ELSEVIER

Searching for new allosteric sites in enzymes

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The discovery of new allosteric sites generates opportunities for the identification of novel pharmaceuticals and increases our understanding of basic biological processes. Increasingly, allosteric sites are being discovered in various families of proteins by several methods, paving the way for the development of entirely new classes of drugs with a wide range of chemotypes. New allosteric sites in enzymes have been discovered both incidentally and by directed means, and the mechanisms by which allosteric activation and inhibition occur at these sites have been investigated. By exploring recent structurally well-characterized examples, trends begin to emerge for both the modes of binding and mechanisms of inhibition.

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Abbreviations

F16BPase	fructose-1,6-bisphosphatase
FVIIa	factor VIIa
GlyP	glycogen phosphorylase
NNRTI	non-nucleoside RT inhibitor
PTP1B	protein tyrosine phosphatase 1B
RT	reverse transcriptase

Introduction

Proteins are dynamic polymers and their conformational flexibility is critical for function. The ability to trap alternate states through binding to sites distal from the active site is one of the most common and powerful means of regulating protein function. Such allosteric regulation has been recognized for half a century, but questions have lingered concerning the mechanics of allosteric regulation, and the structural coupling of allosteric and active sites.

Naturally occurring allosteric sites that affect the regulation of enzymes in central metabolism through feed-back effectors have been extensively studied. Recently, drug discovery efforts have revealed several new allosteric sites that have no known natural effector. Such allosteric sites are of basic interest because they suggest sites that nature may use. These sites are also of pharmaceutical interest because they offer opportunities for discovering new chemotypes for enzymes for which finding drug-like active site inhibitors has been challenging.

Unfortunately, it is not a simple matter to find and characterize new allosteric sites. Although traditional high-throughput screening can readily identify non-competitive inhibitors, many of these work via promiscuous or non-drug-like mechanisms, such as protein aggregation or denaturation, or high-stoichiometry binding [1–3]. Thus, gleaning novel allosteric inhibitors from amongst the mountains of non-specific chaff can be a laborious and intensive process. Nevertheless, within the past few years, several reports have begun to surface on allosteric inhibitors functioning at new allosteric sites. Analysis of these new allosteric sites by X-ray crystallography has given us a detailed view of the molecular mechanisms of inhibition, bringing us closer to a general understanding of the coupling between allosteric and active sites. This review focuses on new allosteric sites that have been observed and structurally characterized in enzymes. A recent review is also available that thoroughly discusses allosteric conformational changes that block protein–protein interactions [4•].

Methods for new allosteric site discovery

Small molecules are often attracted to the active site because active sites are typically located in cavities rich in functional groups that are well suited for binding. There are now several examples of molecules that bind to other sites on the protein surface or in the protein core. In a striking number of these cases, specific binding is sufficient to allosterically exert an inhibitory effect on the bound protein. The site the small molecule reveals may be an allosteric site used by an, as yet, undiscovered natural effector — an orphan allosteric site. In other cases, the allosteric site may not interact with any natural ligand and has only adventitiously been exploited by the small molecule that binds there — a serendipitous allosteric site. In this review, we survey several small molecules that have allowed the discovery of new allosteric sites, but because natural effectors have not been reported for any of these allosteric sites it is not known if they are orphan or serendipitous sites.

Several new allosteric sites have been identified by traditional high-throughput screening followed by X-ray crystallography. This method typically requires rigorous

Table 1

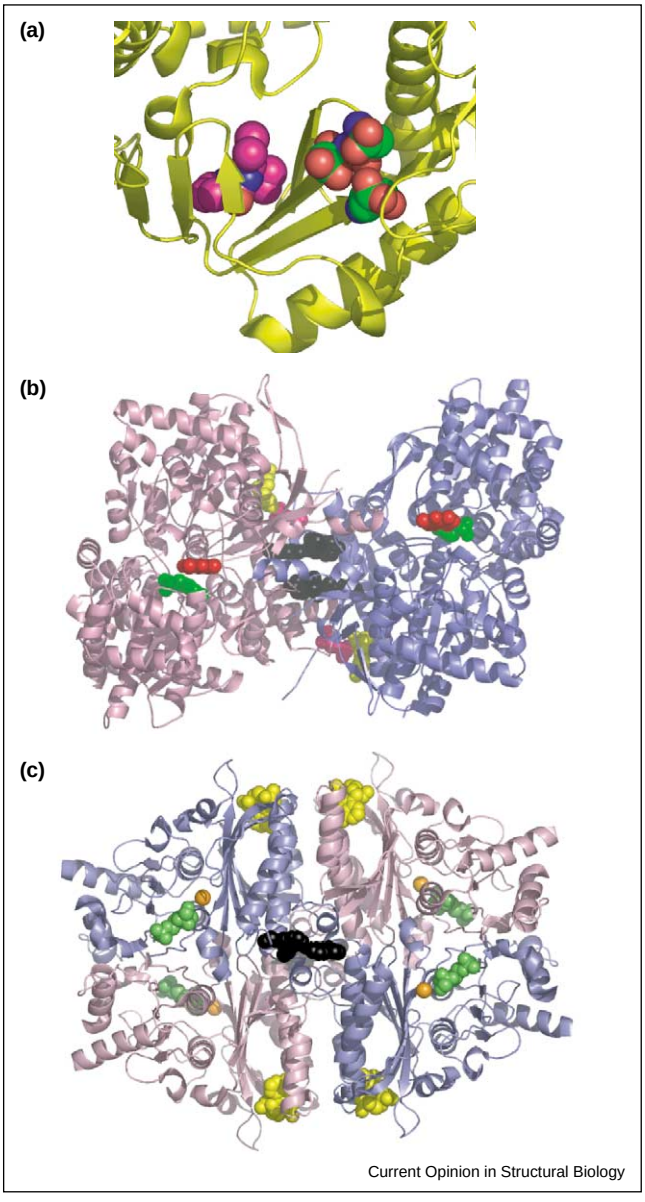
Methods of discovery of new allosteric sites.

High-throughput screening with crystallography	Phage display with crystallography	Tethering
GlyP	FVIIa	Caspase-3
KSP		Caspase-7
F16BPase		
PTP1B		
β-Lactamase		
p38 kinase		
Glucokinase		
HIV-1 RT		

kinetic experiments to determine the mechanism of inhibition before the site of interaction is determined by crystallographic studies. Nevertheless, this method has been widely applied and resulted in the observation of several new allosteric sites (Table 1). One of the first was the allosteric site in HIV-1 reverse transcriptase (RT), which served to nucleate several drug discovery programs for the treatment of AIDS. Three non-nucleoside RT inhibitors (NNRTIs) that bind the novel allosteric site are now commercially available drugs: efavirenz, nevirapine and delavirdine. A host of new NNRTIs are currently being tested in clinical trials. The NNRTIs bind at a site that is near to (10 Å, Figure 1a), but does not overlap the HIV-1 RT polymerase active site [5]. The NNRTIs work by locking the polymerase in an inactive conformation that is also seen in the inactive p51 subunit [6,7]. Binding of nevirapine and other NNRTIs pushes a β sheet as a rigid unit and simultaneously displaces the catalytic Asp186, one of three active site aspartate residues, by 1.9 Å.

Based on the results of screening more than 300 000 compounds [8], researchers at Pfizer and at the National Hellenic Research Foundation in Athens both reported the discovery of a novel allosteric site in human liver glycogen phosphorylase (GlyP) [9,10]. Due to its critical role in controlling blood glucose levels, GlyP is tightly regulated through allosteric activation by AMP or phosphorylation of Ser14, and competitive inhibition by glucose. Interestingly, GlyP is also allosterically inhibited by caffeine. Although the caffeine-binding site was recognized three decades ago [11], the mystery of whether this site evolved as a natural regulatory site or was exploited serendipitously by caffeine remains unanswered. The novel allosteric site binds indole-based compounds (CP-320,626, CP-403,700 and related compounds) and sits more than 30 Å from the catalytic site at the homodimer interface, about 10 Å from the AMP-binding site (Figure 1b). In the absence of the allosteric indole-based inhibitors, the binding site is occupied by water. The authors suggest that this site could not have been predicted based on previous crystal structures, due to the large number of sidechain movements required to reveal the site. Binding of the indole-based inhibitors displaced nine water molecules and buried 85% of the hydrophobic

Figure 1



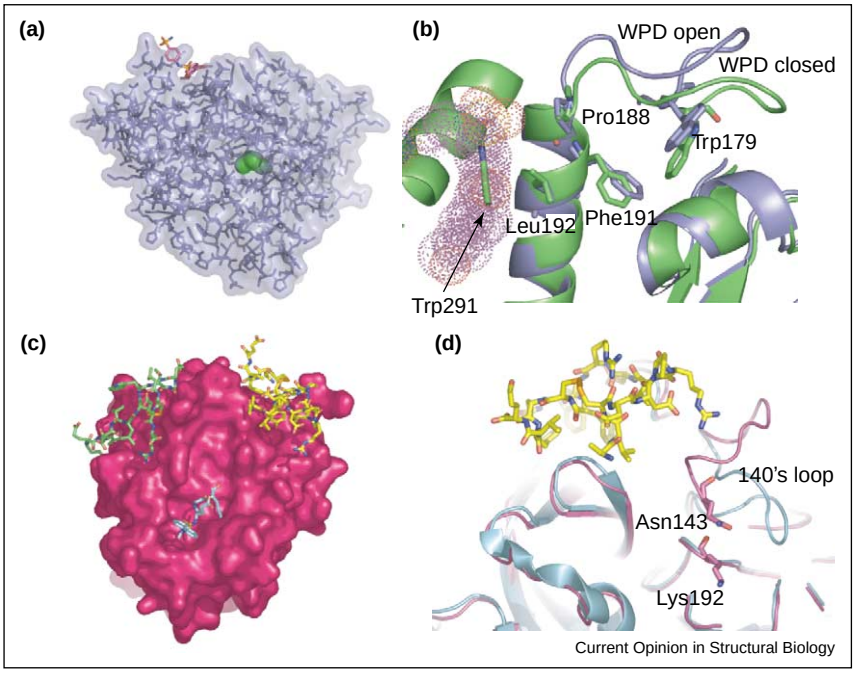
New allosteric sites occur distal from the active site in HIV-1 RT, GlyP and F16BPase. **(a)** The binding site in HIV-1 RT for the NNRTIs (pink spheres) is non-overlapping with the three active site aspartate residues (green spheres). **(b)** GlyP monomers are drawn in pink and blue. The new allosteric site at the dimer interface is shown with the allosteric indole-based compounds drawn as black spheres. The active site is in the region of the pyridoxyl phosphate cofactor (red) and substrate (green). Other regulatory sites are the AMP-binding site (yellow) and the Ser14 phosphorylation site (bright pink). **(c)** The four F16BPase monomers are drawn as pink or blue ribbons. The sites bound by allosteric inhibitors (black spheres) are distal from the AMP-binding site (yellow spheres), the active site manganese (orange) and the fructose-6-phosphate product (green).

ligand surface, forming 6 hydrogen bonds and 111 van der Waals contacts. In the active R (relaxed) state, the two subunits of GlyP are rotated close to one another, but binding of the indole-based inhibitors stabilizes the dimer in the inactive T (tense) state, with the two subunits pushed further apart. A telltale interaction is that of Arg60 and Val64, which are 0.7 Å closer together in the R state. Binding of the allosteric ligands interrupts the intrasubunit hydrogen bond formed by Arg60 and, together with the six ligand–protein hydrogen bonds, locks GlyP apart in the T state conformation. In the T state, the 280's loop near the catalytic site is locked in a closed conformation, promoting glucose binding and synergistic inhibition.

In a case that is strikingly similar to GlyP, a new allosteric site has been observed in fructose-1,6-bisphosphatase (F16BPase), which is also multimeric and allosterically regulated by AMP. F16BPase catalyzes the rate-limiting step in gluconeogenesis, making it important in controlling blood glucose levels. During screening of a library of nucleotide competitive molecules, an additional allosteric site was identified at a region of the homotetramer interface usually occupied by water molecules (Figure 1c) [12••]. The two inhibitor molecules bound at this site are packed against one another and π stack against a neighboring histidine residue. The presence of the allosteric inhibitor causes the two subunits to rotate 17° from their position in the active (R state) structure, mimicking the AMP-inhibited (T state) structure. In the R state, the loop containing residues 52–72 is ordered, allowing residue Asp68 to effectively participate in the coordination of a catalytically required zinc atom. In both the AMP-bound and new allosterically inhibited states, this loop is disordered, preventing catalysis.

Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of insulin receptor phosphorylation and signaling, which makes it a therapeutic target for type 2 diabetes. Although derivatives of benzbromarone discovered by high-throughput screening were suspected to exert their inhibitory effect distal from the active site of PTP1B [13], it was not until these compounds were crystallized with PTP1B that a new regulatory site was identified 20 Å from the active site (Figure 2a) [14••]. PTP1B exists in either an open (inactive) or closed (active) conformation. Formation of the closed state requires interactions between Trp291 from helix α7 and residues from helices α3 and α6. Binding of the

Figure 2



New allosteric sites and mechanisms of inhibition in PTP1B and FVIIa. **(a)** The allosteric compounds (magenta sticks) inhibit PTP1B via an interaction that is 20 Å from the active site cysteine (green spheres). **(b)** Binding of compound 2 (purple dots) to PTP1B (blue ribbons and sticks) affects the conformations of Pro188 and Leu192, which in turn push Phe191 into a position that sterically clashes with the WPD closed conformation of Trp179 (green ribbons and sticks), forcing PTP1B to remain in the WPD open, inactive conformation. **(c)** The binding sites on the heavy chain of FVIIa (bright pink) for the E-76 peptide inhibitor (yellow sticks) and the A-183 peptide inhibitor (green sticks) are spatially separated from one another and distant from the active site, which is modified by the 1,5-dansyl-Glu-Gly-Arg-chloromethyl ketone inhibitor (cyan sticks). **(d)** Binding of the E-76 peptide (yellow sticks) to FVIIa causes disruption of the hydrogen bond between Asn143 and Lys192, liberating the 140's loop to attain the zymogen conformation (purple) rather than the active conformation (cyan).

allosteric inhibitors sterically blocks the required conformation of Trp291. In addition, the allosteric inhibitors trap the open conformation with the catalytic WPD loop blocking the substrate-binding site. The allosteric compounds directly contact Leu192 and Pro188, and subtly shift their conformation. These residues in turn affect the conformation of Phe191, which sterically clashes with the closed conformation of Trp179 in the WPD loop and locks PTP1B in the open inactive conformation (Figure 2b).

The Lazarus group at Genentech used peptide phage display to identify allosteric inhibitors of factor VIIa (FVIIa), a critical protease in the blood-clotting cascade. Two distinct classes of peptides (E and A) that bind to two new and distinct non-overlapping sites were identified (Figure 2c) [15–18]. The two classes of peptides exhibit different modes of inhibition. The E class peptides cause a change in the hydrogen-bonding network near one end of the 140's loop (Figure 2d). Peptide residue Arg7 appears to cause a hydrophobic collapse that involves the position of Leu144, which in turn affects the conformation of Asn143. Asn143 is involved in a backbone hydrogen-bonding interaction with Lys192. Lys192 is adjacent to Gly193, which, together with Ser195, composes the oxyanion hole. This disruption of the oxyanion hole explains the observed effects on V_{max}. In addition, binding of the E peptides forces the 140's loop into a conformation that mimics the inactive zymogen form. The A class of peptide has no effect on the oxyanion hole and does not 'zymogenize' the 140's loop. This class does not affect the V_{max}, but does affect the K_m, indicating a change in the ability of FVIIa to bind substrate. This seems to be accomplished when Trp10 from the peptide pushes Trp61 of FVIIa forward and subtly disrupts the substrate-binding site. The fact that two new allosteric sites have been uncovered on FVIIa for peptides using disparate inhibitory mechanisms may suggest that specific binding and the associated disruption of the dynamics of the enzyme may be more critical to allosteric inhibition than the precise location of the binding site.

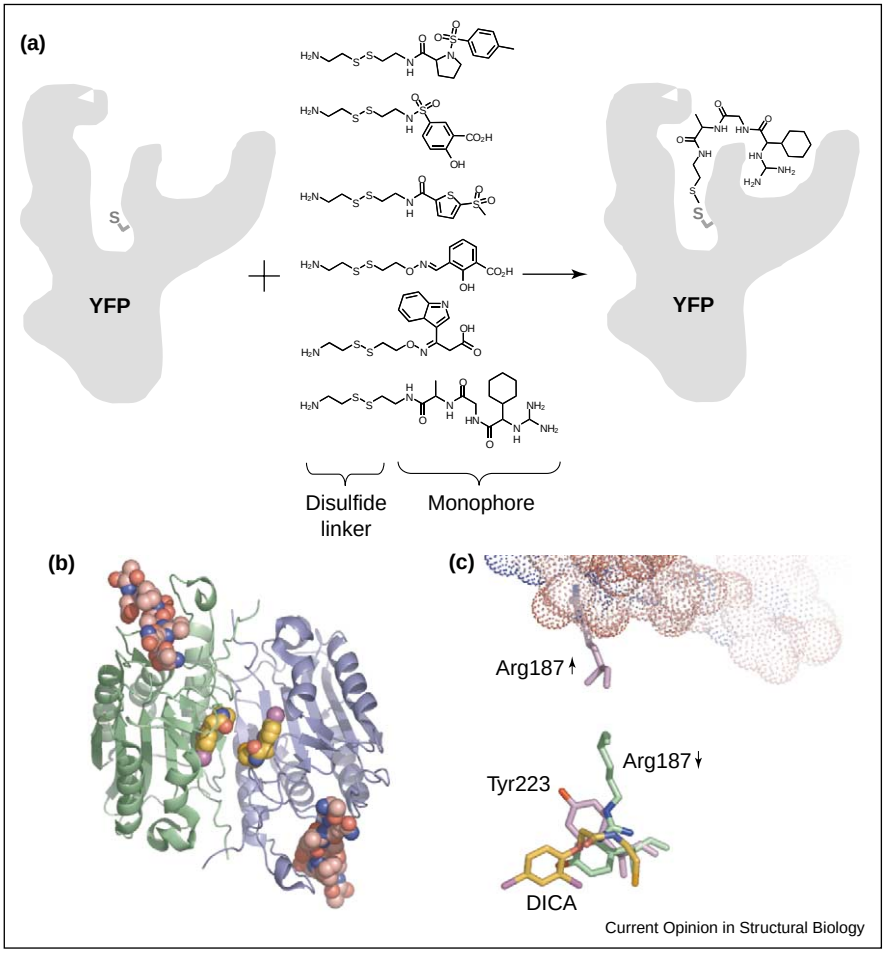
Tethering, Sunesis' proprietary fragment-based drug discovery, offers a site-directed means of probing and discovering new allosteric sites. Tethering takes advantage of native or introduced cysteines on protein surfaces to trap thiol-containing small-molecule ligands. Ligands that bind to orphan or serendipitous allosteric sites can be captured by the formation of a disulfide bond between the small molecule and a cysteine residue in the vicinity of the allosteric site, and rapidly assessed by mass spectrometry and functional modulation (Figure 3a). Due to the formation of a covalent bond between the small-molecule ligand and the protein, Tethering combined with peptide mapping can verify the position of the novel allosteric site without having to solve an X-ray crystal structure. Tethering has led to the discovery of a new allosteric site at the dimer interfaces of caspase-3 and caspase-7, the apoptotic proteases, about 14 Å from the active site (Figure 3b) [19••]. The crystal structure of caspase-7 revealed that the allosteric ligands push Tyr223 at the edge of the allosteric cavity out of its usual position. The new tyrosine conformation prevents Arg187, next to the catalytic cysteine at the active site, from burying itself in the core of the protein (Figure 3c). With Arg187 forced into the 'up' position, caspase-7 is incapable of binding substrate. Additionally, one of the active site loops (L2′) interacts with the allosteric inhibitors, burying the compounds and locking the L2′ loop in the zymogen conformation. Thus, the mature processed form of caspase-7 is effectively 'zymogenized' by the allosteric small molecules.

Strategies of allosteric regulation

The mechanisms by which allosteric inhibition occurs at new regulatory sites are similar to mechanisms used by natural active site and allosteric inhibitors. Moreover, inactivation often occurs at multiple levels for a given inhibitor (Table 2). Indeed, the mechanisms by which allosteric inhibition occurs are overlapping and may be intrinsically related to one another at a biophysical level. In all cases we have considered, an altered state of the enzyme has been captured by the novel allosteric inhibitor. In most cases, these small molecules have captured previously observed conformational states of the enzyme. Classic allosteric effectors, such as BPG for hemoglobin and AMP for GlyP, work by stabilizing the inactive state of the protein. The binding of small molecules to the novel allosteric sites in GlyP and F16BPase also shifts the equilibrium strongly, locking these enzymes in the inactive T state conformation. Many proteases are regulated at the level of zymogen maturation, wherein the inactive zymogen precursor form of the enzyme is activated by proteolysis. Binding at the new allosteric sites in caspase-7 and FVIIa locks each of these enzymes in the inactive zymogen conformation.

In kinases, the conformation of the DFG loop correlates with the activation state of the enzyme. Diaryl urea inhibitors such as BIRB-796 bind to p38 MAP kinase, a serine/threonine kinase, in a new binding pocket and affect the conformation of the DFG loop [20••]. Although the novel pocket is immediately adjacent to the ATP-binding site, none of the inhibitor atoms overlap the ATP site (Figure 4a). BIRB-796 pushes Phe169 (F in the DFG nomenclature) to flip down into the ATP-binding site and distorts the position of Asp168. This prevents binding of the ATP phosphate groups both for steric reasons and by removing Asp168, which helps to chelate magnesium (Figure 4b). The authors [20••] categorize this inhibition mechanism as 'indirectly competing with the binding of ATP'. BIRB-796 is currently in clinical development for use in conditions of inflammation such as arthritis and psoriasis. A well-known marketed drug, Gleevec®, works by a similar mechanism on its targets, Ableson, c-kit and

Figure 3



The use of Tethering to discover a novel allosteric site in the caspases. **(a)** Scheme for using Tethering to discover allosteric sites. Incubation of your favorite protein (YFP) with pools of compounds of unique molecular weight, each composed of a disulfide linker and a monophore group, allows binding of compounds to exposed cysteines. Exposed cysteines can be either native cysteine residues or cysteines that have been introduced specifically to discover small molecules that bind near them. Adjusting the level of reductant during equilibration modulates the stringency of the screen, so that only tight-binding monophores will have the residence time necessary to allow formation of a disulfide bond. Covalently attached compounds can be determined by mass spectrometry and their impact on protein function can be assessed. Peptide mapping by mass spectrometry allows identification of the particular cysteine modified. **(b)** The new caspase-7 allosteric binding site (yellow spheres for compounds) is at the dimer interface of the two caspase monomers (blue and green) and is spatially distant from the substrate-binding groove (peptide inhibitor in orange spheres). **(c)** The allosteric inhibitor DICA (yellow sticks) sterically clashes with the active conformation of caspase-7 (green sticks) and forces it into a zymogenized conformation (purple sticks). This is accomplished when DICA contacts Tyr223 (green sticks), forcing Tyr223 (purple sticks) into the up position. This conformation of Tyr223 is in steric contact with Arg187 (green sticks), which subsequently causes Arg187 (purple sticks) into the up position. The up position of Arg187 (purple sticks) observed in the presence of the allosteric inhibitor is incompatible with the binding of peptide (orange dots) in the active site.

PDGF receptor kinases, which are also stabilized in the inactive 'DFG out' conformation [21] (Figure 4c). Another kinase inhibitor, Bay43-9006, which is in phase III clinical development, binds to Raf kinase in a DGF out configuration [22]. Thus, the active sites of kinases are very adaptive and non-classical inhibitors with drug-like properties can be found that trap them in inactive conformations.

Although allosteric inhibitors that stabilize the inactive state are the most commonly observed new effectors, it is also possible to find new allosteric activators. Extensive studies have yielded several classes of allosteric activators of glucokinase, a protein involved in regulation of glucose metabolism [23,24••]. The human glucokinase monomer is composed of one large and one small domain. Rotation about a hinge between the large and small domains allows interconversion between a super-open inactive form, an open active form and a closed highly active form. The crystal structure of one class of activator identified the allosteric site 20 Å away from the active site at a region formed only in the closed conformation of the enzyme.

Table 2

Properties of serendipitous allosteric sites.

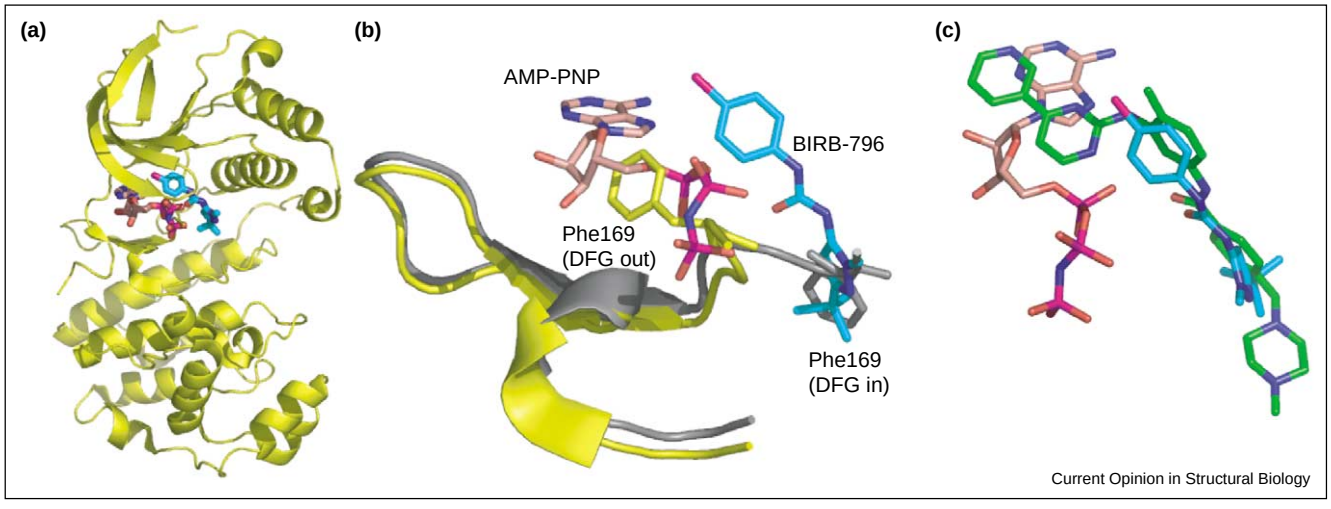
	Binding modes			Seams at which allosteric site is found				Inhibitory conformation	
	Traps altered state	Known inactive state	Existing cavity	Multimer interface	Domain–domain interface	Between secondary structures	Surface	Precludes substrate binding	Alters catalytic machinery
HIV-1 RT	+	+	–		+			–	+
GlyP	+	+	+	+				–	+
F16BPase	+	+	+	+				–	+
PTP1B	+	+	–			+		–	+
FVIIa	+	+/–	–			+	+	–	+
Caspase-7	+	+	+	+				+	+
p38 kinase	+	+/–	–		+			+	+
Glucokinase	+	+	+		+			–	+
KSP	+	+	–			+		–	+
β-Lactamase	+	–	–			+		–	+

Thus, binding of the activator shifts the folding equilibrium to the closed form and increases the catalytic efficiency of the enzyme by disfavoring formation of the super-open inactive conformation [24••].

All of the cases we review require some degree of adaptive fit for binding. In caspase-7, the large central cavity at the dimer interface was readily observable in previous structures and only minor sidechain movements were required to accommodate inhibitor binding. In GlyP and F16BPase, the binding sites at the dimer or tetramer interface were present to some degree in the absence of the compounds, although the sites were filled with water and several significant sidechain movements were required. In other cases, a larger degree of conformational change is associated with presenting a cavity for inhibitor binding. Small molecules often reveal novel allosteric sites by partially reordering the region of the protein into which they tuck themselves. This is possible at domain–domain interfaces, such as between the two lobes of p38 kinase, or between the palm and thumb domains of HIV-1 RT. In other cases, binding sites are created between two elements of secondary structure, as we have already discussed for PTP1B.

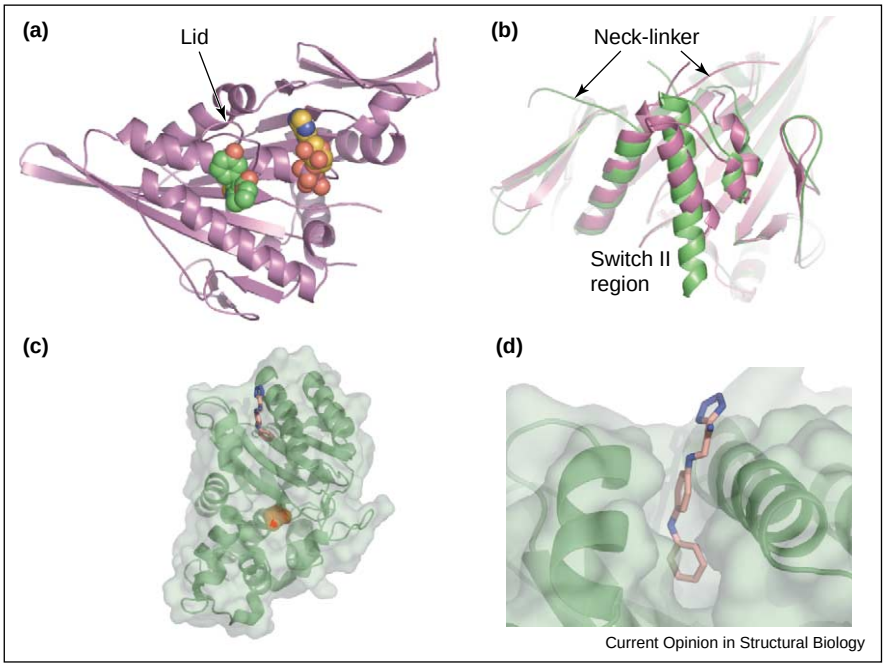
A recent example of adaptive binding between two elements of secondary structure was illustrated by the structure of the motor domain of KSP, a mitotic kinesin, in

Figure 4



The mechanism of inhibition of p38 kinase by BIRB-796. **(a)** The binding sites for ATP (magenta sticks) and BIRB-796 (cyan sticks) in p38 kinase (yellow ribbons) are immediately adjacent but non-overlapping. **(b)** Binding of BIRB-796 (cyan sticks) alters the conformation of Phe169, forcing it into the DFG out conformation (yellow ribbons and sticks). This conformation sterically clashes with binding of ATP (pink sticks), which requires the DFG in conformation (gray ribbons and sticks). Thus, binding of BIRB-796 and ATP are mutually exclusive. **(c)** Comparison of the binding sites for BIRB-796 (cyan sticks), an allosteric inhibitor, with the binding site for Gleevec® (green sticks), an active-site-contiguous inhibitor, which occupies the same binding site in Ableson kinase as ATP (magenta sticks).

Figure 5



Binding between elements of secondary structure in the KSP and β-lactamase new allosteric sites. **(a)** The binding site on KSP (purple cartoon) for monastrol (green spheres) is distal from the binding site for ADP (yellow spheres). **(b)** Comparison of the KSP–ADP–monastrol ternary complex structure (purple) with kinesin KIF1A (green) bound to AMP-PCP (an ATP mimic). The neck-linker and switch II regions are indicated. **(c)** The distance between the β-lactamase allosteric site (pink sticks) and active site Ser70 (red spheres) is shown. **(d)** The surface of β-lactamase (green) is substantially changed by the binding of the allosteric compound (pink sticks). Binding of the allosteric inhibitor in the core of the protein forces the two adjacent α helices apart. Simultaneous with allosteric inhibitor binding is the reordering of one helix (to the left of the allosteric inhibitor in this view) into a more perfect α helix with more regular dihedral angles for helical residues.

complex with a new allosteric inhibitor, monastrol [25••]. Binding of monastrol occurs in a nascently formed binding pocket 12 Å away from the catalytic ATP-binding site, between an α helix and a loop. Locally, monastrol causes ordering of a helix α2 insertion loop, which acts as a lid, closing down on the bound monastrol molecule (Figure 5a). In the absence of monastrol, this α2 insertion loop is one of the most flexible regions of the protein (B-factors are 50% above the average). After binding, this loop becomes rigid (B-factors are 25% lower than the average). Remarkably, binding of monastrol does not cause any alterations to the rigidity of the nucleotide-binding region. Binding of monastrol is also coupled to conformational changes in distal regions of KSP. A gap on the opposite side of the protein opens by about 6 Å, causing conformational changes in the neck-linker region, which is believed to be involved in generating the mechanical force that allows kinesins to process along microtubules. Two residues from the neck-linker rotate by 120° and dock in the larger gap. The monastrol-bound conformation of KSP resembles the 'locked' conformation associated with the binding of ATP compared to the 'unlocked' conformation when bound to ADP (Figure 5b).

A profoundly induced-fit allosteric site occurs in β-lactamase. Horn and Shoichet became intrigued with inhibitors that, based on thermodynamic analysis and mutagenesis, seemed to bind preferentially to an unfolded state of β-lactamase. The crystal structure revealed that these compounds were acting at a novel allosteric site distal from the active site (Figure 5c). The compounds bound between two α helices, forcing them apart (Figure 5d) [26••]. The surface area of the site expands from 14 $Å^2$ to 180 $Å^2$ to accommodate the largest compound. Van't Hoff analysis indicates that 98% of the free energy of the interaction derives from hydrophobic interactions, which is consistent with inspection of the binding cavity. Inhibition by the compound is the result of the displacement and increased mobility of Arg244, a residue shown by mutagenesis studies to be critical for activity due to its direct interaction with β-lactam substrates. This work demonstrates that new binding sites are not limited to preformed cavities on the surface of the protein, but may also occupy favorable sites within the protein core.

Horn and Shoichet suggest that, despite the fact that the binding site itself did not exist in the unliganded

structure, it might have been possible to predict this site. First, the COREX algorithm suggested this site to be unstable. Second, even in the 0.85 Å structure, Leu220, which is at the N terminus of one of the helices affected by allosteric compound binding, exhibited unfavorable phi-psi angles. Interestingly, when the inhibitors bind, Leu220 adopts favorable dihedral angles. Additionally, a crystal structure of a related β-lactamase shows that the same cavity was occupied by a maltoside included in the crystallization buffer. It is not uncommon to see adventitious binding of components of the crystallization buffer to cavities in a protein. Typically these observations are ignored, but these 'crystallization artifacts' recorded in the Protein Data Bank may be our largest repository of information on serendipitous allosteric sites. For example, in the recent crystal structure of cytochrome P450 3A4, the progesterone substrate was bound not at the active site, but at a peripheral site [27]. The role of this site remains unknown, but the authors speculate that it may be a novel allosteric recruitment site. Perhaps additional allosteric sites could be predicted in these ways.

The dynamics of proteins are obviously critical in natural allosteric regulation. Ongoing experimental evidence is accumulating about the precise role of dynamics in known allosteric regulation (for a review, see [28]). To date, little is known about the effects of dynamics on allosteric inhibition at new allosteric sites, but given the various degrees of adaptive fit observed at each of the novel allosteric sites, future work is certain to uncover significant effects of binding at these sites on dynamics. In the meantime, our best indication of the mechanisms of inhibition comes from structural and kinetic information. For p38 kinase, caspase-7, PTP1B and KSP, the mechanism of inhibition seems clear based on the altered conformations of a few critical residues in the available structures. In both p38 kinase and caspase-7, the allosterically induced conformational changes in a single residue (Phe169 in p38 kinase and Arg187 in caspase-7) appear to be sufficient to indirectly block substrate binding. In other cases (GlyP, F16BPase, glucokinase), the structures reveal that more global changes have occurred and that the equilibrium has been strongly shifted to favor a single conformational state. The fact that non-competitive and mixed inhibitors are both observed strongly suggests that effects on both the catalytic machinery itself and the dynamics, allowing substrate binding and product release, occur in these inhibited enzymes. In all cases, the crystal structures revealed distortions in the catalytic machinery.

Independent of the method by which these new allosteric sites were discovered or the mechanism by which they inhibit protein function, the discovery of new regulatory sites always begs the question of whether this new site is used by a natural effector. The identification of these sites should stimulate the search for natural effectors.

Prediction of allostery

Future discovery of new allosteric sites is likely to be aided by computational methods for predicting allosteric regulatory sites. A predictive method called COREX has been successful in recapitulating known allosteric sites, defining pathways that connect the active site to the allosteric site, and predicting effector binding (for a review, see [29]).

Ranganathan and co-workers have developed statistical coupling analysis, which makes use of functional information buried within the evolutionary record from the sequences of a family of proteins. They examine all sequences within a given family, and divide the family into allosterically and non-allosterically regulated family members. By calculating the statistical coupling between pairs of residues between and within these divisions, they are able to pinpoint the residues involved in allosteric signaling. Using this methodology, they have validated previous evidence on the networks of amino acids that transmit allosteric signals in the G-protein-coupled receptor, hemoglobin and chymotrypsin serine protease families [30]. They next predicted and functionally tested the networks of amino acids responsible for the transmission of allosteric signaling in G-protein superfamily members [31] and the RXR nuclear receptor heterodimers [32••].

Evolutionary trace analysis can also be used to find functionally important protein surfaces [33]. By comparing evolutionarily related sequences and structures from the regulators of G-protein signaling (RGS) family, dividing them into allosterically and non-allosterically regulated classes, and analyzing for similarities and differences between the two classes, Sowa and co-workers were able to accurately predict the G_α allosteric effector binding site on RGS9 before it was confirmed by crystallographic studies [34]. Some easy-to-use programs for evolutionary trace analysis are publicly available [35], as are other methods for predicting functional sites on proteins (for a review, see [36]). To date, no novel or orphan allosteric sites have been identified by statistical coupling or evolutionary trace analysis. However, as new allosteric sites are increasingly exploited pharmaceutically, we expect prediction of new sites to emerge as a focus of these types of analyses.

Conclusions

In the discovery of orphan or serendipitous allosteric sites, small molecules themselves can function as the ferrets that sniff out binding sites from which they can affect protein function. In comparing the various new allosteric sites, several points come to light as generalities for these types of sites (Table 2). First, the mechanisms of inactivation observed for new allosteric sites quite often involve stabilization of a naturally occurring conformation rather than use of an entirely new conformation. Second,

the inhibitors are often hydrophobic and the interaction tends to be driven by burial of hydrophobic surface area (e.g. caspase-7, GlyP, β-lactamase), and binding sites are not limited to the surface but can also be found in the core of the protein. Often, binding sites are found at dimerization interfaces (e.g. GlyP, caspase-7, F16BPase, HIV integrase [37••] and a previously reported novel allosteric site in hemoglobin [38]). Interestingly, it is often on proteins with one known allosteric site that another is found (e.g. F16BPase, GlyP, hemoglobin, muscarinic receptors [39,40]). Does this suggest that some proteins are particularly ripe for regulation? Do the dynamics and conformational flexibility of allosterically regulatable proteins make them more prone to disruption and serendipitous inhibition than other classes of proteins?

Third, in most cases, the novel allosteric binding sites could not have been obviously predicted from the unliganded structure. This underscores the fact that most of these sites are tremendously adaptable. Hints such as high-resolution strained dihedral angles and the previous localization of a crystallization artifact in β-lactamase suggest that careful analysis might make prediction of these sites possible. In addition, computational methods based on the evolutionary record may help predict binding sites even in the absence of structural data. These computational methods may also help to distinguish orphan versus serendipitous sites, as the former may be revealed by evolutionary trace or statistical coupling analyses.

Why is it that small molecules can exert such strong effects from unexpected locations? In a naturally occurring allosteric site, to convert an unregulated glucokinase to an allosterically regulated glucokinase, only 11 of 501 total residues (2%) must be changed [41]. Introduction of the allosteric regulation present in crocodile hemoglobin into human hemoglobin also required changing only a few residues [42]. In most serendipitous allosteric sites with clear mechanisms of inhibition, very few residues appear to be involved in the allosteric regulation. Because interaction with or disruption of the conformation or dynamics of just a few residues is sufficient to disrupt activity, it is not surprising that, in these cases, specific binding is sufficient to cause allosteric inactivation.

New allosteric sites have produced great opportunities for drug discovery, as discussed above. Enzymes typically generate charged transition states and the substrates are often not drug like (many are highly charged and some are very non-polar). Thus, producing orally active drug-like inhibitors to complement these sites can be very challenging. These allosteric sites provide opportunities to find not only novel drug-like inhibitors, but activators as well.

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