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p38 α MAP Kinase C-Terminal Domain Binding Pocket Characterized by Crystallographic and Computational Analyses

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Abstract

The MAP kinase protein family has a critical role in cellular signaling events, with MAP kinase p38 α acting in inflammatory processes and being an important drug discovery target. MAP kinase drug design efforts have focused on small molecule inhibitors of the ATP catalytic site, which exhibit dose-limiting adverse effects. Therefore, characterizing other potential sites that bind substrates, inhibitors or allosteric effectors is of great interest. Here, we present the crystal structure of p38 α MAP kinase, which has a lead compound bound in both the active site and in the lipid-binding site of the C-terminal cap. This C-terminal cap is formed from an extension to the kinase fold, unique to the MAP kinase and CDK families, and GSK-3 kinase. Binding of this lead, 4-[3-(4-fluorophenyl)-1H-pyrazol-4-yl]pyridine, to wild-type p38 α induces movement of the C-terminal cap region, creating a hydrophobic pocket centered around residue Trp197. Computational analysis of this C-Terminal domain pocket indicates notable flexibility for potentially binding different shaped compounds, including lipids, oxidized arachidonic acid species such as leukotrienes and small molecule effectors. Furthermore, our structural results defining the open p38 α C-lobe pocket provide a detailed framework for the design of novel small molecules with affinities comparable to active site binders: to bind and potentially modulate the shape and activity of p38 α in predetermined ways. Moreover, these results and analyses of p38 α suggest strategies for designing specific binding compounds applicable to other MAP kinases, as well as the CDK kinase family and GSK-3 β that also utilize the C-terminal insert in their interactions.

Keywords

p38 α ; MAP Kinase Insert; small molecule inhibitor; leukotriene; anisomycin

Protein kinases have critical functions in cellular signaling cascades and their dysregulation is linked to human diseases, including cancer, diabetes, neurodegeneration and inflammatory-based disorders. Concerted characterization of protein kinases is being conducted to define

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Protein Data Bank accession code

Structure factors and coordinates for the p38 α :4-FPP complex have been deposited in the RCSB Protein Data Bank, with the accession code 2P5A.

their molecular mechanisms of action, and for drug design efforts. One group of kinases being studied in detail is the p38 family of proteins, which has key roles in the cell-cycle, inflammation and apoptosis and belong to a larger group of Mitogen-activated protein (MAP) kinases.¹ The p38 kinase family consists of four isoforms, alpha, beta, gamma, and delta, which differ in expression patterns and substrate specificities.² The most thoroughly characterized of these is p38alpha (p38 α), which is expressed in most cell types and is activated in response to cellular stress, including that arising from DNA damage, inflammatory cytokines or ischemia.

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The p38 α MAP kinase pathway is being targeted through significant efforts in the pharmaceutical industry to produce therapeutics treating inflammatory diseases, which include psoriasis, rheumatoid arthritis and chronic obstructive pulmonary disease.^{3,4} These p38 α drug design efforts have predominantly focused on competitive small molecule inhibitors of the ATP catalytic site.^{4,5} A few inhibitors have been produced that also bind the “DFG-out” site; this site is adjacent to the active site and it derives its name from the DFG sequence that is observed to undergo conformational change upon moieties binding this region^{6,7}. However, there are many similarities between these two sites in p38 α and those of other cellular kinases, which can lead to dose-limiting adverse effects of these inhibitors. Therefore, characterizing other potential sites within p38 α that bind substrates, inhibitors or allosteric effectors has recently become a research area of significant interest⁸ to produce a specific inhibitor of p38 α MAP kinase without any off-target activities.

A few allosteric sites have been defined within the larger MAP kinase family through structural and biochemistry-based studies.⁸ This includes a site that binds the linear sequence ‘D-motifs’ present in both substrates and interacting proteins. The D-motif binding site occurs in p38 proteins and also the JNK and ERK MAP kinases^{9–15}; kinase specificity is provided through changes in the hydrophobic groove and adjacent acidic patch that comprise this binding site. A second allosteric site occurs in the C-terminal extension that is unique to MAP kinases and cyclin-dependent kinases (CDKs), and the glycogen synthase kinase 3 (GSK-3). In CDK2, this C-terminal insert forms the binding site for the cell cycle-regulatory proteins Cks1 and Cks2, which modulate the *in vivo* activity of CDK2.^{16,17} Furthermore, this insert in CDK2 is also part of a binding site for the KAP phosphatase that dephosphorylates a key regulatory site of CDK2.¹⁸ In GSK-3 β the C-terminal insert is known to bind either the scaffold protein axin¹⁹, which localizes GSK-3 β with its substrate β -catenin, or with FRAT1 that disrupts GSK-3 β :axin binding. Binding utilizes conformational flexibility and a hydrophobic docking groove in the GSK-3 β insert to mediate these two distinct and exclusive protein partner interactions. In ERK2 MAP kinase, this insert region is known to form a binding site that is utilized by transcription factor substrates and phosphatases, both of which contain a ‘FXF’ binding motif. The function of this C-lobe insert in p38 α is being defined, with one study suggesting that it could function as a ‘FXF’ binding site.²⁰ A second structure-based study indicated that the insert can form a hydrophobic pocket for binding biologically active arachidonic acid metabolites^{21,22}, which are known p38 α allosteric effectors.

Here, we have determined the crystal structure of p38 α in complex with an inhibitor, 4-[3-(4-fluorophenyl)-1H-pyrazol-4-yl]pyridine (4-FPP), which we observe bound in both the active site and the C-lobe pocket. Our computational analyses on this C-lobe pocket indicate that it is flexible and predominantly hydrophobic, though it also contains several potential hydrogen bond acceptors. The C-lobe pocket appears capable of accommodating differently shaped molecules, and in doing so alters the topology of p38 α ; this topology change could potentially affect protein interactions, substrate selection, localization and have allosteric effects on activity. Thus, our structural analyses provide a basis for the *de novo* design of molecules with specificity to the p38 α C-lobe, to determine whether binders to this site can alter the function of p38 α towards a preferred cellular phenotype.

4-FPP Bound at the Active Site

4-FPP was first described in a Searle patent and is a reasonably potent inhibitor of p38 α MAP kinase, p38 α enzyme IC₅₀=600 nM²³, and has been used as a template for inhibitor design by Pfizer Global Research.²⁴ 4-FPP shares similarity to the CSAIDs (cytokine pressive anti-inflammatory drugs) originally developed by SmithKline Beecham (SB). 4-FPP contains a core pyrazol ring, with a 4-fluorophenyl ring at pyrazol C4 and pyridine ring at the C5 position (Fig. 1a). The SB compounds contain a core imidazole ring, a 4-fluorophenyl group, either a pyridine or pyrimidine ring, and another functional group, either at the imidazole C2 or N1 positions (Fig 1b). We compared our 4-FPP structure (Fig. 2a) with structures of p38 α in complex with SB compounds²⁵, including 'SB203580' [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole]. Our p38 α :4-FPP complex structure superimposes onto p38 α :SB203580 complex (PDB code 1A9U) with an RMSD of 1.6 Å. The binding of 4-FPP and SB203580 in the p38 α catalytic ATP-binding site is similar, and the key difference between these two structures is the opening of the C-terminal domain pocket that adjusts to fit 4-FPP. This difference may have occurred through the divergent experimental approaches. We adopted a co-crystallization strategy instead of the inhibitor soaking method used for the SB203580:p38 α complex structure²⁵, and thus SB203580 could potentially bind the C-terminal pocket.

The p38 α catalytic ATP site is formed in a region between the N- and C-terminal domains of the protein, and with the hinge/crossover connection amino acids 106–112 that forms the back of the site.²⁶ 4-FPP binds to this ATP site with an average B-value of 25.7 Å². The pyridine ring of 4-FPP is situated in the predominately hydrophobic pocket that binds the adenine of ATP. This pocket is formed by the side chains of N-terminal domain amino acids Val30, Val38, Ala51 and the crossover Leu108, which occurs on one side of the 4-FPP pyridine ring, and the crossover Met109 and C-terminal domain Ala157 side chains on the other.

The pyridine nitrogen forms a 2.7 Å hydrogen bond with the backbone Met109 nitrogen (Fig. 2b). This bond is similar to the hydrogen bond with the ATP adenine N1 atom in known ATP-kinase complexes, and is also present in many kinase-inhibitor complexes. The 4-fluorophenyl group is in a pocket that is formed by the N-terminal domain Lys53 and Leu104 amino acids, which are present on one side of the pyrazol ring, plus the N-terminal domain Leu75, Ile84 and crossover connector Thr106 side chains on the other. The fluorine atom is within 3.3 Å of the Val105 backbone nitrogen, 3.3 Å of the Leu104 backbone oxygen and 3.7 Å of the Thr106 backbone nitrogen (Fig. 2b), but the geometries are not ideal for hydrogen bonding. As with SB203580, Thr106 has a different rotamer when compared to apo-p38 α , and it now participates along with the neighboring His107 in an extended network of water mediated hydrogen bonds.

The pyrazol ring of 4-FPP occupies a region with the Val38 side chain on one side of this ring, and the Leu167 side chain on the other. A water molecule is 3.9 Å from the pyrazol 1-N atom, and this water forms a 2.9 Å hydrogen bond with the Ser32 side chain oxygen. The pyrazol N2 atom hydrogen bonds with a water at 2.7 Å, and this water forms further hydrogen bonds at 2.5 Å to Asp168 of the 'DFG loop' and 2.6 Å to the Lys53 side chain nitrogen (Fig. 2b). This DFG loop region forms a second binding site for some p38 α inhibitors, and the Lys53 side chain is observed to form a hydrogen bond to certain p38 α inhibitors.²⁵

4-FPP Bound in the C-Terminal Domain Pocket

Surprisingly, refinement of the p38 α complex structure also revealed density for a second 4-FPP molecule in the C-terminal domain pocket (Fig. 3a, b) and movement of α EF/ α F-loop and the EF helix region to accommodate this molecule. This conformation of the pocket is notably different than the apoprotein and the SB203580 complex. Movement of the α EF/ α F-loop alters the conformation of Trp197 and flips Met198 from being buried in the core in the apostructure,

onto the surface of the molecule (Fig. 3c). This suggests that these residues act as a pseudo-ligand in the absence of suitable pocket binding compounds. The electron density for Met198 is weak when it is solvent exposed, while Trp197 is clearly observed, and it stacks against the 4-FPP moiety (Fig. 3a).

4-FPP binds to the C-terminal domain pocket with an average B-value of $30.4 \text{ \AA}^2$, utilizing van der Waals interactions and a few hydrogen bonds (Fig. 3b). The fluorophenyl group binds in a hydrophobic region, stacking between the α EF/ α F-loop Leu195 side chain on one side and the Leu291 side chain of the α H/ α XI-loop on the other. The pyridine ring stacks between the side chains of the α EF/ α F-loop Trp197 and the IL14-helix Ile250. The pyridine nitrogen is within $3.7 \text{ \AA}$ of the Trp197 nitrogen and $3.4 \text{ \AA}$ of a water molecule that forms a hydrogen bond to the Ser252 main chain nitrogen of the α IL14-2L14 loop. The pyrazol ring fits between the Loop L12 Glu192 side chain and the Leu246 IL14-helix side chain. The N1 nitrogen is within $3.2 \text{ \AA}$ of the side chain oxygen and $2.8 \text{ \AA}$ of the backbone nitrogen of loop L15 Ser293. The pyrazol N2 forms a $2.6 \text{ \AA}$ hydrogen bond to the L15 Leu291 backbone oxygen.

In addition to 4-FPP, the detergent β -oligoglucopyranoside (β -OG) has been observed in the C-terminal domain pocket²¹ as has an iododerivative of SB203580.²⁷ We compared the 4-FPP and the β -OG structures, while the pdb deposition of the iododerivative contains only an α -carbon protein backbone, precluding a more detailed comparison. The backbone α -carbon atoms of the 4-FPP and β -OG complex structures (PDB code 2NPQ) superimpose with an RMSD of $1.7 \text{ \AA}$. Interestingly, an increased distance of $2.3 \text{ \AA}$ between the α EF/ α F-loop and the adjacent IL14-helix in the 4-FPP structure (measured between Trp197 and Ser252 C α -atoms) is similar to a $2.6 \text{ \AA}$ opening in the β -OG complex structure, with respect to the apo-enzyme. In the two structures, this opening accommodates the two different ligands in an analogous position in the C-terminal domain pocket (Fig. 3d). The adjacent 2L14-helix in p38 α :4-FPP is comparable to apo-p38 α , whereas this helix is more extended in the p38 α : β -OG complex to accommodate a second bound β -OG. In both of these structures Met198 flips out of the pocket, as compared to apo-p38 α , and Trp197 plays a key role in stacking either of the bound compounds. Also, the 4-FPP fluorophenyl group and the aliphatic chain of β -OG occupy a comparable position within the pocket (Fig. 3d). Additionally, both moieties form hydrogen bonds to the pocket containing Ser293 backbone nitrogen and side chain oxygen.

However, notable differences in binding these distinct moieties in the C-terminal domain pocket are also apparent, including Trp197 that adopts a different rotamer in chelating these two alternate compounds. Also, the hydrogen bonds of 4-FPP with Leu291 and with a water molecule that interacts with Ser252 in the entrance of the pocket are not conserved. Moreover, the head group of β -OG instead binds further to the side of the pocket, forming hydrogen bonds that are not observed in 4-FPP, including those with His199, Asn201, Lys249, Asp292 and Asn294. Together, these variations between the two crystal structures indicate notable plasticity in the C-terminal domain pocket conformation, to accommodate molecules with significantly different shapes and functional groups.

Computational Analyses of the C-Terminal Domain Pocket

To characterize the C-terminal domain pocket binding-site, we employed computational methods to examine both the optimal size and affinity of the site, and the docking affinity of several compounds that could potentially bind in this region. The binding pockets in our p38 α crystal structure were defined by using the AutoLigand code²⁸ and also compared with the differing C-terminal domain pocket region of the β -OG complex. The AutoLigand code searches for pockets on the protein surface and then ‘fills’ them with a contiguous volume that has been molded to fit the pocket. The potential binding affinity of a pocket is then calculated from the total binding energy per volume for a fill, using the AutoDock force-field.^{29,30}

AutoLigand was used to generate a plot of the total binding energy per volume versus the fill volume for our p38 α crystal structure, with the 4-FPP molecules removed. The optimal ligand binding sites are indicated by the minimum point on a curve that describes fill volumes in pockets that were started at different locations. The minimum of this curve is the point at which adding additional volume to the fill does not improve the affinity per volume of the site. By using a search space enclosing the entire p38 α structure in a 1.0 Å grid and generating many different sized fill volumes, this plot (Fig. 4a) revealed several curves with identifiable minima, indicating potential binding sites. Also occurring are small sets of one to three points on the graph (colored circles) with volumes of much less than 100 Å³, which are small cavities that are less likely to be of interest and are often occupied with water molecules. The first curve with the smallest volume (plotted with blue circles) provides information on a group of five structural waters, which are bound in an internal cavity at Ser208, located between the N and C-terminal domains. The second curve (plotted with red circles) relates to a pocket outside the entrance of the active site centered at residue Ile84, with an optimal volume of 196 Å³ and energy per volume of -0.168 kcal/molÅ³. This site has optimal volume of 117 Å³ and an energy per volume of -0.192 kcal/molÅ³. The third curve (plotted with dark green) squares is the p38 α “backside site”, a hinge point between the two kinase lobes, and has an optimal volume of 265 Å³ and an energy per volume of -0.177 kcal/molÅ³. The fourth curve (plotted with red squares) is the p38 α active site, for which AutoLigand analysis calculates a 265 Å³ optimal volume and an energy per volume of -0.187 kcal/molÅ³. The fifth curve (plotted with green squares) with the largest optimal volume of 310 Å³ is the C-terminal domain pocket that has an energy per volume of -0.197 kcal/molÅ³. This increase in energy per volume over the active site is likely due to the more enclosed nature of this pocket.

The AutoLigand fill result for the C-terminal domain pocket is depicted in Fig. 4c, with the bound 4-FPP and the Trp197 side chain also shown. AutoLigand results determine that this pocket is made up of predominantly aliphatic/aromatic sites, with a few hydrogen-bond acceptor sites. These hydrogen bond acceptors include residues at the pocket entrance, the α EF/ α F-loop Asn196, and the flanking α IL14-2L14 loop Ser251 and Ser252. The other hydrogen bond acceptors include residues further inside the pocket that were observed to interact with 4-FPP pyrazol group in our crystal structure: Ser293 side chain and Leu291 backbone oxygen of loop L15.

Interestingly, AutoLigand predicted that the C-terminal domain pocket in the β -OG complex has a more extended shape, and that the pocket has an optimal volume of 446 Å³ and a total energy per volume of -0.192 kcal/molÅ³. AutoLigand revealed that the extended region is of an aliphatic nature, and also includes the Tyr258 side chain at the end of the extension as a potential hydrogen bond acceptor. The alternate conformation of the Trp197 observed in the β -OG complex provides a different shape to this side of the pocket and reveals nearby hydrogen bond acceptors. This includes side chains of loop L12 Glu192, loop L15 Asp294, besides Ser293 that is seen in 4-FPP complex structure. Also included are the hydrogen bond acceptors on the α EF/ α F-loop at the edge of the pocket, the Asn201 and Thr203 side chains and the backbone oxygens of His199, Tyr200 and Asn201.

C-terminal domain insert residues Pro242 to Thr263 contains two α -12 helices (1L14 & 2L14) that are connected by a loop, and this region forms a cap-like structure on the C-terminal lobe of p38 α that rotates to permit the binding of 4-FPP. Therefore, before conducting docking studies, we analyzed the optimal configuration of this cap and the hydrophobic pocket that it creates, through rotation of the cap using FlexTree.³¹ The cap was rotated in 1 degree increments $\pm 15^\circ$ as a rigid structure, about the Pro242 and Thr263 pivot points (Fig. 4b), and each rotation was subsequently analyzed with AutoLigand for the total affinity per volume of the C-terminal domain pocket formed. The best total affinity per volume value was compared for each rotation position. As the cap was moved inward closing the pocket, the total affinity

per volume remained about the same between 0° and -5° with about the same sized fill volumes. As the cap was closed more than -5°, the fill volumes shrank and the total affinity per volume increased. However, the pocket rapidly become too small for any reasonable sized small-molecule ligand to bind, which is the case in the apo-structure. As the cap was moved outward, the pocket became more of a cleft, which allowed the size of the fill volume to increase, but significantly decreasing the total affinity per volume after +3°. The β -OG complex was also moved about the same Pro242 and Thr263 pivot points. Opening the cap region of the β -OG complex up to +15° rapidly reduces the total affinity per volume, similar to the result with the 4-FPP complex structure. Additionally, moving the cap region inwards causes a steric clash with Trp197 at -3°, and with no significant change in shape and values of the fill pocket from 0° to -3°. Thus, the optimal conformation of the pocket is observed in our crystal structure and that of the β -OG complex, which also shares the same configuration of the IL14-helix, and therefore these two conformations were used for our docking analyses.

Ligand Binding Characterized by Computational Docking Analyses

Our crystallography and Autoligand analyses indicate a significant degree of flexibility in the C-terminal domain pocket. Therefore, we conducted docking studies on small molecule compounds to define their suitability for binding the pocket, through clustering analysis and potential binding affinities. These results were compared with those of long chain aliphatic compounds that have been indicated to bind the pocket.²¹ To validate the computational docking re-docking studies of 4-FPP were conducted on both the active site and the hydrophobic C-terminal domain pocket, using AutoDock 4.²⁹ Markedly, we observe that 4-FPP docks into the active site and hydrophobic pocket in the same manner as that observed in our crystal structure. Binding of 4-FPP to the active site occurs with an energy of -6.10 kcal/mol and a clustering of 100 out of 100 (using 2 Å RMSD clusters³²). Binding to the C-terminal domain pocket occurs in two modes. The first conformation is the same as the crystal structure, with 72 out of 100 at -7.68 kcal/mol and the second flipped 180° about the center ring with 28 out of 100 at -7.35 kcal/mol. Thus, these computational analyses suggest the C-terminal pocket has comparable affinities as the active site in binding 4-FPP.

In keeping with 4-FPP binding to the C-lobe, SB203580 docks into the C-lobe pocket, with the 4-fluorophenyl and pyridyl groups pointing into the pocket, and the methylsulfinylphenyl group pointing out; two orientations are observed related by a 180° flip of the fluorephenyl and pyridyl groups are observed. The larger cluster has the 4-fluorophenyl group at the entrance of the cleft (Fig. 4d) in a cluster of 63/100 at -8.6 kcal/mol (as in Fig. 4d) while the second orientation has the pyridyl group at the cleft entrance with a cluster of 28/100 at -8.6 kcal/mol. For our docking studies we also examined another small molecule compound, the antibiotic anisomycin that acts as an agonist in the p38 α cellular pathway.³³ Notably, we observe that anisomycin can dock in the C-terminal domain pocket of our crystal structure in a manner comparable to that of our p38 α :4-FPP complex structure (Fig. 4e). Anisomycin docks with energy of -7.4 kcal/mol and with clustering of 60 out of 100. Trp197 is utilized in a ring stacking interaction and the backbone nitrogens of Ser252 and Ser293 are hydrogen bonded to the anisomycin moiety. In contrast, anisomycin docking to the active site occurs at -6.4 kcal/mol with 15 out of 100 clustering, implying that the C-terminal domain pocket would be a more favored binding site.

Long chain aliphatic compounds may bind to the hydrophobic pocket²¹, and may include ceramide³⁴, leukotriene³⁵⁻³⁷ and phosphatidylinositol ether lipid analogues (PIAs).^{38,39} We observe that leukotriene docks to the extended configuration of this pocket (Fig. 4f), with an energy of -7.6 kcal/mol and with a cluster size of 16 out of 100 dockings at 4 Å RMSD. Cluster grouping is likely lower here due to the marked degrees of conformational freedom on the aliphatic chain, compared to small molecule ligands. The aliphatic chain sits in the extended

hydrophobic pocket and the carboxylate group can potentially form hydrogen bonds with the Ser293 and Asn201 side chains at the edge of the pocket. We also observed a 180° flip in the leukotriene moiety in the next best docking result, with 12 out of 100 dockings and −6.9 kcal/mol. Compounds similar to leukotriene that could potentially bind to the hydrophobic C-lobe pocket, include eicosapentaenoic acid, arachidonic acid and oleic acid.⁴⁰ We observed that these molecules dock to the C-terminal domain pocket in the same manner as leukotriene (data not shown). As a control, the best docking energy of leukotriene bound to the p38α active site was −7.6 kcal/mol, but with poor clustering at 6/100 at 4 Å RMSD. Therefore, the comparatively higher clustering observed for the C-terminal domain pocket suggests that it is a more suitable binding site. Taken together, these docking results agree with the crystallographic analyses, and indicate that despite flexibility in the pocket for binding different shaped compounds, small molecule compounds can bind in a specific conformation and with suitable binding affinities that match or exceed those for the active site.

Conclusions

p38α likely functions as a cellular master key, controlling certain inflammatory processes² and disorders caused by an imbalance of oxidative stress levels⁴¹ or DNA repair mechanisms.^{42, 43} Allosteric changes in p38α promoted by ligand binding may provide powerful useful means to regulate function, and have become of interest to the MAP kinase field, in the last couple of years.^{44,45} This is because inhibitors bound in regions separate from the ATP-binding site could overcome the dose-limiting adverse effects of active site inhibitors, which may act on other kinases. Allosteric activators of p38α may be of therapeutic benefit in the treatment of cancer, because enhanced p38α activity can promote apoptosis.⁴¹ This includes apoptosis induced through chemotherapeutic agents^{46–48} such as oxaliplatin⁴⁹, where p38α regulates the macromolecular machinery associated with DNA double-strand breaks.⁵⁰ Compounds having a negative allosteric affect on p38α are also likely to be of benefit, including inflammatory processes², as p38α plays an additional role in sensing cellular oxidative stress and inhibiting tumor formation.⁴¹ Interestingly, p38α inhibitors may also be of use in the rapid aging disorder, Werner Syndrome (WS)⁵¹. This is because a possible mechanism for the WS phenotype is p38α mediated stress response^{42,43}, and p38α inhibitors have been observed to rescue accelerated replicative decline of WS cells, including rescuing the growth rate and cell morphology.^{43,51}

Recent structural results have determined that kinase interactions with substrates mostly occur in pockets and/or grooves outside of the kinase active site, allosterically regulating kinase activity and promoting pathway specificity (reviewed in^{52,53}). These docking interactions have been well defined for distinct kinases and kinase families, including cAMP or cGMP dependent kinases, GSK-3β and certain members of the MAP and CDK families.^{52,53} One docking region observed in the structures of CDK, MAP and GSK-3β kinases, but not other members of the sizable kinase superfamily, is contained within an insert that forms a cap-like structure on the C-terminal lobe. Where defined, this insert can be critical to substrate or protein partner binding.^{52,53} The function of this insert in p38α is being characterized, with initial reports suggesting a role in binding 'FXF' motifs²⁰ or arachidonic acid metabolites.²¹ Our crystallographic and computational analyses suggest that the p38α C-terminal domain hydrophobic pocket formed by the cap is a flexible binding site, capable of accommodating both long-chain cellular lipid molecules and small molecules that act as p38α modulating compounds. Notably, p38α pocket binding involves side chain movements, such as the Trp197 residue that is observed in different rotamers, emphasizing the importance of allowing side chain rotations for docking, which has also been seen in other systems.⁵⁴ Also, an approach to achieve specificity for binders in the MAP, CDK or GSK-3 kinase insert could employ the 'anchored plasticity method' as developed for nitric oxide synthase, where ligands are designed to favor the side chain movements permitted in the target but not other kinases.⁵⁵ Interestingly,

the movements experimentally observed here also suggest that some pocket residues act as pseudo-ligands, so that ligand binding involves a form of interface mimicry and exchange. Such interface mimicry and exchange has also been observed previously, e.g. in the interactions of peptide binding motifs for PCNA⁵⁶ and the Rad51-BRCA2 interactions.⁵⁷

Binding to the kinase C-terminal domain insert can have different functional consequences, which include localization events. In GSK-3 β , the C-terminal domain insert mediates GSK-3 β localization to a multi-protein complex via its interaction with axin protein. Axin forms a scaffold for the GSK-3 β substrate β -catenin, and thus the GSK-3 β :axin interaction is observed to enhance β -catenin phosphorylation greater than 20,000-fold.¹⁹ Furthermore, the functions of CDK2:Cks1 interaction likely go beyond mediating kinase activity, as the complex has a non-catalytic role in transcription, by mediating the recruitment of proteasomes to coding regions.⁵⁸ Thus, it would be of interest in future studies to determine whether *in vivo* p38 α function can be modulated through C-lobe pocket binding compounds, and our open pocket structure provides a detailed framework for the rational design of such compounds. Binders could disrupt interactions that occur at the pocket site, thus affecting localization events. Binders could also affect potentially important conformational changes that occur in p38 α . This is because the hydrophobic pocket utilizes residues of the α EF/ α F loop that is known to play a key role in the function of the adjacent activation loop.⁵⁹

The C-lobe insert has features that suggest that it is a promising site for the design of inhibitors, particularly in being a hydrophobic pocket rather than flat surface and containing hydrogen bond acceptor sites for specificity, e.g as seen for the binding site of the schistosomal drug praziquantel.⁶⁰ Additionally, our crystallographic and computational analyses suggest that small molecule binders could be designed to tightly bind the p38 α C-terminal domain pocket with affinities comparable to active site binders. A rationally based designed molecule could include a lipophilic region interacting with the predominantly hydrophobic part of the pocket, improving upon the fluorophenyl group of 4-FPP. Also, retaining the hydrogen bonds observed in the pyrazol group of 4-FPP and stacking against Trp197 would be beneficial, as would groups forming hydrogen bonds to the hydrogen bond acceptors at the entrance of the pocket. Overall, the identification and development of new compounds binding alternate sites on p38 α , through methods such as those presented here, are likely valuable to test potential kinase inhibitors as tools and for therapeutic treatment of many kinase-related human disease states. Currently drugs are being developed that bind to the p38 α active site and may also bind the C-terminal domain, as shown here. Thus, characterizing the potential allosteric effects of drug binding to the C-terminal domain pocket is important. Moreover, our results underscore the value of using other methods in addition to high-throughput soaking-based crystallography in drug discovery, including the co-crystallography approach used here or x-ray scattering technologies to identify allosteric inhibitors by changes in shape and conformation in solution.⁶¹

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Figure 1. The molecular structures of (a) 4-[3-(4-fluorophenyl)-1H-pyrazol-4-yl]pyridine (4-FPP) and (b) 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-imidazole (SB203580)

Figure 2. The p38 α MAP kinase:4-FPP complex crystal structure

(a) The p38 α MAP kinase:4-FPP complex structure, with secondary structural elements in green and surface in transparent grey. Two 4-FPP molecules, depicted as spheres, are bound to p38 α . One molecule is bound at the active site and is shown in magenta, the second binds to the C-terminal domain MAP kinase hydrophobic pocket, depicted in salmon. (b) Interactions of 4-FPP in the active site pocket (the surface of p38 α is depicted in transparent grey). 4-FPP forms a 2.7 Å hydrogen bond with the Met109 main chain nitrogen and the pyrazol group forms a hydrogen bond with a water molecule that also forms hydrogen bonds with the Asp168 (of the 'DFG' loop) and Lys53 side chains. (Hydrogen bonds are depicted as yellow dashed line, and distances marked in Å).

Figure 3. 4-FPP Ligand electron density and packing in the hydrophobic pocket compared to β -OG and apo-p38 α

(a) Composite Fo-Fc electron density map at the hydrophobic pocket, contoured at 3σ in blue and 5σ in red, which reveals the presence of 4-FPP inhibitor that is depicted here with carbon atoms colored in salmon. (b) Van der Waal interactions are significant in the binding of 4-FPP to the hydrophobic pocket (p38 α surface depicted in transparent grey) and this includes a stacking interaction with the Trp197 side chain. Hydrogen bonds (yellow dashed lines with distances in Å) include interactions with Leu291 main chain oxygen and Ser293 side chain oxygen and main chain nitrogen, and a water molecule (red sphere) that interacts with the Ser252 main chain nitrogen. (c) Superimposition of the hydrophobic pocket region of p38 α :4-FPP complex structure in green, with the p38 α : β -OG complex structure in yellow and apo-p38 α , PDB code 1P38, in cyan (bound small molecules not shown here for clarity). The α EF/ α F-loop has moved to a similar conformation in the 4-FPP and the β -OG complex structures, compared to apo-p38 α , with the Met198 side chain swinging approximately 180° and Trp197 moving into its place. The opening at helix IL14 is also similar in the two complex structures. However, variations between the two small molecule complex structures are observed. The

rotamers of the Trp197 side chain are different in both cases. Also, the 2L14-helix in p38 α :4-FPP is similar to apo-p38 α , whereas this helix is more extended in the p38 α : β -OG complex to accommodate a second β -OG molecule that binds to this region. This suggests notable plasticity in the pocket conformation to accommodate molecules of significantly different shapes. (d) Overlay of 4-FPP and β -OG bound in the hydrophobic pocket region, between the I114-helix and the α EF/ α F-loop. Notably, the α EF/ α F-loop has opened to a comparable conformation in both the 4-FPP and the β -OG complex structures, to accommodate these two molecules in a similar position in the pocket.

Figure 4. Computational analyses of ligand binding to the C-lobe of p38α

(a) A plot of AutoLigand results of total energy per volume (kcal/mol/Å³) versus volume (Å³), using fill sizes from 20 Å³ to 1150 Å³. The results include a few sets of points that represent small cavities that typically contain bound water molecules, in the crystal structure. In addition, several curves for larger cavities are observed and include the active site region, plotted with red squares, and the C-terminal domain pocket, plotted with green squares. (b) Hydrophobic pocket flexibility analyzed by FlexTree. The p38α molecule is shown as a blue backbone with the cap residues Pro242 and Thr263 depicted in different degrees of orientation; dark blue is the orientation in the crystal structure, yellow is -7°, purple is -15°, red is +7° and cyan is +15°. The bound 4-FPP is depicted in CPK, with the fluorine atom of the 4-fluorophenyl

group colored in green. (c) The AutoLigand result superimposed on the 4-FPP crystal structure. The gray balls are carbon atom centers on a 1 Å grid that depicted the fill and the transparent shell is the molecular surface of all the atom fill points. The 4-FPP moiety in our crystal structure and residue Trp197 are depicted in stick form and the rest of the p38α shown in lines, with the cap forming helices labeled. AutoLigand picks the best atom type with the highest affinity for each point in the fill and colors them gray for aliphatic/aromatic, red for hydrogen-bond acceptor, and cyan for hydrogen-bond donor. The p38α C-terminal pocket is predominantly aliphatic, but with hydrogen bond acceptors at its edges. (d) Docking of SB203580 results in two major clusters, with the larger cluster depicted that has the 4-fluorophenyl group at the entrance of the cleft. The docking was performed using AutoDock4 and the AutoDockTools (<http://mgltools.scripps.edu/>) package was used to generate input files for the docking runs and ligand site analysis. A grid box of 40 × 50 × 50 with a spacing of 0.375 Å was used with an initial population of 150 randomly positioned inputs. The maximum number of energy evaluations was set to 2.5×10^6 and a mutation rate of 0.02 with a crossover rate of 0.8 was used. Clustering was set at 2.0 Å root-mean-square deviation (RMSD). (e) Anisomycin docks to the hydrophobic pocket in a similar orientation and with a similar predicted binding energy as 4-FPP. Docking indicates that hydrogen bonds could be formed between anisomycin and the backbone nitrogens of Ser252 and Ser293, distances labeled in Å. Docking was performed with the same parameters as in (d). (f) The orientation of the largest cluster of leukotriene dockings with the C-terminal domain hydrophobic pocket of the β-OG complex, with the aliphatic chain residing in the extended hydrophobic pocket and carboxylate group at the pocket edge. The AutoLigand analysis highlights the pocket conformation for binding long chain lipophilic molecules. The gray balls shown are carbon atom centers on a 1 Å grid that depicted the fill, and the transparent shell is the molecular surface of the carbon atom fill points. Similar to the 4-FPP structure, the pocket is predominantly aliphatic but has regions at the edge of the pocket containing hydrogen bond acceptors that are depicted in red. The same AutoDock parameters were used as in c above, but due to the many active torsions in the leukotriene ligand, an initial population of 450 randomly positioned inputs was used and the maximum number of energy evaluations was set to 2.5×10^7 . Clustering was set at 4.0 Å root-mean-square deviation (RMSD).

Table 1**Crystallographic data collection, phasing and refinement statistics**

Recombinant p38 α was purified to ~10 mg/ml in 50 mM Tris, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT and was subsequently co-crystallized with 1.9 mM 4-FPP, by established p38 α crystallization conditions.⁶² A diffraction data set was collected at a wavelength of 0.98 Å to 2.1 Å resolution on a single crystal. Diffraction experiments were conducted at beamline 9-1 at SSRL, Stanford (data for the highest resolution shell is shown in parentheses), and data was collected on an ADSC Quantum 210 CCD detector and at a temperature of 100° K. The data set was indexed, merged and scaled with HKL2000.⁶³ Phase information was obtained with the molecular replacement method using the AMoRE program⁶⁴ and an apo-p38 α structure (PDB code 1A9U) as the search model. Our starting model was built into σ A-weighted 2Fo-Fc and Fo-Fc maps using XFIT⁶⁵, and this was followed by several cycles of rigid body and restrained refinement using CNS 1.1⁶⁶, with 5% of data removed for the Rfree calculation.

Data Collection	
Wavelength (Å)	0.98
Space group	P 2 ₁ 2 ₁ 2 ₁
Cell dimensions, <i>a</i> , <i>b</i> , <i>c</i> (Å)	64.94, 74.69, 77.11
α , β , γ , (°)	90, 90, 90
Resolution (Å)	40.0 - 2.1
Unique reflections	21,931 (2,156)
Completeness %	91.9 (88.1)
R _{merge}	5.9 (37.1)
Redundancy	3.2 (3.0)
I/ σ I	18.6 (2.9)
Refinement Statistics	
Resolution (Å)	37.35 - 2.1
Unique reflections	20,679 (2,156)
R _{work} /R _{free} (%)	22.6/26.7
Mean B-factors (Å ²) Protein	
Protein	35.2
Active site 4-FPP	25.7
C-lobe bound 4-FPP	30.4
Water	42.3

Data Collection

No. Atoms	2,645
Protein	2,645
4-FPP	36
Water	193

r.m.s. deviations bond lengths (Å)	0.006
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r.m.s. deviations bond angles (°)	1.20
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Ramachandran plot

Most favored (%)	89.0
Additionally allowed	10.7
Generously allowed	0.3