Bio-Zombie: the rise of pseudoenzymes in biology

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Summary

Pseudoenzymes are catalytically-dead counterparts of enzymes. Despite their first description some 50 years ago, the importance and functional diversity of these 'fit-for-purpose' polypeptides is only now being appreciated. Pseudoenzymes have been identified throughout all the kingdoms of life and, owing to predicted deficits in enzyme activity due to the absence of catalytic residues, have been variously referred to as pseudoenzymes, non-enzymes, dead enzymes, prozymes or 'zombie' proteins. An important goal of the recent Biochemical Society Pseudoenzymes focused meeting was to explore the functional and evolutionary diversity of pseudoenzymes and to begin to evaluate their functions in biology, including in cell signalling and metabolism. Here, we summarize the impressive breadth of enzyme classes that are known to have pseudoenzyme counterparts and present examples of known cellular functions. We predict that the next decades will represent golden years for the analysis of pseudoenzymes.

Main text

The existence of pseudoenzymes was first inferred through a direct comparison of the sequences of lysozyme and α-lactalbumin [1], some time before the dawn of molecular cloning, genomics, proteomics or our appreciation of cell signalling mechanisms or metabolomics. Indeed, this important class of proteins has generally failed to garner the same attention as their active counterparts, appearing to suffer from the stigma that they are merely remnants of evolution, rather than efficient signalling and regulatory entities in their own right. Moreover, their presence in proteomes throughout the kingdoms of life, and their prevalence – with estimates of the order of 10-15% in a typical genome [2] – are illustrative of their fundamental importance in biology. Importantly, an enhanced understanding of pseudoenzyme function and mechanistic adaptations are likely to provide important insights into the rapidly emerging research area that has ascribed non-catalytic functions to 'conventional' signalling enzymes like protein kinases [3], and which might also include 'classical' enzymes such as catalase [4]. This type of discrimination between catalytic and non-catalytic outputs is increasingly important as we seek to untangle the complexity of signalling mechanisms and use this knowledge to focus drug design for therapeutic benefit.

The key goal of the recent Biochemical Society Pseudoenzymes focused meeting held in Liverpool (September 11-14, 2016) was to bring together active researchers focussing on the rapidly emerging pseudoenzyme field. Much of the recent history in this area has been written in the ever-expanding pseudokinases field, where structural, cellular, biochemical and genetic studies have provided a picture of the broad diversity of signalling functions that might be mediated more generally by pseudoenzymes (reviewed in [5-7]).

Owing to defects in catalytic activities arising from the loss of key (conserved) catalytic residues, pseudoenzymes appear to have evolved into important regulatory protein interaction domains [8]. It is important to note that pseudoenzymes are fundamentally distinct from pseudogenes, and should therefore be accorded rather different treatment. In particular, whilst pseudogenes are the non-coding counterparts of conventional genes, pseudoenzymes are transcribed and translated from distinct (often duplicated) genes, and have been shown to perform diverse functions despite catalytic deficiencies. For example, pseudoenzymes have been attributed roles in allosteric regulation of catalytically active cognate (related) enzymes (either in an activating or inhibitory mode) or distinct families of enzymes, in controlling localization of proteins within the cell including by regulating trafficking, and in nucleating the assembly of intracellular signalling hubs. Despite our best efforts, the difficulty in identifying predicted dead enzymes from huge proteomes and the diversity of pseudoenzymes (summarized in Table 1) makes this overview far from comprehensive; instead it should be considered as a work in progress. Indeed, the functions of pseudoenzymes in biological networks are still emerging in most research fields, and identifying examples of 'naturally-occurring' defective enzymes based upon the vast enzyme and signalling literature poses a substantial challenge. In part this owes much to the historic lack of interest in proteins that have lost catalytic activity, but is also complicated by the diversity of ways that have been used to identify such proteins, which include terms such as non-enzymes, prozymes, dead enzymes, and catalytically-defective enzymes. However, we predict that the uptake of the term "pseudoenzyme", backed up with an up-to-date open access website (https://en.wikipedia.org/wiki/Pseudoenzyme), should aid their identification and a rapid expansion to include many more examples in the future. Nonetheless, the wealth of published examples that we have identified underscores their importance in biology and their exceptionally broad mechanistic diversity.

In terms of their evolutionary trajectory, seminal bioinformatic studies argue for the evolution of most (but perhaps not all) pseudoenzymes from ancestral active enzyme counterparts [9, 10]. This conclusion was reached through the common loss of evolutionarily-traceable mechanisms in pseudoenzymes, including mutations that cause active site occlusion, but especially *via* mutation of one or more of the key catalytic residues and motifs recognised in related enzyme counterparts [10]. Intriguingly, whether a pseudoenzyme can be reverted to an ancestral function varies widely between protein families and superfamilies [8]. For example, biologically-relevant levels of catalytic activity can often be restored to pseudophosphatases through simple mutations (such as facile reintroduction of an absent catalytic Cys residue), perhaps indicative of selective pressures to retain the phosphopeptide binding site for their biological function in both an enzyme and pseudoenzyme mode. On the other hand, it appears to be more tricky to 'resurrect' pseudokinases into catalytically-active kinases, even when multiple conventional catalytic motifs are reintroduced, as illustrated for RYK [11]. One possibility is that resurrection of activities among enzymes with complex catalytic mechanisms, such as kinases, relies on extensive conformational changes that are not necessary to restore phosphatase activities to other pseudoenzymes, such as pseudophosphatases. This finding is consistent with the idea that the protein kinase fold has been widely co-opted for divergent protein interaction functions.

Pseudoenzymes have been proposed to arise most commonly following gene duplications, allowing the enzyme to be retained for a catalytic role, so that additional 'copies' are liberated to evolve new functions without the requirement to maintain active site geometry for catalysis. In some cases, the duplication has led to introduction of a tandem domain architecture, where a pseudoenzyme domain has arisen adjacent to the catalytic counterpart, and the pseudoenzyme domain has acquired a function as an allosteric regulator of the conventional enzyme domain (Janus Kinases [12], GCN2 [13], EccC ATPase [14]). In terms of pseudokinases and pseudophosphatases, multiple examples of binary signalling polypeptides containing both enzyme and pseudoenzyme sequences arranged in series are known, many in the context of tyrosine (de)phosphorylation [7, 15]. Interestingly, recent structural evaluation of the specialised RBR (RING-BetweenRING-RING) family of 13 human Ubiquitin E3 ligases [16] has revealed an analogous tandem domain arrangement. This family, which includes the linear ubiquitylation E3 ligase HOIP and the Parkinson's disease-associated ligase Parkin, feature two domains of similar fold in a tandem array: the required-for-catalysis, Rcat (also known as RING2), domain that is preceded by the pseudoenzyme 'Benign' Rcat, BRcat (also known as InBetweenRING, IBR) domain [17-21].

The evolution of new functions following gene duplication has led to a number of cases where a pseudoenzyme functions within the same 'pathway' as the ancestral enzyme, most commonly to become an allosteric activator or suppressor and thus contributing an important layer of regulation. As noted by others [22], such a shared pathway might be predicted, since the duplicated gene product could be co-expressed both temporally and spatially alongside the conventional enzyme following duplication of the enzyme gene locus.

An important outcome of the 'Pseudoenzymes' meeting was the appreciation (or perhaps re-discovery) that a very wide range of pseudoenzymes has appeared across the kingdoms of life. Most importantly, pseudoenzymes have been described in various microbes, model prokaryotes, unicellular protists and across the eukaryotes, including in yeasts, plants, invertebrates and vertebrates. In some cases, evolution has produced ubiquitous pseudoenzyme subfamilies, in others either a specific niche has been defined by a

pseudoenzyme, or we remain in the dark as to how sequence variation dictates the required transition between enzyme and pseudoenzyme: only future experimentation will reveal these mechanisms. In Table 1, we summarize the enormous diversity among pseudoenzyme classes, which now includes pseudokinases, pseudo-Histidine kinases of the 'two-component' family, pseudophosphatases, pseudoproteases, pseudoDUBs, pseudo-Ubiquitin ligases, pseudonucleases, pseudoATPases, pseudoGTPases, pseudochitinases, pseudosialidases, pseudolyases, pseudotransferases, pseudoHATs, pseudophospholipases, pseudooxidoreductases and pseudodismutases. Whilst these types of pseudoenzyme are distinct with respect to their evolutionary origins, and their cellular mechanisms of action are often poorly understood, it is clear that they perform biological functions, including allosteric regulation of *bona fide* enzymes, regulation of protein localisation/trafficking, or nucleation of signalling complexes. Intriguingly, despite extensive searches, some classes of enzyme do not have readily identifiable pseudoenzyme counterparts. A notable example is the HECT E3 Ubiquitin ligase family, a critical group of E3 ligases distinct from the more common 'scaffold-like' RING-type E3 ligases whose catalytic residues are notoriously difficult to predict from sequence [23]. HECT E3 ligases possess an invariant Cys residue in the catalytic centre that forms a direct covalent ubiquitin intermediate after transfer from an E2 ligase [24]. We speculate here that this critical mechanistic Cys is fundamental to the function of this enzyme class, and not appropriate for a 'pseudoenzyme' niche in cell signalling.

Finally, we anticipate that as we learn more about pseudoenzymes from detailed cellular and molecular studies, the molecular basis for pseudoenzyme evolution will be slowly revealed, enabling the diverse mechanisms by which pseudoenzymes operate in cells to become clearer.

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Table 1: Diversity amongst pseudoenzymes

Class	Function	Examples	References
Pseudokinase	Allosteric regulation of conventional protein kinase	STRADα regulates activity of the conventional protein kinase, LKB1	[5, 25]
		JAK1-3 and TYK2 C-terminal tyrosine kinase domains are regulated by their adjacent pseudokinase domain	[26] [27]
		KSR1/2 regulates activation of the conventional protein kinase, Raf	
	Allosteric regulation of other enzymes	VRK3 regulates activity of the phosphatase, VHR	[28]
	Protein interaction domain	MLKL pseudokinase regulates exposure of the executioner four-helix bundle domain, and engagement of HSP90:Cdc37	[29-31]
	Scaffold for assembly of signalling complexes	Tribbles proteins nucleate assembly of a complex between a substrate (C/EBPα) and the E3 Ubiquitin ligase, COP1	[32, 33]
Pseudo-Histidine kinase	Protein interaction domain	*Caulobacter* DivL binds the phosphorylated response regulator, DivK, allowing DivL to negatively regulate the asymmetric cell division regulatory kinase, CckA	[34]
Pseudophosphatase	Occlusion of conventional phosphatase access to substrate	EGG-4/EGG-5 binds to the phosphorylated activation loop of the kinase, MBK-2	[35, 36]
		STYX competes with DUSP4 for binding to ERK1/2	[37]
	Allosteric regulation of conventional phosphatases	MTMR13 binds and promotes lipid phosphatase activity of MTMR2	[38]
	Regulation of protein localisation in	STYX acts as a nuclear anchor for ERK1/2	[37, 39]

	a cell		
	Regulation of signalling complex assembly	STYX binds the F-box protein, FBXW7, to inhibit its recruitment to the SCF Ubiquitin ligase complex	[39, 40]
Pseudoprotease	Allosteric regulator of conventional protease	cFLIP binds and inhibits the cysteine protease, Caspase-8, to block extrinsic apoptosis	[41]
	Regulation of protein localisation in a cell	Mammalian iRhom proteins bind and regulate trafficking single pass transmembrane proteins to plasma membrane or ER-associated degradation pathway	[22, 42-44]
Pseudodeubiquitinase (pseudoDUB)	Allosteric regulator of conventional DUB	KIAA0157 is crucial to assembly of a higher order heterotetramer with DUB, BRCC36, and DUB activity	[45]
Pseudoligase (pseudo-Ubiquitin E2)	Allosteric regulator of conventional E2 ligase	Mms2 is a ubiquitin E2 variant (UEV) that binds active E2, Ubc13, to direct K63 ubiquitin linkages	[46]
	Regulation of protein localisation in a cell	Tsg101 is a component of the ESCRT-I trafficking complex, and plays a key role in HIV-1 Gag binding and HIV budding	[47]
Pseudoligase (pseudo-Ubiquitin E3)	Possible allosteric regulator of conventional RBR family E3 ligase	BRcat regulates interdomain architechure in RBR family E3 Ubiquitin ligases, such as Parkin and Ariadne-1/2	[17, 48]
Pseudonuclease	Allosteric regulator of conventional nuclease	CPSF-100 is a component of the pre-mRNA 3′ end processing complex containing the active counterpart, CPSF-73	[49]
PseudoATPase	Allosteric regulator of conventional ATPase	EccC comprises two pseudoATPase domains that regulate the N-terminal conventional ATPase domain	[14]
PseudoGTPase	Allosteric regulator of conventional	GTP-bound Rnd1 or Rnd3/RhoE bind p190RhoGAP to	[50-52]

	GTPase	regulate the catalytic activity of the conventional GTPase, RhoA	
	Scaffold for assembly of signalling complexes	MiD51, which is catalytically dead but binds GDP or ADP, is part of a complex that recruits Drp1 to mediate mitochondrial fission	[53]
		CENP-M cannot bind GTP or switch conformations, but is essential for nucleating the CENP-I, CENP-H, CENP-K small GTPase complex to regulate kinetochore assembly	[54]
	Regulation of protein localisation in a cell	Yeast light intermediate domain (LIC) is a pseudoGTPase, devoid of nucleotide binding, which binds the dynein motor to cargo. Human LIC binds GDP in preference to GTP, suggesting nucleotide binding could confer stability rather than underlying a switch mechanism.	[55]
Pseudochitinase	Substrate recruitment or sequestration	YKL-39 binds, but does not process, chitooligosaccharides via 5 binding subsites	[56, 57]
Pseudosialidase	Scaffold for assembly of signalling complexes	CyRPA nucleates assembly of the *P. falciparum* PfRh5/PfRipr complex that binds the erythrocyte receptor, basigin, and mediates host cell invasion	[58-60]
Pseudolyase	Allosteric activation of conventional enzyme counterpart	Prozyme heterodimerisation with S-adenosylmethionine decarboxylase (AdoMetDC) activates catalytic activity 1000-fold	[61-63]
Pseudotransferase	Allosteric activation of cellular enzyme counterpart	Viral GAT recruits cellular PFAS to deaminate RIG-I and counter host antiviral defence	[64]
		T. brucei deoxyhypusine synthase (TbDHS) dead paralog,	[65]

		DHSp, binds to and activates DHSc >1000-fold.	
Pseudo-histone acetyl transferase (pseudoHAT)	Possible scaffold for assembly of signalling complexes	Human *O*-GlcNAcase (OGA) lacks catalytic residues and acetyl CoA binding, unlike bacterial counterpart	[66]
Pseudo-phospholipase	Possible scaffold for assembly of signalling complexes	FAM83 family proteins presumed to have acquired new functions in preference to ancestral phospholipase D catalytic activity	[67]
	Allosteric inactivation of conventional enzyme counterpart	Viper phospholipase A2 inhibitor structurally resembles the human cellular protein it targets, phospholipase A2	[68]
Pseudo-oxidoreductase	Allosteric inactivation of conventional enzyme counterpart	ALDH2*2 thwarts assembly of its "wild-type" counterpart, ALDH2*1, into a fully-active homo-tetramer	[69]
Pseudo-dismutase	Allosteric activation of conventional enzyme counterpart	Copper chaperone for superoxide dismutase (CCS) binds and activates catalysis by its enzyme counterpart, SOD1	[70, 71]
Pseudo-dihydroorotase	Regulating folding or complex assembly of conventional enzyme	*Pseudomonas* pDHO is required for either folding of the aspartate transcarbamoylase catalytic subunit, or its assembly into an active oligomer	[72]

Pseudoenzymes are shown in blue, while conventional enzymes are shown in black text.