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Are all regions of folded proteins that undergo ligand-dependent order-disorder transitions targets for allosteric peptide mimetics?[†]

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Abstract

Although the classical view of how proteins function relied on well folded structures, it is now recognized that the function of many proteins is dependent on being intrinsically disordered. The primary consideration in this work is the intermediate group of proteins that are overall well folded, but which contain small regions that undergo order/disorder transitions. In particular, the current focus is on those order/disorder transitions that are energetically coupled to ligand binding. As exemplified by the case of human liver pyruvate kinase (hL-PYK), peptides that mimic the sequence of the order/disorder region can be used as allosteric regulators of the enzyme. Based on this example and others reported in the literature, we propose that a similar use of peptides that mimic protein regions that experience ligand-dependent order-disorder transitions can be a generalized initiation point for the development of allosteric drugs.

Classic views of protein functions were based entirely on well organized, “properly folded” protein structures. In contrast, many proteins are now recognized to be intrinsically disordered (1). Between the extremes of well organized folded proteins and those that are completely disordered, many folded proteins have small regions that undergo order/disorder transitions due to some external stimulus. The group of proteins considered in this work is those that are overall well folded that also have small regions that experience order/disorder transitions in response to ligand binding. Although such order/disorder regions often contain sites for covalent modification (2, 3), there are also many ligand-dependent order/disorder transitions that are not associated with known covalent modification sites. Using the example of human liver pyruvate kinase (below), we propose that peptides that mimic these regions of a protein that undergo order/disorder transitions in response to ligand binding can be used as a starting point for the development of allosteric drugs. This use of peptide mimetics remains distinct from the common use of peptide mimetics to block protein-protein interactions.

The regulatory role of the N-terminus of human liver pyruvate kinase in the diabetic liver

The diabetic liver responds to hormone signals communicating an energy deficiency in tissues that are dependent on insulin for glucose transport. In response, the regulatory cascade that includes cyclicAMP-dependent protein kinase is activated, resulting in

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phosphorylation of numerous proteins. Included in this number, several regulatory enzymes in glycolysis and gluconeogenesis become phosphorylated. The outcome of the overall change in phosphorylation status of these glucose utilizing enzymes in the liver is the shift from glucose catabolism to gluconeogenic biosynthesis of glucose. In a diabetic, when this synthesized glucose enters the blood it compounds the problem of already high blood sugar rather than relieving the energy deficiency represented by the original hormone signal. Inhibitory phosphorylation of human liver pyruvate kinase (hL-PYK for human or L-PYK for generic mammal) is thus a component of an overall response by the liver to perceived needs of the body as a whole. This role in liver function has been well characterized, including very detailed studies rates of phosphorylation and dietary stimuli that promote this modification (4–15). In addition, the site of phosphorylation on hL-PYK at Ser12 has been known for some time (16–24). In fact the N-terminal peptide from L-PYK has been the initiation point for characterizing the substrate recognition specificity of the cyclicAMP-dependent protein kinase (4). Despite the extensive knowledge of the physiological role of phosphorylation of L-PYK, the details of the molecular mechanism by which phosphorylation modifies the affinity of the protein for PEP substrate have only recently been investigated (25–28). Such knowledge is needed to facilitate the design of drugs that prevent inhibition and/or activate hL-PYK as a means of counteracting hyperglycemia associated diabetes.

At the onset of studies in my laboratory, a structure had already been reported for a truncated form of the PYK isozyme expressed in erythrocytes (R-PYK) (29). L-PYK and R-PYK are two gene products that result from the use of alternative start sites. As a result, hL-PYK is 31 amino acids shorter than the R-PYK variant. However, the first ordered residue in the R-PYK structure was equivalent to Gln26 of hL-PYK. Therefore, the preexisting structure of R-PYK did not reveal details related to regulation by phosphorylation. The only other clue into the molecular level mechanism was the observation that, like phosphorylation, proteolytic removal of the N-terminus results in decreased PEP affinity (30).

Recent studies directed at understanding the molecular mechanism of this N-terminal regulation include a truncation series, designed point mutations surrounding the phosphorylation site, alanine-scanning mutagenesis using single, double consecutive, and triple consecutive alanines, mutant cycle analysis, and crystallographic studies of mutant protein (25–28). Data from these studies are consistent with the N-terminus binding to the main body of the protein in the absence of phosphorylation (or oxidation). This activating interaction results in a long-range, through-protein increase in substrate, phosphoenolpyruvate (PEP), affinity. Phosphorylation at Ser12 and oxidation at Cys436 on the main body of the protein both prevent this activating interaction to result in the apparent modification-dependent reduction in PEP affinity (Figure 1). There is now sufficient information to assign detailed functions to residues in the 25 amino acid N-terminus of hL-PYK:

MEGPAGYLLRRASVAQLTQELGTAFF

The initial six residues have no role in energetic coupling with PEP binding (26, 28). Residues 7–10 define a binding motif, although it is still unclear exactly where on the main body of the protein this motif docks. Interestingly, this 7–10 binding motif is also the recognition sequence for cyclicAMP-dependent protein kinase (4). Within this binding motif Faustova et al. found that many single amino acid substitutions could be accommodated with only moderate influences on PEP affinity; the primary exception was a mutation that altered the side-chain charge at position 9 (25). These findings are consistent with

observation from studies in my laboratory that each residue in the 7–10 binding motif contributing to binding affinity, rather than the motif working as an all-or-none cooperative unit (28). Further into the N-terminus, Leu20, Phe24, and Phe25 all make favorable interactions with the side-chain of position 436 (28). Therefore, it seems likely that oxidation of Cys436 interferes with the ordering of additional N-terminal residues as compared to those that become disordered as a result of phosphorylation at Ser12.

Allosteric nature of peptide mimetics

Consistent with this developing model of the regulation of hL-PYK, peptide mimetics of the non-phosphorylated N-terminus that include the sequence of residues 6–12 activate the S12D (phosphorylation mimic) inhibited protein (27). Since these peptides are designed to bind at a site distant from the active site, they modify substrate affinity through an allosteric mechanism. As previously outlined, allosteric drugs have an intrinsic safety benefit. Mainly, once all effector binding sites are filled, no additional regulator effect will result as the concentration of the drug is raised even higher (i.e. a concentration-independent limit to function) (31,32). This intrinsic limit to the maximal effect is a safety advantage over more traditional drug designs such as those based on competitive binding.

In summary, the N-terminal based peptide activators of hL-PYK 1) exemplify mimetics that are designed on a ligand-dependent order/disorder transition, 2) act as allosteric effectors, and 3) are a starting point for drug design to counteract hyperglycemia. Like the example of hL-PYK, there are likely many systems that have regions distant to the ligand binding site that undergo order/disorder transitions. Peptide mimetics of these regions will also function through allosteric mechanism. Therefore, the benefits of allosteric drugs discussed here will be generally applicable to most peptide mimetics based on ordered/disordered regions.

Example of peptide mimics of order/disorder elements in the literature

Although there was no intent here to provide an exhaustive list, there are other examples in the literature of peptide mimetics of ligand induced order/disorder regions of a protein. Although an early example was the regulation of glycogen phosphorylase by a phosphopeptides mimic of the N-terminus of that protein (33), the interpretation of that data has now been questioned (34). Therefore, other examples will be considered. Also, looking beyond examples that are consistent with our very focused use of peptides to mimic order/disorder in overall well folded proteins, there are many examples of peptides based on proteins that seem to function in a similar manner even if structural/mechanistic details have not been reported (35, 36).

One example from the literature is the activation of p53 by peptides that mimic the negative regulatory domain of the p53 protein (37). p53 is composed of a N-terminal transactivation domain, a proline-rich domain, the core DNA binding domain, the tetramerization domain and the C-terminal negative regulatory domain. Despite the general consideration of p53 as an intrinsically disordered protein (38), the core DNA binding domain can form structure that has been solved and this structural domain plays a key role in p53 function (39). Hupp *et al* demonstrated that peptides derived from the negative regulator domain could activate p53 activity (37). Furthermore, the authors demonstrated that these peptides function through an allosteric mechanism. Therefore, allosteric activation of p53 by C-terminal peptides offers an example of mimetics of a order/disorder transition.

A second example provided in the literature is the activation of the insulin receptor by tandem hormone-binding elements. The nomenclature of tandem refers to the inclusion of “Site 1” and “Site 2” elements within the activator peptides (40–43). There is now evidence that the Site 1 element functions by mimicking and, thereby, displacing the -CT segment

from interacting with the remainder of the protein (44). Although the -CT segment is known to be required for insulin binding (45–48), it was omitted from the original structural model (43). This -CT segment has now been shown to form an -helix, although the final nine residues of the -CT remain disordered. Therefore, Site 1 insulin-mimetics are another example of peptide ligand that mimic a region of a protein.

Commonality of order/disorder regions in folded proteins

The current focus is on a very narrowly defined group of proteins, those that are overall well folded with only small regions that experience order/disorder transitions. To justify a focus on this group and the potential of using peptide mimics of the regions that undergo order/disorder transitions to initiate drug development, we need to consider the representation frequency of this group of proteins. Several studies estimate this occurrence to be very high. 1) Bioinformatic surveys have been used to estimate that >30% of eukaryotic proteins contain a disordered segment (49, 50). 2) Realize that this narrowly defined group of proteins is often included in a broader category of intrinsically unstructured proteins. The Disprot database attempts to link structure/function relationships for unstructured proteins (no discrimination between partially and completely unfolded). The 6.01 release of the Disprot database (51) (current as of this writing) includes 684 disordered proteins that include 1513 disordered regions. The large variation in the degree of disorder included in this database has now been sharpened by the finding that N- and C-terminal regions of proteins are more likely to be disordered (52). 3) Sufficient numbers of proteins with ligand-dependent order/disorder to facilitate theoretical considerations of the roles of such energetic coupling within protein systems (53, 54). 4) Finally, NMR descriptions of ligand dependent changes in the dynamical properties of regions far removed from the ligand binding site are now common. Taken together, we conclude that order/disorder transitions in small regions/segments of a protein are common and there are several cases where these order/disorder transitions have been tied to ligand binding events.

Potential challenges

The most likely challenge to be encountered in attempts to design peptides mimetics based on regions of folded proteins that undergo ligand-dependent order-disorder transitions is poor binding affinity. If order/disorder regions are in a binding equilibrium then the proximity of the “unbound” (i.e. disordered region) to the binding site must of necessity contribute to entropy in a way that favors “binding” (i.e. the transition from disordered to ordered). Removing the tether between the main body of the protein and the peptide of interest will modify this entropy component. Therefore, initial studies with peptides that mimic regions that undergo order-disorder transitions are very likely to display poor binding (27). In turn, initial steps of mimetic development will require working with very high concentrations of peptides, the solubility of which may be a limiting factor.

Summary

In addition to our work with human liver pyruvate kinase, there are other examples for which peptides mimic a region of the protein that undergoes a ligand-dependent order/disorder transition. Furthermore, order/disorder within overall folded proteins is likely common and many of these are likely energetically coupled to ligand binding. Therefore, we conclude that peptides that mimic these regions of a protein that undergo order/disorder transitions in response to ligand binding can be used as a starting point for the development of allosteric drugs. However, poor binding affinities of peptides used in initial steps of mimetic development should be anticipated.

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Figure 1.

Based on 4IMA (28), the N-terminus of one subunit (black) crosses a subunit interface to interact with a second subunit (gray). The docking site for the first 17 residues has not yet been resolved. However, it appears that phosphorylation at Ser12 (denoted as P) results in the N-terminus transitioning from a bound ordered state to an unbound disordered state. Oxidation at Cys435 (spacefill on black subunit) also likely causes an order to disorder transition of the N-terminus (not shown), although it is likely that a larger region is interrupted by oxidation in comparison to the disorder caused by phosphorylation (28). Only one full subunit (gray) and a portion of a second subunit (black) of the homotetramer are included here.