreme conditions

Catalytic nucleic acids: ribozymes and dnazymes

Catalytic nucleic acids are nucleic acid molecules (RNA or DNA) that fold into specific 3-dimensional shapes and perform chemical reactions without proteins. The idea that nucleic acids could function as catalysts was once considered unlikely, yet this view changed sharply after the identification of naturally occurring ribozymes. Early work showed that the *Tetrahymena* rRNA intron could excise itself without any protein help, providing the first clear demonstration of an RNA molecule acting as an enzyme (Cech, 1990) [9]. This discovery expanded the concept of biological catalysis and supported the long-standing hypothesis of an ancient “RNA world,” in which RNA stored information and performed metabolic reactions (Cech, 2024) [10].

Ribozymes are catalytic RNA molecules that can speed up biochemical reactions without the need for proteins. Ribozymes are able to catalyze reactions because RNA can fold into elaborate tertiary structures pseudoknots, bulges, stacked helices bring reactive groups into precise orientations (Doherty & Doudna, 2000) [17]. One of the strongest pieces of evidence for RNA-based catalysis in modern cells is the ribosome. Ribozymes catalyze reactions such as (Lee & Lee, 2017) [37]:

- Self-splicing (cutting and rejoining RNA strands)
- Cleaving other RNA molecules
- Ligation (joining RNA pieces)
- Peptide bond formation (in the ribosome)

Table 4: Examples of ribozymes (Symons & Uhlenbeck, 1991; Tanner, 1999) [45].

Type	Function	Example
Self-splicing introns	Remove introns from RNA	Group I & Group II introns
Hammerhead ribozyme	RNA cleavage	Plant virus RNAs
RNase P (RNA component)	tRNA maturation	Bacterial RNase P
Ribosomal RNA	Peptide bond formation	23S/28S rRNA in ribosome

DNazymes, also known as deoxyribozymes or catalytic DNA, are single-stranded DNA molecules that can catalyze chemical reactions much like protein enzymes or ribozymes. The 1994 study by Breaker and Joyce marked the first demonstration that single-stranded DNA can function as a true enzyme. Using an *in vitro* selection approach, they

screened vast libraries of random DNA sequences until they isolated a molecule capable of site-specific RNA cleavage a reaction previously believed to require either proteins or catalytic RNA. The resulting DNzyme showed sequence-dependent folding that created an active site able to accelerate phosphodiester bond cleavage. This discovery

challenged long-held assumptions about DNA’s role as merely a genetic storage molecule and opened the door to engineering DNA-based catalysts for sensing, therapeutics, and molecular engineering. Their work remains a foundation for the rapidly expanding field of catalytic DNA. While ribozymes occur naturally, DNazymes (also known as deoxyribozymes) are mostly created in the laboratory. Using large random DNA libraries and iterative selection, Breaker & Joyce isolated the first DNA enzyme capable of cleaving RNA a landmark finding that proved DNA, despite lacking a 2’-OH group, can still support catalytic function when properly structured (Breaker & Joyce, 1994) [7]. Since then, dozens of DNazymes have been engineered with

activities ranging from RNA cleavage to metal-ion sensing. Many of these catalysts show exquisite specificity; for example, certain DNazymes respond only to Pb²⁺ or Zn²⁺, which makes them valuable in environmental monitoring and biosensor development. The versatility of DNazymes has been extensively documented, with Silverman’s review summarizing how catalytic DNA has evolved into a powerful tool for synthetic chemistry, diagnostics, and molecular engineering (Silverman, 2016) [44]. DNazymes are attractive because they are stable, inexpensive to synthesize, and can be tuned by altering sequence motifs or structural elements traits that are often harder to control in protein enzymes.

Table 5: Types of dnazymes

Type of DNAzyme	Main Reaction Catalyzed	Key Features / Examples	Applications	References
RNA-cleaving DNazymes	Site-specific RNA cleavage	10-23 and 8-17 DNazymes; metal-ion dependent (Mg ²⁺ , Pb ²⁺ , Zn ²⁺)	Gene silencing, diagnostics	Breaker & Joyce, 1994 [7].
Ligation DNazymes	Joining of RNA/DNA fragments	Catalyze phosphodiester bond formation	RNA assembly, nanotechnology	Bartel & Szostak, 1993 [4].
Metal-ion-dependent DNazymes	Metal-triggered cleavage or folding	Pb ²⁺ -, Zn ²⁺ -, UO ₂ ²⁺ -specific DNazymes	Environmental sensors, water quality testing	Lan & Lu, 2011[36].

Declarations

Ethical approval

This article does not contain any studies involving human participants or animals performed by the author. Therefore, ethical approval was not required.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Competing interests

The author declares that there are no competing interests of a financial or personal nature that could have influenced the work reported in this manuscript.

Author contributions

The review framework, conducted the literature analysis, drafted the manuscript, and approved the final version for submission.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials

Not applicable. This manuscript is a review article based on published literature, and no new datasets were generated or analyzed.

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