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REVIEW ARTICLE

Enzymes, pseudoenzymes, and moonlighting proteins: diversity of function in protein superfamilies

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As more genome sequences are elucidated, there is an increasing need for information about the functions of the millions of proteins they encode. The function of a newly sequenced protein is often estimated by sequence alignment with the sequences of proteins with known functions. However, protein superfamilies can contain members that share significant amino acid sequence and structural homology yet catalyze different reactions or act on different substrates. Some homologous proteins differ by having a second or even third function, called moonlighting proteins. More recently, it was found that most protein superfamilies also include pseudoenzymes, a protein, or a domain within a protein, that has a three-dimensional fold that resembles a conventional catalytically active enzyme, but has no catalytic activity. In this review, we discuss several examples of protein families that contain enzymes, pseudoenzymes, and moonlighting proteins. It is becoming clear that pseudoenzymes and moonlighting proteins are widespread in the evolutionary tree, and in many protein families, and they are often very similar in sequence and structure to their monofunctional and catalytically active counterparts. A greater understanding is needed to clarify when similarities and differences in amino acid sequences and structures correspond to similarities and differences in biochemical functions and cellular roles. This information can help improve programs that identify protein functions from sequence or structure and assist in more accurate annotation of sequence and structural databases, as well as in our understanding of the broad diversity of protein functions.

Introduction

The increasing speed of DNA sequencing has yielded a vast number of genome sequences, but there is a large question of what functions and biochemical pathways are imparted by the millions of proteins they encode. The main method of estimating the functions of new proteins is through amino acid sequence alignment with the sequences of proteins with known functions. However, the accuracy of this method is limited because similar proteins, even proteins with very high degree of amino acid sequence identity, can vary significantly in function. Enzyme superfamilies can have members that share sequence and structural homology

Abbreviations
AdhE, *E. coli* aldehyde alcohol dehydrogenase; ALDH1, aldehyde dehydrogenase 1; ALDH2*1, human mitochondrial aldehyde dehydrogenase active subunit; ALDH2*2, human mitochondrial aldehyde dehydrogenase inactive subunit; CCS, copper chaperone for superoxide dismutase; EhADH2, *Entamoeba histolytica* alcohol dehydrogenase 2; ER, endoplasmic reticulum; ERAD, ER degradation pathway; IRP1, iron-responsive protein 1; IRP2/IREB2, iron-responsive element-binding protein 2; LA, alpha-lactalbumin; PhP, phoenix proteins; PLP, pyridoxal 5'-phosphate; PutA, proline dehydrogenase/proline oxidase from *S. typhimurium* and *E. coli*; ROS, reactive oxygen species; SOD, superoxide dismutase.

yet catalyze different reactions or act on different substrates. The enolase superfamily contains thousands of proteins with similar structures and that use similar chemistry in the catalytic mechanism, an α-proton abstraction of a carboxylate substrate generating an enediolate intermediate that then undergoes epimerization, racemization, or β-elimination. However, the proteins in different subgroups catalyze different reactions: enolase (conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis), muconate lactonizing enzyme (breakdown of aromatics derived from lignin, 1,2-dihydroxybenzene and a dihydroxybenzoic acid, to citric acid cycle intermediates in the β-ketoadipate pathway), mandelate racemase (interconversion of (S)-mandelate and (R)-mandelate), 3-methylaspartate ammonia lyase (breakdown of L-threo-3-methylaspartate to mesaconate and ammonia), D-mannonate dehydratase (breakdown of D-mannonate to 2-dehydro-3-deoxy-D-gluconate and water), D-glucarate dehydratase (breakdown of D-glucarate to 5-dehydro-4-deoxy-D-glucarate and water), and D-galactarate dehydratase (breakdown of D-galactarate to 5-dehydro-4-deoxy-D-glucarate and water) [1–4]. Also challenging in determining protein function is that a small change in the amino acid sequence, often in or near the active site, could appear to result in a loss of catalytic function but instead creates variations of function, such as a change in catalytic mechanism or substrate. In recent years, it has become clear that differences in protein function within an enzyme superfamily can extend beyond differences in catalytic activity. Moonlighting enzymes have one or more noncatalytic functions in addition to a catalytic function, and pseudoenzymes have no catalytic function at all. In this review, we discuss examples of protein superfamilies that contain catalytically active enzymes, moonlighting proteins, and pseudoenzymes.

Definition and examples of moonlighting proteins

In moonlighting enzymes, the protein has a catalytic activity and a second, unrelated activity [5,6]. Over 300 moonlighting proteins have been identified [7]. In pathogens and probiotic bacteria, dozens of enzymes that are known to have a catalytic function inside the cell are also found displayed on the surface of the cell where they have a second function as a receptor or adhesin to host proteins and tissues. These include some of the enzymes in the enolase superfamily described above. Several enolase enzymes that catalyze the conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis in the cell cytoplasm are also found as adhesins on the cell surface of bacteria (e.g., Oral streptococci [8], Bifidobacteria [9]), yeast (*Candida albicans* [10]), worms (*Onchocerca volvulus* [11]), and mammals (humans [12], rats [13]). The taxon-specific crystallins include enzymes that were adopted to perform a second function in the lens of the eye in several species. For example, in birds, argininosuccinate lyase is the delta 2 crystallin [14] and lactate dehydrogenase is the epsilon crystallin [15,16] Other enzymes, referred to as trigger enzymes [17], have a second function in which they bind to transcription factors or directly to DNA or RNA to regulate transcription or translation. Still, other moonlighting proteins have other combinations of catalytic and/or noncatalytic functions. For example, cytochrome C has several noncatalytic functions. When inside mitochondria, it transfers electrons between components of the respiratory chain. In response to an accumulation of unfolded proteins, DNA damage, or other significant cell stress, it is released into the cytoplasm where it binds to other proteins to form a complex, the apoptosome, that activates caspases that promote apoptosis [18]. As will be shown below, homologues of moonlighting proteins can have the same functions, only one of the functions, or different combinations of functions.

Definition and examples of pseudoenzymes

Instead of having a function in addition to a catalytic function, a pseudoenzyme is a protein, or a domain within a protein, that has an amino acid sequence and three-dimensional fold that resembles a conventional catalytically active enzyme, but does not have catalytic activity [reviewed in Refs. 19–24]. Their lack of catalytic activity resulted in their being called dead enzymes, zombies, or recently, phoenix proteins (PhP) because the loss of catalysis during evolution is accompanied by the birth of new vital function and role in cell. Perhaps surprisingly, pseudoenzymes are found in a wide variety of enzyme families. They often function as molecular switches or information integrators to regulate signaling pathways, transcription, or translation. Their functions often occur in the same cell types and within the same pathways as their catalytically active enzyme homologues. In many cases, a pseudoenzyme subunit interacts with catalytically active homologues—through direct binding and regulation of catalysis or by serving as a scaffold for assembly of a multiprotein complex containing active homologues. The three-dimensional structure can be close enough to that of an active homologue that they often still

bind substrate, cofactor, or metal ions, but instead of using those molecules in a catalytic activity, ligand binding is used as a regulator of another function.

The first pseudoenzyme to be reported was alpha-lactalbumin (LA), a homologue of lysozyme that is expressed in the mammary gland and acts as a regulatory subunit of lactose synthase [25]. Lactose synthase is a 1:1 complex of a catalytic β-1,4-galactosyltransferase subunit and a noncatalytic LA subunit. Without LA, β-1,4-galactosyltransferase transfers the galactosyl residue of UDP-galactose to N-acetylglucosamine or oligosaccharides containing N-acetylglucosamine. When LA binds to β-1,4-galactosyltransferase, it helps glucose bind in the enzyme's active site by forming a hydrogen bond to the sugar's O1 hydroxyl group and also by blocking oligosaccharides from binding. These interactions reduce the Km for glucose from approximately 2 molar to 1000-fold less, which promotes the transfer of the galactosyl residue to glucose instead of to N-acetylglucosamine and results in the synthesis of lactose (milk sugar) by the mammary glands [26,27].

Pseudoenzymes are now known in dozens of protein families, including pseudokinases, pseudoubiquitin ligases, pseudodeubiquitinases, pseudonucleases, and pseudoNTPases. Pseudoproteases contain examples in both soluble protease families and transmembrane protease families. iRhom is a transmembrane protein in the endoplasmic reticulum (ER) that is an inactive homologue of the rhomboid serine proteases [28,29]. The active rhomboid proteases and iRhom bind to the same client proteins, but cleavage of the client proteins results in the release of the cytoplasmic or extracellular domains, and binding by the inactive iRhom results in sending the intact client proteins to the ER degradation pathway (ERAD). Further examples of pseudoenzymes from these and other protein families are reviewed by Murphy, Mace, and Eyers [30] and are available online at https://en.wikipedia.org/wiki/Pseudoenzyme.

All found in some families

Proteins in a superfamily can be very diverse in functions because one family of proteins with homologous amino acid sequences and structures can contain various types of enzymes with different substrates and catalytic activities as well as pseudoenzymes and moonlighting proteins (Table 1). A cartoon illustrating the relationship or evolution of various enzymes, moonlighting enzymes, and pseudoenzymes within a protein superfamily is shown in Fig. 1. The amino acid sequence or three-dimensional structural motif that enables a catalytic function in an ancestral enzyme can be maintained in evolution to yield an active enzyme or modified to result in an enzyme with an altered catalytic activity or a pseudoenzyme. In some branches of the evolutionary tree, a second functional motif is gained to result in a moonlighting protein. Subsequent loss of the catalytic or noncatalytic function from the moonlighting protein can result in an enzyme that resembles the ancestral enzyme or a pseudoenzyme with a function that appears unrelated to the canonical catalytic function of the enzyme superfamily. In the examples of enzyme families discussed below, some of the pseudoenzymes appear to have evolved from moonlighting enzymes that lost the catalytic activity because the pseudoenzyme shares the noncatalytic function of the moonlighting enzyme. Other pseudoenzymes might have evolved from a path separate from the evolution of the moonlighting enzymes in the superfamily because the pseudoenzyme function does not correspond to the 'second' function of the moonlighting enzyme. In the cartoon, the multiple versions of proteins in an enzyme superfamily can represent proteins in different species as well as multiple proteins within one species that arose by duplication of an ancestral gene before sequence and functional divergence.

Table 1. Examples of enzyme families containing pseudoenzymes and/or moonlighting enzymes.

Enzyme family	Pseudoenzyme[a]	Moonlighting enzyme[a]
Argininosuccinate lyase	delta 1 crystallin	delta 2 crystallin/ argininosuccinate lyase
Lysozyme	alpha-lactalbumin	
Aldehyde dehydrogenase	ALDH2*2	ALDH1/epsilon crystallin
Superoxide dismutase	CCS	*S. cerevisiae* SOD1
Galactokinase	Gal3	Gal1
Aminotransferases	GabR	
Kinases	TRIB1	Human Erk2

[a]In some enzyme superfamilies, there are many pseudoenzymes and/or moonlighting enzymes, but only one example is given in the table.

Argininosuccinate lyase

Argininosuccinate lyase is found in many species as an enzyme in the urea cycle. In several avian species, the enzyme gained a second function as a crystallin in the lens of the eye [31]. In ducks, gene duplication of the moonlighting protein and then loss of enzyme function in one homologue resulted in a pseudoenzyme, the

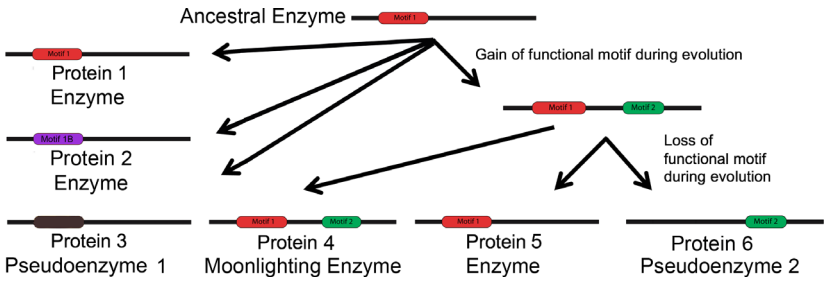

Fig. 1. An ancestral enzyme can lead to proteins with a variety of functions during evolution. Extant proteins can have the same catalytic function as the ancestral enzyme, symbolized by the red oval labeled motif 1 and represented by Protein 1. Changes in protein sequence and structure during evolution could result in an enzyme that catalyzes a different reaction (motif 1B in Protein 2) or that has lost catalytic activity to become a pseudoenzyme (black motif in Protein 3) with a function that shares some characteristics with the ancient catalytic function. In some evolutionary branches, the ancient enzyme gained a second function, symbolized by motif 2, so that some members of the protein family are moonlighting enzymes (Protein 4). In subsequent evolutionary changes, some of the moonlighting proteins have lost the noncatalytic function to yield a protein similar to the ancestral monofunctional enzyme (Protein 5) or lost the catalytic function to yield a pseudoenzyme (Protein 6) with a function that is not related to the ancient catalytic function.

delta 1 crystallin. The delta 1 crystallin pseudoenzyme shares 93% amino acid sequence identity to the argininosuccinate lyase/delta 2 crystallin moonlighting protein.

Dehydrogenases

Human mitochondrial aldehyde dehydrogenase is a tetrameric enzyme that can be found in two forms in some populations: a catalytically active subunit, ALDH2*1, and an inactive or pseudoenzyme subunit, ALDH2*2. Individuals can be homozygous for either type of subunit or can be heterozygous. In heterozygotes, the ALDH2*2 form can be considered a pseudoenzyme because it inhibits the tetrameric assembly of its active counterpart ALDH2*1. This noncatalytic function is similar to those seen in many other pseudoenzymes because it is derived from an aspect of the function of the catalytic homologue, namely tetramerization. Within the same aldehyde dehydrogenase superfamily, there are also other variations. Elephant shrews (*Elephantulus edwardii*) have a moonlighting aldehyde dehydrogenase 1 (ALDH1) with 65% amino acid identity to human ALDH2. The elephant shrew ALDH1 has a different kind of noncatalytic function from the human pseudoenzyme—it is also a crystallin [32]. More broadly in the dehydrogenase superfamily, there are also other moonlighting enzymes with a noncatalytic function that is different from that of the ALDH2*2 pseudoenzyme function, but these dehydrogenases share much less sequence identity to ALDH2: The PutA proline dehydrogenase/proline oxidase from both *S. typhimurium* and *E. coli* shares 29% amino acid sequence identity to human ALDH2. Both are moonlighting enzymes that are also transcription factors [33–35]. The intestinal pathogen *Entamoeba histolytica* alcohol dehydrogenase (EhADH2) has 22% amino acid sequence identity to human ALDH2 and has a second function as a cell surface protein that binds to fibronectin, laminin, and type II collagen in host tissues [36]. *E. coli* aldehyde alcohol dehydrogenase (AdhE) [37] shares 23% amino acid sequence identity with human ALDH2 and has additional functions in which it binds to the ribosome and affects translation through RNA unwinding activity and RNA helicase activities [38].

The superoxide dismutase superfamily also contains catalytically active enzymes, a pseudoenzyme, and a moonlighting enzyme, although, like in the dehydrogenases described above, the pseudoenzyme function does not appear to have evolved from a moonlighting function. The copper chaperone for superoxide dismutase (CCS) is a pseudo-dismutase [39]. It binds and allosterically activates catalysis by SOD1, its active superoxide dismutase enzyme counterpart with which it shares 50% amino acid sequence identity. The copper/zinc [Cu-Zn]-type superoxide dismutase (Sod1) in *Saccharomyces cerevisiae* (baker's yeast) [40] shares 44.9% amino acid sequence identity with human CCS and 56% amino acid sequence identity with human SOD1. It is a moonlighting enzyme, but its noncatalytic function is not related to the function of the human CCS pseudoenzyme. In the cytoplasm, it acts as a superoxide dismutase, but in response to H_2O_2, a reactive oxygen species (ROS), it moves to the nucleus where it binds to DNA and acts as a transcription factor [41]. It regulates the expression of genes involved in oxidative resistance and DNA damage repair, including RNR3 and GRE2. Another *S. cerevisae* protein, CCS1, has 34% amino acid sequence identity to

human SOD1, 26% to human CCS, and 29% to S. cerevisiae SOD1 and does not have catalytic activity but delivers copper ions to SOD1.

Aconitase, an enzyme in the citric acid cycle, is the same protein as the iron-responsive protein 1 (IRP1). Aconitase requires a 4Fe-4S iron–sulfur cluster in the active site for catalytic activity. When cellular iron levels are low, the enzyme loses the iron–sulfur cluster, changes conformation, and becomes able to bind to mRNA encoding genes involved in iron uptake and utilization [42–44]. Other aconitases have the same catalytic function but a different second function—the yeast enzyme has a second function in the maintenance of mitochondrial DNA [45]. The aconitase/IRP1 protein also has a pseudoenzyme homologue that shares 54% amino acid sequence identity, the iron-responsive element-binding protein 2 (IRP2/IREB2). IRP2 binds to mRNA but lacks aconitase catalytic activity.

Galactokinase

The *S. cerevisiae* Gal1 galactokinase is both an enzyme and a transcription factor. Gal3 is a related protein with 74% amino acid sequence identity to Gal1 but is only a transcription factor and does not have catalytic activity [46–48].

PLP-binding proteins

Pseudoenzyme transcription factors are also found in the superfamily of proteins that bind pyridoxal 5′-phosphate (PLP) as a cofactor in the active site. The MocR/GabR subfamily includes GabR and several other transcription factors that possess a C-terminal aminotransferase-like domain that binds PLP but is catalytically inactive and an N-terminal winged-helix domain that binds DNA [49–56]. It is likely that the DNA-binding domain was added to an ancestral aminotransferase enzyme to result in an intermediate moonlighting protein before loss of catalytic activity to result in the pseudoenzyme transcription factor. However, no such extant moonlighting protein has been confirmed to date for this gene family.

Kinases

Pseudoenzymes are estimated to make up over 10% of the kinase superfamily [57,58]. Kinases often function as multimers or in multiprotein complexes, and the pseudoenzyme subunits generally have functions that have some relationship to the function of their active enzyme counterparts—for example, they act as molecular scaffolds of a multiprotein signaling complex or function in allosterically regulating the catalytic activity of the active kinase homologues [59,60]. In the Janus tyrosine kinases (JAK1, JAK2, JAK3, and Tyk2), a polypeptide chain can contain both an active kinase domain and a pseudokinase domain. The tribbles pseudokinases (TRIB1, TRIB2, and TRIB3) have an added C-terminal docking domain for binding to an E3 ligase, and the pseudokinase domain binds to the substrates of the ligase to regulate their ubiquitinylation. The human Erk2 kinase is a catalytically active cytoplasmic serine/threonine kinase with a wide variety of protein substrates [61]. It is a moonlighting enzyme with a second function that is not related to its kinase catalytic activity. It functions as a DNA-binding transcriptional repressor in the nucleus [62] and represses expression of gamma interferon-induced genes. Unlike the MocR/GabR protein family, there are no examples known in the kinase protein family of pseudokinases with only a transcription factor function.

These examples illustrate that proteins with significant levels of amino acid sequence and structural conservation within an enzyme superfamily do not necessarily have the same function; instead, they can include a variety of enzymes, pseudoenzymes, and moonlighting proteins. At this time, far less is known about the molecular mechanisms of pseudoenzyme functions and the noncatalytic functions of moonlighting enzymes than of the catalytic functions within a protein family. However, it is clear that pseudoenzymes can share a high percentage of amino acid sequence identity with an active enzyme and have relatively small changes in sequence or structure that prevent catalytic activity. Changes can include replacement of amino acid residues essential for catalysis or binding of substrate or cofactor, but in some cases the catalytic amino acids are retained and there are other changes that prevent catalysis such a loop that blocks the entrance of ligands into the active site pocket. More biochemical studies and X-ray crystal structures with sufficient resolution to visualize details of the active site structure (approximately 2.5 Å resolution or better) are needed for improving our understanding of how to distinguish pseudoenzymes from their active counterparts as well as for accurately predicting what pseudoenzymes do and understanding how they do it. In addition to understanding when sequence and structural changes do or do not correspond to a loss of the catalytic function, more information is needed about the types of modifications in sequence and structure that enable development of a noncatalytic function, which can share aspects of the catalytically active enzyme homologue's function such

as binding to substrate, allosteric stabilization of an active site, or assisting with assembly of multiprotein complexes.

Perhaps even more challenging is to determine the various functions of moonlighting proteins, in part because diverse combinations of functions are found in homologous moonlighting proteins. Most noncatalytic functions of moonlighting enzymes have been found through serendipity, and as of yet no common sequence or structural characteristics have been identified for moonlighting proteins. Many of the noncatalytic functions are not associated with known sequence or structural motifs or other characteristics, in part because many of these functions involve protein/protein interactions, but attempts are being made to use computational methods to develop ways to identify moonlighting proteins from amino acid sequences and structures. Some of the known moonlighting proteins have been included in the most recent Critical Assessment of protein Function Annotation algorithms (CAFA) contest, a large-scale test of computer-based methods to predict protein function based on sequence [63]. As for the pseudoenzymes, identification of protein functions and cellular roles through biochemical and biophysical tests of function, additional X-ray crystal structures, and other analyses are needed to help improve function prediction algorithms.

Conclusions

Moonlighting proteins and pseudoenzymes are found in species throughout the evolutionary tree and in many diverse protein families. There is a growing appreciation of the wide variety of functions that can be found among proteins within a superfamily, even among closely related proteins, and of the important cellular roles of pseudoenzymes and the noncatalytic functions of moonlighting proteins. They are often very similar in sequence and structure to their monofunctional and catalytically active counterparts. The examples above illustrate that functions (or absence of function) can be shared with varying levels of amino acid sequence identity—closely related proteins can share multiple functions, only one function, or no functions. A greater understanding is needed about how similarities and differences in sequences and structures relate to similarities and differences in biochemical functions, mechanisms of action, and cellular roles. Because the differences among enzymes, pseudoenzymes, and moonlighting proteins can be relatively small, more work is needed on elucidating the detailed information about the similarities and differences in the sequence and structure motifs related to the catalytic and noncatalytic functions within a protein family. There is still much to be learned from comparative structure and function studies to help elucidate these characteristics as well as for improving our understanding of the full diversity of biochemical and signaling pathways in health and disease and identification of novel drug targets. For example, targeting a pseudoenzyme that regulates a pathway might be more specific and less toxic than binding to the active site of an enzyme homologue. Understanding which function of a moonlighting protein is involved in a disease could help avoid side effects that could be caused by targeting the wrong function.

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Conflict of interest

The authors declare no conflict of interest.

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