Protein Species and Moonlighting Proteins: Very Small Changes in a Protein's Covalent Structure Can Change its Biochemical Function

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Abstract

In the past few decades, hundreds of moonlighting proteins have been identified that perform two or more distinct and physiologically relevant biochemical or biophysical functions that are not due to gene fusions, multiple RNA splice variants, or pleiotropic effects. For this special issue on protein species, this article discusses three topics related to moonlighting proteins that illustrate how small changes or differences in protein covalent structures can result in different functions. Examples are given of moonlighting proteins that switch between functions after undergoing post-translational modifications (PTMs), proteins that share high levels of amino acid sequence identity to a moonlighting protein but share only one of its functions, and several “neomorphic moonlighting proteins” in which a single amino acid mutation results in the addition of a new function.

Introduction

Over the past few decades, more and more proteins have been identified that perform two or more distinct and physiologically relevant biochemical or biophysical functions that are not due to gene fusions, multiple RNA splice variants, or pleiotropic effects. These moonlighting proteins [1] are found throughout the evolutionary tree, from eukaryotes, including mammals, yeast, worms, and plants, to bacteria, archea and even viruses. Some of the first examples to be discovered were soluble enzymes that were adopted for a second function as structural proteins in the lens of the eye (crystallins) [2,3]. More recently many other examples have been found that include cytosolic enzymes and chaperones that moonlight as receptors on the cell surface or as secreted cytokines, components of the cytoskeleton, transcription factors, translation factors, adhesins or scaffolds. Over a dozen ribosomal proteins have been found to moonlight as transcription factors or translation factors, sometimes as members of other protein complexes [for reviews see 4-23]. The online MoonProt Database, which includes information about those moonlighting proteins for which biochemical or biophysical evidence supports the presence of at least two biochemical functions in one polypeptide chain, includes hundreds of moonlighting proteins [24, moonlightingproteins.org], and the list is continuing to grow every year, so it is likely that many other proteins also have additional functions that have not yet been found.

For this special issue on protein species [25,26], this article discusses three topics related to moonlighting proteins that illustrate how small changes or differences in protein covalent structures can result in different functions. Examples are given of

moonlighting proteins that switch between functions after undergoing post-translational modifications (PTMs), proteins that share high levels of amino acid sequence identity to a moonlighting protein but share only one of its functions, and several "neomorphic moonlighting proteins" [27] in which a single amino acid mutation results in the addition of a new function (Figure 1).

PTMs can cause a switch between the different functions of a moonlighting protein

Post-translational modifications (PTMs) are widespread in proteins and can be a means of regulating function, including increasing or decreasing the rate of enzyme catalysis or altering information flow through a signaling pathway. In the case of moonlighting proteins, some PTMs have been identified that cause a switch between two of the functions of the protein, in effect serving as a toggle between functions (Figure 2, Table 1A). These post-translational modifications can be especially important regulatory mechanisms for moonlighting proteins that function in two different pathways and can enable a switch between the activities of the two pathways. Because they can be dynamic and reversible, and are added much more quickly than synthesizing a new protein, PTMs on moonlighting proteins can contribute significantly to a cell's ability to adapt quickly to changes in the environment.

In response to interferon-gamma of human myeloid cells, ribosomal protein L13a becomes phosphorylated, which causes its release from the ribosome [28] (Figure 2). L13a becomes a translation factor as part of IFN-gamma-activated inhibitor of

translation (GAIT complex) consisting of ribosomal protein L13a, glutamylprolyl tRNA synthetase (EPRS), NS1-associated protein-1 (NSAP1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAIT complex binds to 3'-UTR noncoding regions of mRNAs for VEGF-A, ceruplasmin, and other inflammation-related proteins to regulate (decrease) their expression and limit or terminate inflammation.

When not serving as part of the GAIT complex, the EPRS amino acyl-tRNA synthetase ligates an amino acid to its cognate tRNA molecule for use in protein synthesis. Phosphorylation of the human EPRS in response to interferon-gamma releases it from the tRNA multi-synthetase protein complex so that it can become part of the GAIT complex [29,30] (Figure 2). Initial phosphorylation of Ser886 in the linker between the glutamyl rRNA synthetase and prolyl-tRNA synthetase domains of EPRS and a second phosphorylation at Ser999 cause release from the tRNA multi-synthetase complex and enables binding to the NS1-associated protein, followed by binding to ribosomal protein L13a and GAPDH for formation of the GAIT complex.

GAPDH in glycolysis also has an additional function related to those above. rpL13a is unstable and subject to proteolysis once released from the ribosome, but GAPDH serves as a chaperone to extend its half-life. Under oxidative conditions, which can result from the inflammatory response, oxidatively modified LDL (LDLox) can cause a post-translational modification of Cys247 of GAPDH, resulting in S-nitrosylated GAPDH (SNO-GAPDH) [31]. SNO-GAPDH cannot bind to and protect rpL13a from degradation.

Ribosomal proteins rpL10A and S3 (rpS3) have functions in the nucleus when released from the ribosome. rpL10A helps protect plants from gemniviruses [32,33] (Figure 2). Phosphorylation by NSP-interacting kinase (NIK) directs rpL10A to the nucleus where it interferes with gemnivirus proliferation or movement to other cells. rpS3 participates in repair of DNA damage by serving as an endonuclease that acts on damaged DNA. Protein kinase C-delta (PKCdelta) phosphorylation of rpS3 causes it to move to the nucleus to repair damaged DNA [34]. The phosphorylation is only observed in the free, non-ribosomal associated protein, and repair endonuclease catalytic activity increases after phosphorylation. rpS3 also serves as a component of some NF-kappaB transcription factor complexes and provides specificity for binding of the complex to specific DNA sequences and target genes (Figure 2). For this function, rpS3 becomes phosphorylated at Ser209 by the kinase IKKβ (inhibitor of κB (IκB) kinase-β) [35]. This phosphorylation causes an increase in binding of rpS3 to importin-alpha, which promotes RPS3 translocation into the nucleus.

Mouse estrogen receptor alpha is a transcription factor in the nucleus, but some of it is palmitoylated and targeted to the plasma membrane (Figure 2). The membrane-bound receptor participates in extranuclear functions in MISS (membrane initiated steroid signaling) signaling pathways that cause rapid dilatation of the vasculature and increased endothelial repair, as well as an increase in phosphorylation of the endothelial NO synthase [36]. ER-alpha with a mutation at the palmitoylation site (C451A-ERα) is not targeted to the plasma membrane and does not have the functions associated with

signaling at the plasma membrane functions, but it still exhibits the nuclear functions.

An isoform of pyruvate kinase also has both cytoplasmic and nuclear functions. Pyruvate kinases transfer a phosphoryl group from phosphoenolpyruvate to ADP in glycolysis in the cytoplasm. Pyruvate kinase isoform M2 was found to also function in the nucleus, where it has several functions, including as a protein kinase that phosphorylates STAT3, which activates the transcription of MEK5, and an activator of beta-catenin, which induces c-Myc expression [37]. Yang and coworkers demonstrated that ERK2 translocation to the nucleus requires phosphorylation of PKM2 at Ser 37 by EGFR-activated ERK1/2 [38]. Phosphorylated PKM2 recruits the cis-trans isomerase PIN1 that promotes isomerization of PKM2, so that it can bind to importin alpha5 and be translocated to the nucleus. Luo and coworkers demonstrated another role for PTMs in regulating PKM2 activity [39]. PKM2 binds to the alpha subunit of hypoxia-inducible factor 1 (HIF-1) to increase HIF-1 binding and p300 recruitment to hypoxia response elements. The binding of PKM2 to HIF-1 is enhanced by hydroxylation on prolines 403 and 408.

Leukotriene A4 hydrolase (LTA-H; EC 3.3.2.6) has two different enzymatic activities. It is a zinc metalloenzyme that catalyzes the hydrolysis of LTA4 to yield the dihydroxyarachidonic acid metabolite, LTB4, the last step in the biosynthesis of leukotriene B4. It is also an aminopeptidase. Rybina and coworkers found that phosphorylation of the enzyme at Serine 415 inhibits the epoxide hydrolase activity, and

dephosphorylation by a phosphatase activates the hydrolase activity. However, phosphorylation does not affect the aminopeptidase activity of the enzyme [40].

Many other moonlighting proteins have also been found to be post-translationaly modified, however, for a lot of these modifications it is not known if or how they affect function. Many of the modifications were identified in large-scale proteomics studies, and the effects of the observed PTMs on protein function were not assayed. For example, phosphotyrosine, phosphothreonine, and N6-acetyllysine were identified as modifications of peroxiredoxin in humans [41-44]. Human Hsp60 has several sites of lysine acetylation [44]. Human enolase is acetylated on several serines and lysines [44], as well as phosphorylated on tyrosine [41]. Human leukotriene A-4 hydrolase [44] and phosphoglycerate kinase [41,44] were also found to be modified in proteomics studies. Many other moonlighting proteins might also be post-translationally modified, but not all the PTMs have been identified to date, and in many cases it is not known which PTMs affect function.

Homologues of Moonlighting Proteins might not have both functions

Two protein species with a high degree of amino acid sequence identity often share the same function. However, there are many cases where two proteins have different functions due to differences in just a small percentage of the amino acids in the sequence. In the case of moonlighting proteins, close sequence homologues may share one, both, or even no functions (Table 1B).

For example, the delta 1 and delta 2 crystallins are highly expressed proteins found in the lens of the eye of ducks. Both proteins evolved from the ubiquitous enzyme arginosuccinate lyase. They share 94% amino acid sequence identity, differing in only 27 amino acid residues, but only the delta 2 isoform is a moonlighting protein that retains arginosuccinate lyase catalytic activity; the delta 1 protein is not catalytically active [45-48]. Mutation of the delta 1 protein to increase its similarity to the delta 2 isoform resulted in recovery of arginosuccinate lyase activity [49]. Replacement of two loops (residues 22-31 and 74 to 89) and an additional single amino acid change of M9W resulted in recovery of a small amount of arginosuccinate lyase activity, with a relative catalytic efficiency 3% of wild type. A significant 8.5-fold increase in catalytic efficiency was then achieved by adding a E115D substitution. The E115D change appears to aid in formation of the correct conformation and stability of a region of the protein involved in substrate binding and catalysis, although it does not interact directly with the substrate. The changes in the N-terminal region of the protein appear to be necessary for correctly sequestering the substrate from solvent because the N-terminal region undergoes a conformational change and moves into the active site cleft upon substrate binding.

The glycolytic enzyme enolase moonlights by targeting a portion of the cytoplasmic pool of a tRNA, tRNALys acceptor 1 (tRK1), to the mitochondrion for the translation of some mitochondrial proteins in yeast cells [50]. Enolase binds to and causes a large conformational change in the tRK1 so that it can be passed to the mitochondrial lysyl-tRNA synthetase (preMSK), which aids in its passage into the

mitochondrion. Although their sequences are 95% identical, only the yeast Eno2p enolase isoform performs this binding and chaperone function, and the yeast Eno1P isoform exhibits only minimal activity in this pathway [50]. Most of the 21 amino acid differences (416/437 amino acid residues) between Eno1P and Eno2P are located on the solvent exposed protein surface, distant from the active site, as is usually the case in such close sequence homologues when both proteins retain enzyme activity. It is interesting to note that one-third of the differences are near each other on the Eno1 protein structure (PDB ID = 1L8P), with L62M, H63N, K66N, D70N and P74A located on the same solvent-exposed face of a surface helix, and G51E and I81L located in loops at either end of the helix, but it is not known if this helix is involved with interactions with the tRNA or synthetase.

Antlions, which are the larval stage of insects in the *Myrmeleonidae* family, have endosymbiotic *Enterobacter aerogenes* bacteria in their salivary glands that secrete a homologue of protein folding chaperonin 60 (GroEL/Hsp60) to be used as an insect neurotoxin to incapacitate prey insects [51]. The homologous chaperonin 60 protein from *E. coli*, GroEL, has no neurotoxic action, but a single amino acid substitution, I100V, T101N or G471A in the solvent exposed surface of the equatorial domain or E338D in a surface exposed loop of the apical domain, can turn this protein into a potent insect neurotoxin [51]. All of these mutations are located in positions that are far from the ATP binding site or the central cavity in which substrate proteins become folded. Many members of the GroEL/Hsp60 chaperonin family have a moonlighting function outside the cell as a cell-signaling molecules and bind to receptors on human

epithelial cells, fibroblasts, and white blood cells [4-7]. The *Enterobacter aerogenes* GroEL homologue may also act as a toxin by binding to a receptor or protein in the prey insects.

Even a single amino acid change can alter protein function

In some cases, even less than one percent amino acid sequence difference in a protein can make a difference in protein function. In fact, several diseases are caused by single amino acid changes that result not in a loss of enzyme function, but in the gain of a new function, referred to as a "neomorphic moonlighting functions" [27] (Table 1C).

Single amino acid differences in isocitrate dehydrogenase (IDH) can result in a different catalytic activity that promotes cancer [52,53]. IDH1 and IDH2 catalyze the oxidative decarboxylation of isocitrate to alpha-ketoglutarate in the Krebs cycle. A single amino acid substitution at R132 in the catalytic pocket of IDH1 was found to be the cause of some gliomas and some cases of acute myeloid leukemia (AML). A single substitution at the equivalent R172 position in IDH2 was also found to cause some gliomas. These substitutions do not prevent catalytic activity. Instead the mutant enzymes reduce the product, alpha-ketoglutarate to the R-enantiomer of 2-hydroxyglutartate ((R)-2HG R(2)-2-hydroxyglutarate (2HG; [54-56]. 2HG is an oncometabolite that inhibits alpha-ketoglutarate-dependent dioxygenases, including several proteins that function in histone and DNA demethylation. This inhibition affects the epigenetic state of the cells and blocks differentiation.

A single amino acid substitution can also add a catalytic activity to dihydrolipoamide dehydrogenase (DLD), a flavin-dependent oxidoreductase that is found in several multienzyme complexes, including the pyruvate, alpha-ketoglutarate, and branched chain amino acid dehydrogenase complexes. DLD is normally a dimer that converts dihydrolipoic acid to lipoic acid along with the reduction of NAD+ to NADH. Some single amino acid substitutions located in the homodimer subunit interface cause a decrease in dimer formation. The protein then exhibits protease activity due to a normally hidden protease active site [57,58]. The proteolytic activity is independent of the DLD activity because replacing the serine in the catalytic dyad of the protease active site with alanine (S456A), results in a loss of protease activity, but the enzyme maintains DLD activity.

Conclusions

The examples above illustrate that even in an average protein of hundreds of amino acids, a relatively small difference in sequence or PTMs can result in a large difference in function. Regulation of activity by PTMs makes use of several protein characteristics. Proteins are dynamic and can shift between different states through relatively small modifications. In addition, interactions of proteins with other molecules often involve patches of solvent exposed surfaces with specific shape, charge, polarity, and hydrophobicity requirements that can be altered by the presence of PTMs. For example, the addition of a bulky and negatively charged phosphoryl group can interfere with two proteins interacting, even if the protein-protein interaction surface involves dozens of amino acids. A single lipid group can provide enough of a hydrophobic “tail”

to cause a protein to interact with the hydrophobic portion of a lipid bilayer. The ongoing annotation of the effects of PTMs on protein function in databases such as the UniProtKB [59] can be helpful in understanding the functions of proteins under different conditions.

Differences in function between protein species can also be due to minor differences in amino acid sequence because the amino acids determining protein function and specificity of interactions with other proteins or molecules can be a relatively small number out of the hundreds of amino acids in an average protein. Alteration of one or a few amino acids during evolution can result in the loss of one of the functions of a moonlighting protein as different protein species arise. Even more dramatically, as illustrated by mutant forms of IDH, a single amino acid difference between protein species can result in a change in an active site pocket that results in the gain of a new function, or, as illustrated by a mutant form of DLD, even a small change at an oligomeric interface can affect a protein's oligomeric structure enough to reveal a hidden pocket that can serve as a second active site.

With such small differences in protein covalent structure resulting in differences in protein function, the prediction of protein function by searching for amino acid sequence homologues, although a very powerful and often successful method, can sometimes result in an incorrect prediction, and testing the predictions in vitro or in vivo is still preferable, although far more difficult to do. Because the amino acids that determine function and specificity for some types of protein functions (i.e. enzymes) are

293 often relatively few and clustered in active sites or other binding sites, the ongoing
294 development of function prediction algorithms that rely on detailed three-dimensional
295 functional motifs or protein-protein docking methods may aid in increasing the accuracy
296 of function prediction over the use of sequence homology alone for distinguishing
297 between the functions of different proteins species in the future.
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Table 1. Examples of Moonlighting Proteins and Related Proteins Discussed

A. Moonlighting Proteins with PTMs that cause switch or activation of one or more functions

Protein/one function	Another function	PTM
Ribosomal protein L13a	translation factor in GAIT complex	phosphorylation
EPRS amino acyl-tRNA synthetase	part of the GAIT complex	phosphorylation
GAPDH	chaperone for rpL13a	S-nitrosylation
Ribosomal protein rpL10A	interfere with gemnivirus	phosphorylation
Ribosomal protein rpS3	endonuclease and component of transcription factor complexes	phosphorylation
Mouse estrogen receptor alpha transcription factor	membrane initiated steroid signaling	palmitoylation
Pyruvate kinase isoform M2	protein kinase	phosphorylation
Pyruvate kinase isoform M2	binds HIF-1 to increase HIF-1 binding and p300 recruitment to hypoxia response elements	proline hydroxylation
Leukotriene A4 hydrolase aminopeptidase	epoxide hydrolase	phosphorylation

B. Moonlighting Proteins and Non-moonlighting Homologues

Protein/One function, Another function	
Duck delta 2 crystallin	arginosuccinate lyase
Duck delta 1 crystallin	not catalytically active
Yeast Eno2p enolase	targeting tRNA to the mitochondrion
Yeast Eno1p enolase	not active in tRNA targeting
Enterobacter aerogenes chaperonin 60/GroEL	insect neurotoxin
E. coli chaperonin 60/GroEL	no neurotoxic action

C. Neomorphic Moonlighting Proteins

Enzyme, New function, Cause of new function

Isocitrate dehydrogenase (IDH1)	new enzyme product	mutation at R132
Isocitrate dehydrogenase (IDH2)	new enzyme product	mutation at R172
Dihydrolipoamide dehydrogenase	protease activity	mutations causing decreased dimerization

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Figure Legends

Figure 1. Small differences between "protein species" can correlate with differences in moonlighting protein functions. A. PTMs can cause moonlighting proteins to toggle between two functions (i.e. phosphorylation (red star) resulting in the switch from an enzyme function in the top panel to a transcription factor function in the bottom panel). **B.** A homologue of a moonlighting protein might have only one of the two functions (i.e. enzyme function in both proteins, but the protein-protein interaction function is found only in the protein in the top panel). **C.** A single amino acid substitution (red X) can result in the gain of a neomorphic moonlighting function (i.e. a new enzyme catalytic activity, with a new product (green triangle)).

Figure 2. Post-translational modifications can trigger a change in function of moonlighting proteins. Upon phosphorylation, ribosomal proteins L13a, L10a, and S3 dissociate from the ribosome to perform other functions in the cell. S3 enters the nucleus to function as an endonuclease in DNA damage repair. It also works with NF-KappaB as a transcription factor. L10a moves to the nucleus to interfere with gemnivirus reproduction. L13a joins the GAIT complex on 3' UTRs of mRNAs. Phosphorylation also triggers glutamylprolyl tRNA synthetase to move from a multiprotein complex to join the GAIT complex. A different PTM, palmitoylation, causes some of a nuclear receptor, the estrogen receptor, to partition to the plasma membrane where it participates in a membrane-associated signaling pathway.

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