Brought to life: targeted activation of enzyme function with small molecules

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Abstract Cell-permeable small molecules that are capable of activating particular enzymes would be invaluable tools for studying protein function in complex cell-signaling cascades. But, is it feasible to identify compounds that allow chemical–biology researchers to activate specific enzymes in a cellular context? In this review, we describe some recent advances in achieving targeted enzyme activation with small molecules. In addition to surveying progress in the identification and targeting of enzymes that contain natural allosteric-activation sites, we focus on recently developed protein-engineering strategies that allow researchers to render an enzyme of interest “activatable” by a pre-chosen compound. Three distinct strategies for targeting an engineered enzyme are discussed: direct chemical “rescue” of an intentionally inactivated enzyme, activation of an enzyme by targeting a *de novo* small-molecule-binding site, and the generation of activatable enzymes via fusion of target enzymes to previously characterized small-molecule-binding domains.

Keywords Enzyme activation · Allosteric · Chemical genetics · Protein kinases · FLaSH · Chemical inducers of dimerization

Introduction

Chemical biologists often analogize the activities of the compounds they discover to genetic mutations. Indeed, the

term “chemical genetics” (roughly 50,000 Google hits at the time of writing) arose to emphasize precisely this point: A small molecule can, in principle, like a mutation, control gene-product activity with high specificity, allowing for precise elucidation of the target protein’s (and, thus, the encoding gene’s) function(s). Furthermore, the chemical geneticist will argue, small molecules can be used in varying concentrations, and small molecules act rapidly and reversibly, allowing for greater experimental and temporal control than genetic mutations afford. Taking the analogy a bit further, one can imagine two general classes of chemical-genetic experiments: loss-of-function (using small-molecule inhibitors to remove a specific protein’s function) and gain-of-function (using small-molecule activators to turn on a target protein’s activity). The default biologically active small molecule (i.e., “drug”) is the inhibitor: The most intuitive way to modulate the activity of a cellular enzyme is to design and synthesize a compound that competitively binds to the target’s active site. In principle, however, target-specific activators could prove equally powerful: What better way to deconvolute a complex cell-signaling pathway than to have a way to specifically upregulate its component activities, one enzyme at a time?

It is not by accident or oversight that most protein-level chemical-genetic approaches focus on the disruption, as opposed to activation, of protein function. It is inherently more straightforward to design small molecules that “gum up the works” of a functioning protein than to find compounds that “fix” a broken one. Indeed, the very term “target” implies a functioning unit waiting to be destroyed by a small-molecule warhead.

So, is it possible to discover compounds that specifically *activate* target enzymes? And, is it feasible to devise general methods that allow chemical–biology researchers

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to activate specific enzymes of interest? To approach answers to these questions, we will limit our scope to small molecules that activate enzymatic function at the level of protein—that is, compounds that activate an enzyme by a protein/small-molecule interaction. We will focus on protein targeting, as opposed to DNA- or mRNA-targeting drugs, because compounds that directly activate a target enzyme maximize the key advantages that small-molecule biological probes hold over genetic mutations—namely, rapidity and reversibility of action. (That said, small-molecule control of gene expression at the DNA level is itself a rich and exciting field of chemical biology [1, 2].) In this short review, we will describe some notable successes in achieving targeted enzyme activation with small molecules, with a particular focus on recently developed protein-engineering strategies that allow researchers to render an enzyme of interest “activatable” by a pre-chosen drug.

Allosteric activation of natural enzymes

While much of our attention will center on mutagenic means to *make* an enzyme activatable, we must first give Nature its due: Many enzymes possess natural allosteric-activation sites that can be targeted by the standard tools of medicinal chemistry. (The question of “How many?” is extraordinarily difficult to answer; general means for identifying allosteric sites are not known, and allosteric sites are often not readily apparent by inspection of a crystal structure [3, 4].) In the last few years, high-throughput screening (HTS) of small-molecule libraries has been

effective for identifying allosteric activators of several natural enzyme targets, including glucokinase (GK), AMP-activated protein kinase (AMPK), p300 histone acetyltransferase, RNase L, and the sirtuin family of NAD⁺-dependent protein deacetylases.

The recently discovered GK activators provide excellent examples of the impact that direct enzyme activators can have on chemical biology and medicine. GK, which enhances insulin secretion from pancreatic β -cells and glucose metabolism in the liver [5], contains an allosteric binding site that normally binds to the glucokinase-regulating protein (GKRP) [6]. At low glucose concentrations, GK is localized in the nucleus and binds to GKRP in an inactive form, while at high glucose concentrations, the GK–GKRP complex dissociates, and GK moves to the cytoplasm to phosphorylate glucose [7]. By screening 120,000 structurally diverse synthetic compounds, Grimsby and co-workers [8] discovered a GK activator, compound **1** (Fig. 1), that dramatically reduces GK’s Michaelis constant (K_M) for glucose and also reverses the inhibitory action of GKRP. The authors went on to show that treatment with **1** lowered blood-glucose concentrations and improved glucose tolerance in rodent type 2 diabetes models [8]. These seminal results have opened an entire field of GK activators as chemical tools for probing glucose metabolism and potential anti-diabetes therapeutics, and several classes of GK activators have now been identified [9–14]. Thanks to the work of Kamata and co-workers, we now understand the structural basis of GK activation in some detail [15]. These authors solved the structure of GK bound to activator compound **2** (Fig. 1). Comparison of the GK/2 co-structure (Fig. 2a) with that of the apoenzyme

Fig. 1 Chemical structures of the small-molecule enzyme activators discussed in the text

Fig. 2 Structural basis for allosteric activation of GK by compound 2. **a** Co-crystal structure of the GK/2/glucose complex, in which GK adopts the active “closed” conformation (PDB: 14VS [15]). GK is shown as a *magenta ribbon*. For perspective, two regions of the enzyme (amino-acid residues 64–71 and 443–461) that undergo major conformational changes upon binding to **2** are colored in *green*. Space-filling representations of glucose and compound **2** are colored by element: *gray* for carbon, *red* for oxygen, *blue* for nitrogen, and *yellow* for sulfur. **b** Crystal structure of apo GK, in which GK adopts the inactive “super-open” conformation (PDB: 14VT [15]). Protein coloring scheme is identical to that of **a**. Images were generated using the Chimera software package (<http://www.cgl.ucsf.edu/chimera>)

(Fig. 2b) revealed that the GK activator stabilizes a “closed” and activated form of the enzyme by binding to an allosteric site 20 Å removed from GK’s active site. In the absence of compound, a “super-open” and inactive form of GK is observed.

HTS on synthetic compound libraries has also proven to be an effective means for the discovery of small molecules that selectively activate other natural enzymes (Fig. 1): Compound **3** binds to the auto-inhibitory domain of AMPK, thereby releasing the auto-inhibition and activating the enzyme [16]; compound **4** alters the structure of p300, causing a fivefold increase in the turnover number (k_{cat}) of the protein’s histone-acetylation activity [17]; and compound **5** induces the dimerization of RNase L and thereby produces a sevenfold rate enzymatic enhancement [18]. Finally, it has been found that natural-product polyphenols such as resveratrol (**6**) activate sirtuins in vitro and enhance the life span of yeast [19], as well as that of metazoans [20], though the causal connection between sirtuin activation and life-span enhancement is not clear [21].

Small-molecule activation of engineered proteins

The examples of small-molecule-induced activation discussed in the previous paragraphs all involve enzyme targets that possess natural allosteric-control sites. Most enzymes, however, do not have (known) allosteric sites, and each allosteric site mentioned above is targeted by a different family of compounds, each with greater or lesser degrees of potency and specificity. The world of proteins is dazzling in its structural diversity, and no *general* approach for targeting natural allosteric enzymes is known; indeed, it is difficult to imagine a more efficient means than HTS for doing so. But, can we imagine more general and systematic means of discovering small molecules that can selectively activate *modified* enzymes? Some recent advances in protein-target engineering allow for an answer of a qualified “yes.” It is, at least, conceivable to use rational protein design to change enzyme targets that do not possess natural allosteric-control sites into non-natural activatable mutants that do. Given certain constraints, one can use site-directed mutagenesis, peptide insertion, or the generation of protein chimeras to create mutant enzymes that can be selectively activated by small molecules. The general methods, described to date, for activating engineered proteins are the focus of this review. While these approaches differ substantially in ease and generality of use, what they share is that the enzyme being activated is an intentionally mutated protein target, not the wild-type protein. (It should be noted that while such systems may have profound effects in chemical–biology studies, they of course do not provide directly applicable medicinal tools, as the mutated target protein would not be present in a human patient.) Three general approaches for targeting an engineered enzyme will be discussed: direct chemical complementation of a mutation in or near an enzyme active site, activation of an enzyme by targeting a de novo non-natural

small-molecule binding site, and the generation of activatable enzymes via fusion of target enzymes to small-molecule-binding domains.

Direct chemical rescue of enzyme activity

The most intuitive manner in which to make an enzyme activity dependent on the presence of a small molecule is simple chemical complementation, in which an enzymatically deleterious mutation is overcome by the addition of a small molecule that can functionally replace the “lost” amino-acid side chain. Chemical complementation and its cousin, substrate-assisted catalysis (in which the enzyme’s substrate provides the missing functionality) [22], have a long and important history in the study of enzyme mechanisms and protein folding. These contributions have been ably reviewed elsewhere [23, 24], and our focus will be on the prospect of using chemical complementation as a tool for chemical *biology*—that is, a means to study biological pathways by specifically turning on an enzyme’s function in whole cells and/or model organisms.

In principle, any cellular enzyme that one wished to study could be catalytically compromised via mutation and subsequently re-activated by addition of a suitable small molecule in a cellular model system. In practice, the rational design of such mutant/activator pairs is extremely difficult. Moreover, if one wishes to use the activator in cells, chemical-complementation strategies are subject to many of the same cell permeability, bioavailability, and toxicity constraints that limit pharmaceutical discovery. To our knowledge, only one bona fide cellular strategy for direct chemical rescue of an engineered signaling enzyme has been described. Fortuitously, this one example targets the protein kinases, one of the largest (over 500 encoded by mammalian genomes) and most important families of signaling enzymes [25].

It was nearly a decade ago that Cole and co-workers first showed that a mutated protein kinase [C-terminal Src kinase (Csk)] could be activated by the addition of small molecules [26]. These authors mutated a conserved arginine residue of the Csk catalytic loop to alanine and found that the resulting “dead” kinase (R318A Csk: 3,000-fold lower k_{cat} than wild-type Csk) could be activated by a range of compounds that possess imidazole rings or guanidinium-like functional groups. The strongest activation observed was with imidazole itself: a 60-fold increase in relative velocity at 50 mM imidazole [26]. These authors, as well as other investigators, later used the activatable Csk mutant to probe the role of this kinase in actin-stress-fiber formation [27, 28]. There do exist, however, complications in the use of imidazole to turn on Csk activity. Even in the presence of 50 mM imidazole (which is significantly cytotoxic [29]),

R318A Csk activity is only about 5% of the wild-type’s activity [26], highlighting the difficulty in finding small molecules that act as true “on switches.”

What is most exciting about the kinase-activity-rescue approach devised by Cole and co-workers is its potential to be generalized. The protein-kinase catalytic loop is highly conserved, and most protein kinases have an arginine or lysine residue at a position either two or four residues to the C-terminal side of an invariant aspartate residue [25]. Thus, the homology of the kinase superfamily allows, in principle at least, for the application of a Csk-like chemical-rescue strategy to any protein kinase through a relatively straightforward series of steps: identify the positively charged residue that corresponds to R318 of Csk using a primary sequence alignment [26], mutate the corresponding residue in the kinase of interest to alanine, and investigate the alanine mutant’s ability to be activated by imidazole or related compounds.

Indeed, it was the application of imidazole-induced rescue not to Csk but to a different protein kinase, c-Src, that has provided the most unambiguous example of the power of chemical rescue for studying kinase function. Following from their groundwork on Csk, Cole and co-workers created a catalytically compromised mutant of c-Src kinase (R338A c-Src) that could be rescued by imidazole (Fig. 3) [30]. Serendipitously, the robustness of R338A c-Src rescue exceeded that of R318A Csk rescue: The inherent k_{cat} value of R338A c-Src is roughly 200-fold reduced from that of wild-type c-Src, and in the presence of imidazole, its activity increases two orders of magnitude to 30–50% of the wild-type value. Importantly, half-maximal recovery of R338A c-Src activity was attained at 2.5 mM, a concentration at which imidazole is not strongly toxic.

Cole and co-workers then expressed R338A/Y527F c-Src (the second mutation was designed to prevent down-regulation of Src function by Csk phosphorylation [31]) in a SYF (Src, Yes, and Fyn deleted) mouse embryonic fibroblast cell line [30]. As a control, they expressed D386N/Y527F c-Src in another fibroblast cell line; the D386N mutation renders c-Src essentially non-functional and unresponsive to imidazole [30]. After incubation with imidazole, tyrosine phosphorylation levels were significantly higher in the R338A/Y527F c-Src-expressing cells than in the D386N/Y527F c-Src expressers. This upregulation of phosphotyrosine levels was unambiguously due to Src activity, as incubation of R338A/Y527F c-Src-expressing cells with both imidazole and PP1 (a Src-family inhibitor [32]) did not produce an increase in tyrosine phosphorylation relative to that of the D386N/Y527F c-Src-expressing cells. More importantly, the authors used affinity chromatography and mass spectrometry to identify 18 novel potential c-Src targets. Additionally, the authors provided new evidence that Src is a positive effector of PDGF-

Fig. 3 Structural basis for the chemical rescue of R338A c-Src by imidazole. **a** ATP binding by wild-type c-Src (PDB: 2SRC [53]). The c-Src protein surface is shown in *magenta*, with the portion of the surface comprising R338 highlighted in *yellow*. The bound non-hydrolyzable ATP analogue is colored by element as in Fig. 2. **b** The R338A “hole” is filled by imidazole. A model of R338A c-Src was generated from PDB 2SRC by deletion of the R338 side-chain atoms beyond the β -carbon. Protein and ATP analogue representations are identical to those of **a**

stimulated MAPK activation [30]. It remains to be seen whether or not imidazole rescue will prove to be a widely used method for the study of kinase-signaling pathways. But the stunning results of Cole and co-workers on c-Src, coupled

with the potentially general targeting method by which rescue was achieved, certainly provide a positive outlook.

FLAsH-induced “rescue” of an enzyme containing a de novo activation site

While protein-kinase rescue provides a remarkable example of the potential of small-molecule activators in biological studies, it offers little guidance for how to proceed in identifying activators for other enzyme families. Could there exist a means to introduce de novo “activation loops” in enzymes for which other chemical-rescue strategies have not been developed? A recent report has shown that insertion of a short peptide into a protein domain can indeed be used to generate an activatable enzyme variant [33]. This study utilized the cell-permeable small-molecule fluorescein arsenical hairpin binder (FLAsH), which is known to bind specifically to peptides containing tetra-cysteine motifs [34, 35]. Liscovitch and co-workers inserted a FLAsH-binding peptide (Cys-Cys-Pro-Gly-Cys-Cys) at various locations within the bacterial antibiotic-resistance enzyme TEM-1. FLAsH has no significant affinity for wild-type TEM-1 (which contains no CCPGCC motifs), but binds to and modulates the activity of mutants containing CCPGCC. The authors identified several FLAsH-responsive mutants, most of which are inhibited by FLAsH binding [33]. By contrast, the TEM-1 mutant with a CCPGCC insertion at amino acid 215 (4C-215 TEM-1) is specifically *activated* by FLAsH. 4C-215 TEM-1 is significantly impaired in the absence of FLAsH (37-fold reduction in $V_{\max}$ compared with the wild-type protein), but incubation with 10 μ M FLAsH for 2 h leads to a roughly 11-fold increase in TEM-1 activity (Fig. 4). Although the authors do not show that TEM-1 activation can occur in living cells, the cell permeability of FLAsH has been well established in previous work [36–38]; thus, it is likely that intracellular 4C-215 TEM-1 activation could indeed be

Fig. 4 Specific TEM-1 activation via mutagenic introduction of FLAsH affinity. Wild-type TEM-1 (**a**) is modified to contain a cysteine-rich FLAsH-binding peptide (**b**). FLAsH does not bind to the wild-type enzyme, whereas it specifically binds to the engineered loop of 4C-215 TEM-1 and activates the enzyme

effected with FAsH. Liscovitch and co-workers go on to argue that their FAsH-based approach could constitute a general method for ligand-sensitive protein engineering. This is true up to a point. It is no doubt the case that there is nothing “special” about TEM-1; any enzyme could in principle be rendered activatable by insertion with a FAsH-binding peptide. [Indeed, our lab has recently found that insertion of an analogous FAsH-binding unit renders a protein tyrosine phosphatase activatable by FAsH (Chen and Bishop, unpublished results).] The clear limitation of FAsH-based activation, however, is that 4C-215 TEM-1 activation provides no sequence guidance for how to extend the approach to a different family of enzymes. That is, if one wanted to generate FAsH-activatable mutants of an enzyme that is not homologous to TEM-1, it is not clear where in the enzyme one would place the potentially FAsH-binding insertion. For most enzyme targets, a new panel of insertion mutants, in addition to a labor-intensive process of purifying and testing each mutant for FAsH activation, would be required [39].

Enzyme activation using chemical inducers of dimerization

Chemical inducers of dimerization (CIDs) have arguably proven to be the single most powerful tool in the chemical-genetic analysis of engineered cellular systems [40–43]. Briefly, CIDs are small molecules (usually based on the natural products rapamycin or FK506) that specifically bind to two proteins [usually the 12-kDa FK506-binding protein (FKBP12) and/or the FKBP-rapamycin-binding domain (FRB)], rapidly bringing together those proteins in a ternary protein–CID–protein complex. If the CID-binding proteins are fused to other proteins of interest (POIs), then the target proteins will also be brought together, effecting small-molecule-induced protein-complex formation and/or changes in cellular localization. CIDs do not generally directly activate enzymes by inducing allosteric changes. However, some enzymes can be activated by the physical processes of plasma-membrane localization (Fig. 5a) or dimerization (Fig. 5b); for such enzymes, CIDs can be used as activating small molecules. Additionally, a general strategy has recently been developed in which a CID can be used to rescue a POI from proteasomal degradation, effectively allowing for small-molecule activation of target proteins (Fig. 5c). Our discussion of CIDs will focus on these specific strategies in which CID addition gives rise to a targeted increase in enzyme activity.

A number of proto-oncogenic protein tyrosine kinases are activated when they are translocated to the plasma membrane and/or dimerized. For these enzymes, fusion to CID-binding domains represents a powerful strategy for engineering small-molecule-inducible activation, and, thus,

for probing the mechanisms of oncogenic transformation. The signaling pathways of the Src-family kinases have been studied by inducing their activation via localization to the plasma membrane [44]; likewise, activity of the anti-apoptotic kinase Akt can be induced via membrane localization [45]. CID-induced homo-dimerization can also be sufficient to activate some target enzymes, either in an intracellular or membrane-bound context. Intracellular activation has been used to study caspase activation in apoptosis [46], and Smith and Van Etten have shown that the dimerization-induced activation of the non-receptor tyrosine kinase c-Abl confers interleukin-3-independent growth in Ba/F3 cells [47]. Moreover, the CID-induced dimerization of membrane-bound receptor tyrosine-kinase (RTK) activity provides a general paradigm for activation through dimerization: engineered forms of FGFR [48], ErbB [49], c-kit [50], PDGFR [51], and the insulin-receptor [51] tyrosine kinases have all been used in signaling studies that use CID-activatable chimeras of the target RTKs.

Muir and co-workers have recently reported a CID-based system that is, in principle, even more generalizable in that it does not require that localization or multimerization of a particular POI is inherently activating [52]. In this approach, the addition of a CID produces a dimerization event that rescues the target protein from proteolysis. Two chimeric proteins are generated (Fig. 5c): The first contains the POI attached to the C-terminal half of ubiquitin, which is in turn attached to FRB and a “degron” that targets the fusion protein to the proteasome; the second chimera contains the N-terminal half of ubiquitin attached to FKBP. In the absence of CID, the two chimeras will stay separate, and the degron-POI chimera is degraded. Addition of CID, however, brings the two halves of ubiquitin together, allowing ubiquitin-specific proteases to act on the complex, thus releasing the POI from the remainder of the protein complex. Freed from its previously attached degron partner, the POI can now fold and function normally. Thus, in and only in the presence of the CID will the POI function in a cell. Muir and co-workers successfully used this “proteolytic shunt” to rescue three enzymes—luciferase, v-Src, and caspase-3 (as well as a transcription factor, Smad3)—from degradation in living cells, and the fact that the shunt works on very different proteins highlights its general utility.

Although the CID-induced proteolytic shunt does not involve activation of an enzyme per se (it does not directly improve the catalytic properties of the POI), it is an example of a drug-controlled enhancement of a POI’s biological activity through a protein-level interaction. That said, the practical outcomes of CID-induced rescue arguably have more in common with DNA-level gene-expression activation than with other protein-targeting small-molecule approaches. Most fundamentally, the proteolytic shunt controls the *amount* of the target protein in a CID-treated cell (as does

Fig. 5 Three strategies for CID-induced activation of enzyme function. **a** Activation through membrane localization. Application of a CID induces a hetero-dimerization of a membrane-anchored chimera and an intracellular chimera containing the target enzyme. Membrane localization “activates” the target enzyme, usually due to an increase in local concentration of potential enzyme substrates. **b** Activation through dimerization. Application of a CID induces homo-dimerization of a membrane-bound enzyme, usually a receptor tyrosine kinase. Multimerization at the membrane leads to target-enzyme activation. **c** Activation via a “proteolytic shunt.” Addition of CID leads to hetero-dimerization of two protein chimeras: one containing a degron, a CID-binding domain, the C-terminal half of ubiquitin (Ub_C), and the target enzyme; the second containing a CID-binding domain attached to the N-terminal half of ubiquitin (Ub_N). In the absence of CID, the degron-containing chimera is degraded by the proteasome. Addition of CID induces hetero-dimerization, bringing the two halves of ubiquitin together; ubiquitin-specific proteases release the target enzyme

transcriptional upregulation) rather than the specific activity of the POI molecules that are present at the time of drug treatment. In addition, a 24-h CID incubation is necessary for full POI rescue, a rather long timescale that is comparable to the duration of drug treatment required for transcription-level upregulation [2].

Conclusion

In surveying the landscape of chemical–biology tools for activating enzyme function, the inadequacy in the term

“chemical genetics” becomes apparent. There exists no single unifying strategy for targeting proteins that approaches the universality of genetic modifications. The molecular-recognition problem of protein/small-molecule interactions is simply too complex: Every enzyme target and every small-molecule effector is different, necessitating a rich array of organic-chemistry and biochemical tools, each of which is capable of targeting some fraction of the proteome. The four complementary enzyme-activation strategies discussed here—targeting of natural allosteric sites, direct chemical rescue, de novo allosteric-site engineering, and CID-induced rescue—may enable chemical–biology

researchers to activate hundreds of cellular enzymes in a target-specific manner. As such, these tools provide a partial solution to one of the central challenges of chemical biology: bringing the activities of specific gene products under chemical control.

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