The demise of catalysis, but new functions arise: pseudoenzymes as the phoenixes of the protein world

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Abstract

Pseudoenzymes are noncatalytic homologues of enzymes and are found in most enzyme families. Although lacking catalytic activity and sometimes referred to as “dead” enzymes, they instead resemble phoenixes because the loss of a catalytic function during evolution was associated with the development of vital new functions. They are important in regulating the activity and location of catalytically active homologues, scaffolding the assembly of signaling complexes, and regulating transcription or translation. They are key actors in cell proliferation and differentiation, proteostasis, and many other biochemical pathways and processes. They perform their functions in diverse ways, but many retain some aspect of the function of their catalytically active homologues. In some pseudoenzymes, their functions are very different from other members of their protein families, suggesting some arose from ancient moonlighting proteins during evolution. Much less is known about pseudoenzymes than their catalytically active counterparts, but a growing appreciation of their key roles in many important biochemical processes and signaling pathways has led to increased investigation in recent years. It’s clear that there is still much more to learn about the structures, functions and cellular roles of these phoenix-like proteins.

Introduction

Most enzyme families contain pseudoenzymes, a protein or domain with a fold that resembles a conventional catalytically active enzyme but that has negligible catalytic activity [1,2]. Pseudoenzymes can share a high level of amino acid sequence identity with an active enzyme but have sequence or structural changes that prevent activity, such as amino acid residues essential for catalysis are missing in the active site pocket, the entrance to the pocket is blocked, or residues needed for substrate binding are altered [3,4]. Although found more often in metazoans than in single celled organisms, pseudoenzymes are found in all kingdoms of life and in many protein families. They are estimated to comprise 10% or more of the members of the kinase, phosphatase, and protease protein families [4]. Pseudoenzymes are not the same as pseudogenes, which are genes with homology to expressed protein coding genes but have disabled open reading frames that are not translated to make proteins. Pseudoenzymes are expressed proteins and have functions as signaling molecules, scaffolds, transcription factors, translation factors, regulators of protein localization, or noncatalytic subunits of heterooligomeric enzymes that may include a homologous catalytically active subunit. Many pseudoenzyme domains still bind substrate, metal ions, or cofactors and use that ability to regulate their interactions with other macromolecules. They are often expressed in the same tissues and in the same cellular locations as their active homologues and can be involved in the regulation of biological processes in which their active homologues act. Many pseudoenzymes that don’t have a known function must have some function in the cell because the proteins are expressed and they have close sequence homologues among metazoan species.

Without a function there would be no ongoing selective pressure to maintain expression of the protein during evolution, and there would be no requirement to maintain amino acids important for a biological function, either through canonical catalytic activity, non-canonical catalytic activity, or for a catalysis-independent function.

The first report of a pseudoenzyme was in 1967, when alpha-lactalbumin, which has no catalytic activity, was found to be homologous to lysozyme, the enzyme that cleaves the β-1,4-sugar linkages in bacterial cell walls. Instead of being an enzyme, alpha-lactalbumin is a regulatory subunit of lactose synthase [5]. It is also a noncatalytic subunit of β-1,4-galactosyltransferase in mammal milk and changes the substrate specificity of β-1,4-galactosyltransferase to glucose [6]

Many pseudoenzymes are pseudokinases. The pseudokinases are found scattered throughout the subfamilies of the protein kinase superfamily and are predicted to make up 10% of the superfamily [7,8]. Some pseudokinases bind ATP even though they do not use it as a substrate. ATP binding might be important for the protein's role in a signaling pathway because it could trigger conformational changes in the protein that could in turn affect a pseudokinase's interactions with other proteins [9]. Some pseudokinases contain just a pseudokinase domain, but others are much larger with additional domains [10]. They often form multimers or complexes with active kinases and function in activating kinase catalytic activity or serve as a scaffold. The Janus tyrosine kinases (JAK1, JAK2, JAK3 and TYK2) contain a pseudokinase domain and a functional kinase domain within the same polypeptide chain. Tribbles pseudokinases

(TRIB1, TRIB2, TRIB3) have a serine/threonine pseudokinase domain and a C-terminal docking domain for interacting with a E3 ubiquitin ligase. The pseudokinase domain binds to protein substrates of the E3 ligase and regulates their ubiquitinylation by the ligase [11]. Ubiquitinylation targets the substrates to degradation by the proteasome. The Tribbles also act as molecular scaffolds for assembly and regulation of multiprotein complexes of active kinases in signaling pathways for cell proliferation and differentiation and transcription.

There are also many kinds of pseudoproteases, including transmembrane pseudoproteases. iRhom is an inactive homologue of rhomboid serine proteases [12] that regulates the fate of transmembrane proteins in the endoplasmic reticulum (ER) [13]. In the ER, active rhomboid proteases cut transmembrane helices within client proteins, releasing the cytoplasmic or extracellular domains. The iRhoms resemble active rhomboid proteases, except that they have an extended C-terminus and a cysteine-rich luminal loop between the first two transmembrane helices. They still bind to client proteins, but instead of releasing the cytoplasmic or extracellular domains, they target the intact proteins to the ER degradation pathway (ERAD).

Pseudodeubiquitinases are found in multiple subfamilies of deubiquitinases, [14]. They often are found as a subunit of a heterodimer or as a scaffold subunit in a larger signaling complex that includes active deubiquitinases. Their functions include allosteric activation and/or stabilization of active deubiquitinases. Similarly, pseudophosphatases have functions as regulatory subunits of active phosphatase subunits in a multimer or in

protecting substrates from dephosphorylation. There are also many more types of pseudoenzymes with a large diversity in functions and molecular mechanisms, including pseudoubiquitin ligases, pseudonucleases, pseudoNTPases, pseudochitinase, pseudosialidase, pseudolyase, pseudotransferase, pseudo-histone acetyltransferase, pseudophospholipase, pseudo-oxidoreductae, pseudodismutase, pseudodihydroorotase, and a pseudo-RNase. A more extensive list with further examples and their functions was published by Murphy, Mace, and Eyers [15] and is online at https://en.wikipedia.org/wiki/Pseudoenzyme. In general, pseudoenzymes participate in a variety of roles that often involve promoting or inhibiting their active enzyme homologues or regulating signaling pathways or other processes in which their catalytically active homologues are found. The molecular mechanisms by which they perform these roles are diverse and include allosteric regulation of other enzymes, scaffolding the assembly of signaling complexes, binding and inhibiting active enzymes, stabilizing the fold of an active enzyme, regulating protein localization, recruitting or sequestering of substrates, and regulating folding of a conventional enzyme.

Evolution of pseudoenzymes

During evolution, gene duplication events enabled some enzyme family members to lose their catalytic function to specialize in another function as a pseudoenzyme, but the protein could not have simply lost its catalytic function and then later in evolution evolved a new function. When a mutation occurs that knocks out the protein function, the selective pressure is lost, and the enzyme would be lost as a pseudogene unless the protein already has a second function in the cell. That means some aspect of a new

function must have been present in the ancestral enzyme (Figure 1 A and B). Indeed, many pseudoenzymes retain some aspect of the function of their active enzyme homologues, even though they themselves are not catalytically active. For example, many pseudoenzymes are found as subunits in multimers with their conventional enzyme homologues. Others bind to substrates of the catalytically active homologues, for example, to protect a phosphorylated site from phosphatases or help to recruit client proteins. As mentioned above, some enzymes bind to ATP as a conformational switch [9], and others use cofactors in a similar way [16,17]. The pseudoenzymes are also often involved in the same signaling pathway as the catalytically active homologues. In general, the pseudoenzyme's function is an exaptation of a property that was already present in the ancient catalytic function but then became the primary function.

There are some cases where the function of a pseudoenzyme appears completely unrelated to the catalytic function of the other proteins in the protein family. So how did these proteins lose their catalytic function and gain a completely different function? Because loss of the catalytic function during evolution before gain of the noncatalytic function would have risked loss of the protein-encoding gene (decay to a pseudogene), the ancestral enzyme was likely to have been a moonlighting protein. Moonlighting proteins contain two or more biochemically or biophysically relevant functions within one polypeptide chain [18,19]. Over 350 examples of moonlighting proteins are annotated in the MoonProt Database [20, 21]. They include enzymes that have a second, independent function, such as transcription factors, cell surface receptors, chaperones, or cytoskeleton subunits. For example, in bacterial pathogens

and symbionts, several dozen intracellular enzymes, including the glycolytic enzymes enolase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), have a second function on the cell surface where they act as receptors for host cells and proteins [22].

In this scenario, an ancestral enzyme first gained a second, unrelated and independent function to become a moonlighting protein. The functional sites on a moonlighting protein can be located distant from each other on the protein structure [23] (Figure 1C). In some gene lineages the catalytic activity was then lost, but the newer, noncatalytic functional site remained and became the only function of the protein, now a pseudoenzyme (Figure 1D). In some cases, gene duplication enabled one copy of the protein to lose the catalytic function and the other copy to lose the noncatalytic function, which is referred to as subfunctionalization, complementary degenerative mutations [24] (Figure 2). In the examples below, pairs of homologous proteins exist where one protein is a moonlighting protein and the other is a pseudoenzyme that retains only the noncatalytic function (Table 1).

Crystallin(s)

The taxon specific crystallins include moonlighting enzymes that have been adopted for a second, noncatalytic function in the lens of the eye [25]. They include enzymes in ducks, reptiles, platypus, elephant shrew and several other species. Arginosuccinate lyase is a ubiquitous protein in the urea cycle (Figure 3). In ducks, it was adopted to serve as a crystallin, so the duck protein is also called the delta 2 crystallin [26]. There is another protein, the delta 1 crystallin, that shares 93% amino

acid sequence identity with the delta 2 crystallin but it does not have catalytic activity and only has the noncatalytic crystallin function.

Enzyme and regulatory protein

Many moonlighting enzymes, also referred to as trigger enzymes, have a catalytic function and another, noncatalytic, function in which they regulate translation or transcription by binding to RNA or DNA or to other proteins that are translation or transcription factors [27]. As explained by Commichau and Stulke, enzymes are the cellular components that are best sensors of the availability of their substrates and products, so it makes sense that an enzyme with a second function in regulation of the translation or transcription of enzymes in that biochemical pathway would benefit the organism [27]. The PutA proline dehydrogenase and BirA biotin-protein ligase are examples of trigger enzymes, moonlighting proteins that respond to the level of ligands in the cell and bind to DNA to regulate transcription [28-31]. It appears that some trigger enzymes were the sources of pseudoenzymes that have lost the catalytic function but retain the ability to regulation translation or transcription. In some cases, this model is supported by the presence of a homologue that is a moonlighting enzyme/regulator.

Homologous pair binding RNA

Human cytosolic aconitase is the same protein as the iron-responsive element binding protein 1 (IRP1/IREB1) [32-34]. When cellular iron levels are high, the protein binds an 4Fe-4S iron sulfur cluster in the active site and acts as an enzyme in the citric

acid cycle. When iron levels are low, the iron-sulfur cluster is removed. Loss of the iron-sulfur cluster results in the protein changing conformation to perform a second function: it binds to mRNA encoding genes involved in iron uptake and utilization. Iron-responsive element binding protein 2 (IRP2/IREB2) is a pseudoenzyme that shares 54% amino acid sequence identity to IRP1 and binds to mRNA, but it does not bind an iron sulfur cluster and is not active as an aconitase.

Homologous pair binding transcription factor

S. cerevisiae Gal 1 and its pseudoenzyme homologue Gal3 [35] regulate transcription of the galactose/melibiose regulon in response to the presence of specific carbon sources through binding to the transcriptional regulator Gal80p [36]. Gal80p forms a complex with the DNA binding transcriptional activator Gal4 and inhibits its activity. Binding of Gal3 or Gal1 to the Gal80p/Gal4p complex results in the release of Gal4p and activation of gene expression. Even though the proteins share 74% amino acid sequence identity, and both can interact with Gal80, only Gal1 is also galactokinase, which adds a phosphoryl group to galactose in the first step in galactose metabolism. Gal3 is a pseudoenzyme that only retains the transcription regulation activity [37].

GabR/MocR family of pseudoenzymes

The *Bacillus subtilis* GabR protein is a transcription factor in γ-aminobutyric acid (GB) metabolism that contains a dead aminotransferase enzyme-like domain [38,39]. The C-terminal aminotransferase domain retains its ability to bind the pyridoxal 5'-

phosphate (PLP) cofactor required for aminotransferase catalytic activity but cannot catalyze the aminotransferase reaction. Instead the domain uses the PLP binding ability to regulate an N-terminal winged helix-turn-helix DNA binding domain. In the presence of PLP and γ-aminobutyric acid (GABA), GabR activates the gabTD operon, so that the bacterium can use GABA as a nitrogen and carbon source. Structural studies of GabR determined that two factors prevent the protein from being an active enzyme [16,17]. First, a tyrosine residue blocks the lysine that is required in the aminotransferase catalytic mechanism. Second, the binding site binding specificity is only for GABA and not alpha amino acids and dicarboxylic acids. Several other pseudoenzymes have a similar dead aminotransferase domain or another pseudoenzyme domain and a winged helix DNA binding domain, including PdxR proteins from *Bacillus clausii* [40] and *Listeria monocytogenes* [41], and *Brevibacillus brevis* ddlR [42]. The PurR purine repressor of *Bacillus subtilis* contains a domain that is homologous to phosphorobosyltransferases and is also connected to a winged helix DNA binding domain [43]

The winged helix-turn-helix DNA binding domain in these proteins appears to have been added on to an enzyme domain during evolution. Loss of the catalytic function before gain of the DNA binding function would have been likely to result in loss of selective pressure and loss of the protein because of decay of the gene to a pseudogene. There must have been an ancestral homologue, or perhaps there still is an extant homologue, that was/is a moonlighting protein with an active aminotransferase catalytic activity and a DNA binding activity.

Enzyme and unknown function

Alt1/Alt2

Through yeast genetics and biochemical characterization, the Gonzalez lab showed that the alanine aminotransferases in two yeast species, *Lacchancea kluyveri,* LkALT1, and *Kluyveromyces lactis,* KlALT1, are moonlighting proteins with alanine aminotransferase activity and a second, noncatalytic activity (Figure 4) [44, 45]. A third yeast species, *S. cerevisiae,* inherited two alanine aminotransferases during an ancient allopolyploidization event, in which the genomes of two different ancestral yeast species were brought together. ScAlt1 and ScAlt2 are homologous proteins that share 65% amino acid sequence identity. Only ScAlt1 has retained alanine aminotransferase activity. Alt2 appears to have diverged to become a pseudoenzyme without catalytic activity. Interestingly, the results of genetics knockout experiments suggest the noncatalytic activity of Alt2 is different from the noncaltalytic function(s) of LkALT1 and KlALT1. This suggests that the Alt2 noncatalytic function continued to diverge during evolution after the ancestral protein lost its catalytic function.

Perspectives

Recognition of the significant roles pseudoenzymes play in key cellular pathways and processes is continuing to grow, but their great importance is sometimes not perceived by the wider scientific community perhaps because of the confusion of the term with pseudogenes or referring to them as “dead” enzymes leads to connotations of insignificance. However, as in the apocryphal story about Mark Twain (the pseudonym of Samuel Clemens) that after a newspaper prematurely published his obituary, he

replied “the report of my death has been greatly exaggerated”, the lack of catalytic function of pseudoenzymes is often greatly misconstrued. Studies over the last few decades have proven that, like a phoenix in Greek mythology that starts a new life arising from the ashes of its dead precursor, the “death” of a pseudoenzyme’s catalytic function can be the start of a vital new function. Renaming these proteins as phoenix proteins (PhP) would emphasize their significant new functions, their mechanism of evolution, and the potential importance of their study.

Most of the proteins traditionally classified as pseudoenzymes appear to have evolved from active enzymes by maintaining and developing some aspect of the original enzyme function, for example protein-protein interactions. A smaller group of pseudoenzymes evolved from moonlighting proteins containing a catalytic function that was distinct from the current function. The number of known proteins in this second group is likely to grow in part because the number of experimentally confirmed moonlighting proteins is growing rapidly [20,21]. Moonlighting proteins are found in all enzyme classes and throughout the evolutionary tree and include proteins with diverse combinations of a catalytic function and an additional function. The examples described above illustrate that they can lead to pseudoenzymes through loss of the catalytic function. It’s not clear why we don’t see more homologous pairs of moonlighting and pseudoenzyme proteins. It’s possible that they have not been searched for, or the sometimes small differences between pseudoenzymes and their active homologues make it difficult to identify the homologous pairs. Another possibility is that subfunctionalization takes place relatively rapidly after duplication, perhaps because

subfuctionalization enables optimization of each of the two proteins and independent regulation of their functions and expression levels.

In spite of their wide importance in signaling, regulation, and coordination of many important pathways and processes, pseudoenzymes have been studied far less than their catalytically active counterparts. Much more information is needed about the structures, biochemical functions, and cellular roles of these phoenix-like proteins. In addition, because pseudoenzymes often closely resemble their catalytically active counterparts, a better understanding is needed of the similarities and differences between proteins within a superfamily, including clarification of the changes that render pseudoenzymes catalytically inactive as well as changes that have merely resulted in variations on a catalytic mechanism, for example, the SelO pseudokinase binds ATP in an unusual conformation in its active site and AMPylates its substrates instead of phosphorylating them [46]. In addition to increasing our understanding of the many cellular processes in which these proteins participate, this information is greatly needed for identifying the functions of the millions of proteins encoded in the thousands of genome sequences becoming available.

Conflicts of interest

The author has no conflicts of interest

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Figure Legends

Figure 1. Comparison of traditional pseudoenzymes and pseudoenzymes evolved from moonlighting proteins. In traditional pseudoenzymes, the protein function is derived from part of the main function of the ancestral protein. Other pseudoenzymes arise from moonlighting enzymes that have lost the catalytic function. **A.** In a kinase, binding to another protein (the substrate, brown oval), and transfer of a phosphoryl group (red star) from ATP to the substrate are aspects of the catalytic activity. **B.** In a pseudoenzyme related to the kinase in A, the transferase function has been lost, and the protein binding activity is repurposed as the protein activity. **C.** In a moonlighting enzyme, the catalytic function (hexagon converted to triangle) in the active site is separate from and independent of another function, such as binding to another protein (brown oval). **D.** In a pseudoenzyme evolved from the moonlighting protein, the catalytic activity has been lost, and the remaining activity is different from other members of the enzyme protein family.

Figure 2. Evolution of pseudoenzymes from moonlighting proteins. During evolution an enzyme with a catalytic functional motif (motif 1) can gain a second functional motif (motif 2) to become a moonlighting protein. Gene duplication and subsequent evolution can result in subfunctionalization with duplicated genes each losing one functional motif. The resulting homologous proteins in the protein family, which can be in the same or different species, can include proteins that evolved from proteins that never contained the motif 2 (protein 1), a moonlighting protein that has two functions symbolized by motif 1 and motif 2 (protein 2), proteins that evolved from

proteins that gained but then lost motif 2 (protein 3), and pseudoenzymes that evolved from proteins that gained motif 2 but lost motif 1 (protein 4).

Figure 3. The relationship between arginosuccinate lyase and the delta 1 and delta 2 crystallins. Top. Arginosuccinate lyase is an active enzyme in many species. **Middle.** In ducks, the delta2 crystallin is a crystallin in the lens of the eye and an active enzyme in other tissues. **Bottom.** Also in ducks, the delta1 crystallin shares 94% amino acid sequence identity with the delta2 crystallin but does not have arginosuccinate lyase catalytic activity.

FIgure 4: Yeast alanine aminotransferase homologues. A. A comparison of enzyme homologues through yeast genetics showed that some enzymes have a second, moonlighting function. A gene knockout results in a knockout phenotype (phenotype 1), which is restored to the wild type phenotype by addition of the wild type gene. Addition of a catalytically dead enzyme partially restores the wild type phenotype (phenotype 2), suggesting that there is a second function that the catalytically dead mutant protein can still perform. Addition of a homologue with catalytic function restores different aspects of the wild type phenotype (phenotype 3), suggesting that the homologue does not perform the moonlighting function. **B.** Through yeast genetics studies, the KlAtl1 and LkAtl1 proteins were shown to be moonlighting proteins with alanine transferase activity and another function. In *S. cerevisiase,* where there are two alanine transferase homologues, ScAlt1 has alanine transferase activity and ScAlt2 has a different function.

Table 1. Pseudoenzymes with Moonlighting Protein homologues

Pseudoenzyme	**Moonlighting protein**
Duck delta1 crystallin	duck delta 2 crystallin/arginosuccinate lyase
S. cerevisiae Alt2	KlAtl1, LkAtl1 alanine aminotransferases*
S. cerevisiae Gal3	Gal1 galactokinase/transcription regulator
mammalian IRB2	mammalian IRB1/aconitase
No extant moonlighting homologue?	
GabR DNA binding	ancient aminotransferases with DNA binding domain?
PdxR DNA binding	ancient aminotransferases with DNA binding domain?
PurR DNA binding	ancient phosphoribosyltransferase with DNA binding domain?

* The noncatalytic function(s) of KlAtl1and LkAtl1 might be different from the function of ScAlt2