Structural Basis for AMPK Activation: Natural and Synthetic Ligands Regulate Kinase Activity from Opposite Poles by Different Molecular Mechanisms

Matthew F. Calabrese,1,2 Francis Rajamohan,1,3 Melissa S. Harris,1 Nicole L. Caspers,1 Rachelle Magyar,1 Jane M. Withka,1 Hong Wang,1 Kris A. Borzillieri,1 Parag V. Sahasrabudhe,1 Lise R. Hoth,1 Kieran F. Geoghegan,1 Seungil Han,1 Janice Brown,1 Timothy A. Subashi,1 Allan R. Reyes,2 Richard K. Frisbie,1 Jessica Ward,2 Russell A. Miller,2 James A. Landro,2 Allyn T. Londregan,1 Philip A. Carpino,2 Shawn Cabral,1 Aaron C. Smith,1 Edward L. Conn,1 Kimberly O. Cameron,2 Xiayang Qiu,1 and Ravi G. Kurumbail1,*

1Worldwide Research and Development, Pfizer Inc., Eastern Point Road, Groton, CT 06340, USA
2Worldwide Research and Development, Pfizer Inc., 610 Main Street, Cambridge, MA 02139, USA
3Co-first author
*Correspondence: ravi.g.kurumbail@pfizer.com
http://dx.doi.org/10.1016/j.str.2014.06.009

SUMMARY

AMP-activated protein kinase (AMPK) is a principal metabolic regulator affecting growth and response to cellular stress. Comprised of catalytic and regulatory subunits, each present in multiple forms, AMPK is best described as a family of related enzymes. In recent years, AMPK has emerged as a desirable target for modulation of numerous diseases, yet clinical therapies remain elusive. Challenges result, in part, from an incomplete understanding of the structure and function of full-length heterotrimeric complexes. In this work, we provide the full-length structure of the widely expressed α1β1γ1 isofrom of mammalian AMPK, along with detailed kinetic and biophysical characterization. We characterize binding of the broadly studied synthetic activator A769662 and its analogs. Our studies follow on the heels of the recent disclosure of the α2β1γ1 structure and provide insight into the distinct molecular mechanisms of AMPK regulation by AMP and A769662.

INTRODUCTION

AMP-activated protein kinase (AMPK) is a central monitor and regulator of cellular energy status in eukaryotes. From a functional perspective, conditions of energy depletion cause activation of AMPK that leads to upregulation of catabolic processes (fatty acid oxidation and glycolysis) and a corresponding down-regulation of anabolic processes (cholesterol and lipid synthesis and gluconeogenesis). The net effect is to replenish ATP at times of stress so that cellular energy balance is restored (Carling et al., 2011; Hardie, 2007; Oakhill et al., 2012; Steinberg and Kemp, 2009). Given this central function in metabolism, AMPK has emerged as an attractive target for diabetes and cancer (Hardie, 2013; Viollet et al., 2007). Pharmacological activation of AMPK has long been sought as a therapeutic approach in these disorders, yet clinically viable direct small molecule activators of AMPK have to date remained elusive. This is, in part, due to the complexity of this enzyme and the lack of detailed understanding of its molecular mode of action.

In recent years, there has been significant progress in elucidating the structural basis of these complex and critical functions. AMPK is a ~145 kDa heterotrimeric serine/threonine kinase comprised of one catalytic α subunit in complex with regulatory β and γ subunits. The α subunit includes an N-terminal kinase domain (KD), which is the catalytic engine of the enzyme, and a C-terminal subunit-interacting domain (SID) that plays a structural role in mediating interactions with β and γ. Between these regions lies an auto-inhibitory domain (AID) comprised of a three-helical bundle, as well as regulatory motifs termed the α regulatory subunit-interacting motifs (RIMs), which appear to have a critical role in allosteric regulation of AMPK by nucleotides (Chen et al., 2013; Xiao et al., 2011). α-N-myristoylation of the β subunit may facilitate reversible binding to membranes and activation by upstream kinases (Oakhill et al., 2010; Steinberg and Kemp, 2009; Warden et al., 2001). The unstructured N-terminal portion of this domain is followed by a β sheet rich carbohydrate binding module (CBM). This motif bears sequence similarity to starch binding domains in other proteins and interacts with glycogen and β-cyclodextrin, a cyclic form of maltoheptaose (Polekhina et al., 2005). The most C-terminal element of the β subunit serves as a scaffold, forming an extended β sheet with α and γ subunits within the regulatory core. Finally, the γ subunit is comprised of four tandem cystathionine β synthase (CBS) motifs, which are structural modules of ~60 amino acids organized into a two-stranded β sheet with two surrounding α helices. A pair of CBS modules forms a “Bateman” domain, which possesses two nucleotide binding sites, one on each side. The presence of multiple gene products (α1, α2; β1, β2; γ1, γ2, γ3) theoretically allows subunits to be mixed-and-matched to create twelve unique heterotrimers. The distinct activities of each complex are not fully understood, but derive, in part, from differential expression patterns across tissues. For example, γ1 and γ2 appear to be widely expressed, while γ3 appears restricted to skeletal muscle (Birke and Wojtaszewski, 2006; Cheung et al., 2000). In addition, expression patterns may differ
across species, as human liver predominantly contains the α1β2γ1 heterotrimer, while rat and dog livers mainly contain α2β1γ1 and α1β1γ1 respectively (Wu et al., 2013). A full understanding of the biological function of AMPK will require structural characterization of the different heterotrimers and assignment of their cell- and tissue-specific functions.

Activity of AMPK has generally been thought to require phosphorylation on a conserved threonine (T172) within the activation loop by upstream kinases, LKB1, or CaMKKβ, leading to >-500-fold stimulation of its activity (Hawley et al., 1996, 2005; Hurley et al., 2005; Shaw et al., 2004; Woods et al., 2003a, 2005). However, recent work has revealed that under certain conditions, AMPK that is unphosphorylated on T172 of the activation loop is still competent to phosphorylate SAMS peptide (Scott et al., 2014). Catalytic activity can be further modulated by interactions with adenine nucleotides in the γ subunit. Binding of ATP is regarded as inhibitory, while AMP and ADP appear stimulatory. Stimulation occurs principally through protection of T172 from dephosphorylation by phosphatases, with additional allosteric activation triggered by AMP (Gowans et al., 2013; Hardie, 2011; Steinberg and Kemp, 2009).

The capacity to be activated by nucleotides was an original defining feature of AMPK (Carleng et al., 1989; Steinberg and Kemp, 2009), but the first synthetic activator was not reported until 2006 (Cool et al., 2006). When administered to ob/ob mice, this thienopyridine derivative, termed A769662, was observed to lower blood glucose, decrease body weight, and reduce circulating levels of triglycerides. A769662 was shown to mediate its effects through direct interaction with AMPK with in vitro potency yielding a functional enzyme with activity comparable to full-length wild-type (WT), but nearly devoid of heterogeneity as assessed by mass spectrometry (Figure S1 available online).

We crystallized AMPKγ1 in the presence of AMP and staurosporine, a potent kinase inhibitor, and determined the structure at 3.4 Å resolution (Table 1). α1β1γ1 adopts an extended conformation similar to that seen for α2β1γ1 (Figures 1 and 2). The α and β subunits dock atop the γ subunit where the C-terminal region of the β subunit is sandwiched between the top of the γ domain and the C-terminal region of the α subunit. The KD of the α1 subunit is located against the core, and the activation loop encompassing pT172 is well ordered and positioned as previously described (Xiao et al., 2011) (Figure S2A). The C-helix of the KD adopts a conformation consistent with an active kinase in which a critical salt bridge between K45 and E64 is intact. The regulatory segment of the α subunit, which is partially modeled in α2β1γ1, is disordered in our structure.

The CBM of the β1 subunit sits atop the N-lobe of the KD of the α1 subunit, where each domain contributes a β sheet in a near-orthogonal orientation to create a clam-shell-shaped interface. The conformation and location of the CBM is similar to that observed for α2β1γ1 (Figure 2), but differs dramatically from that observed for the yeast homolog, where the CBM was shown to interact with the γ subunit (Amodeo et al., 2007). The CBM-KD interface is stabilized by electrostatic interactions between two conserved lysines in the α1 subunit (K29, K31) and the phosphomimetic aspartic acid at position 108 of the β1 subunit (Figure 1C). Note that position 108 is a serine in WT AMPK, which can be phosphorylated in vivo (Mitchelhill et al., 1997; Warden et al., 2001;
Structure of AMPK α1β1γ1

Woods et al., 2003b). The S108D mutation led to greater homogeneity and, consequently, was the construct that first enabled crystallization. We have subsequently reinstated the native S108 and, after in vitro phosphorylation (>75% pS108), crystallized the pS108 α1β1γ1 construct and collected a data set to 3.9 Å resolution. Although the lower resolution precluded extensive model building and refinement, maps were of sufficient quality to allow visualization of the phosphate in a difference electron-density map. The phosphate occupies a similar location as the carboxylate in S108D and generates an analogous orientation of the CBM-KD interface (Figure S2B). These observations confirm that the α/β interface observed in the S108D crystals is not an artifact of the phosphomimetic mutation. There is an additional sphere of electron density visible within the CBM-KD interface which we have modeled as a sulfate due to its proximity to positively charged amino acids (αK29, αK51, βR83) and the presence of >1 M sulfate in the crystallization conditions (Figure S3). This ion is likely a consequence of crystallization and not of physiological or mechanistic relevance. Additional weak density observed in this cavity is likely due to water or buffer components, which have not been modeled.

The β1 subunit further contributes a novel helical motif (recently termed the “C-interacting helix”), which extends from the C-terminal end of the CBM and packs against the B- and C-helices of α1, generating a pseudo-helical bundle (Figures 1B and S2C). This helix has not been observed in previous studies of the isolated CBM, but was recently observed in α2β1γ1 (Xiao et al., 2013). The C-interacting helix comprises residues 162–171 and modeling resumes at residue 201 as the structure transitions into the core particle. There exists a stretch of partially continuous electron density (varying in clarity across structures), which likely accounts for a portion of this linker. We have attempted to build this segment, but have ultimately chosen to exclude it from the final model, as we were unconvinced of amino acid registration or identity.

AMPK α1 and α2 share a sequence identity of ~77%, and the global architecture between the α1β1γ1 and α2β1γ1 structures appears highly conserved. Overall shape and topology are alike, with docking of the CBM of the β subunit atop the KD (Figure 2A). Both structures contain an ordered C-interacting helix which packs against the N-lobe of α1 or α2, and both structures contain an active conformation of the regulatory spine as seen in other kinases (Figure 2B) (Taylor and Kornev, 2011). Despite these similarities, these structures possess several important differences. First, although the α2β1γ1 structure contains the phosphorylated activation loop (pT172), and is bound to an activator, a critical salt bridge between K45 and E64 in the N-lobe is broken due to displacement of the αB and αC helices of α2 (Figure 2C). This salt bridge is thought to be a requisite for a catalytically competent kinase and is maintained in our α1β1γ1 structures. In addition, the KD and CBM are significantly offset with respect to the regulatory core. This is caused by a ~15° rigid body rotation near the base of the KD (Figure 2D). With alignment based on the N-lobe of the KD, we see that although the overall shape of the CBM-KD interface is preserved, the β1 CBM docks differently atop α1 versus α2 (Figure 2E). Although slight variation occurs even within the α2β1γ1 structure between the two copies in the asymmetric unit, differences are more pronounced in comparisons between α1β1γ1 and α2β1γ1 (global root-mean-square deviation [rmsd] ~1.5 Å).

A769662 Binds at a Novel α/β Allosteric Site

AMPK α1β1γ1 was initially crystallized in the presence of AMP, but in the absence of the synthetic activator, A769662.
The intervening nitrile group faces solvent and likely perturbs the pK_a of the adjacent hydroxyl, increasing its acidity. The nitrogen of the pyridine core donates a hydrogen bond to D88 of a1 and disrupts an electrostatic interaction between D88 (a1) and R83 (b1) observed in the apo structure (Figures 3 and S4). The repositioned R83 stacks ~4 Å above the Cl-A769662 core, forming a cation interaction with the ligand, contributing to its overall free energy of binding. In addition, the side chain guanidinium group of R83 forms an ion-pair with a1 D217 from a neighboring molecule in the crystal. From the bottom, I46 of a1 forms a pedestal upon which the 6-5 core of the ligand rests. The central phenyl ring occupies a narrow channel, which is lined in the back by hydrophobic residues and in the front by K31. K31 participates in two additional interactions; it simultaneously forms an electrostatic interaction with D108 of b1 and donates a hydrogen bond to the hydroxyl group of the phenol in the tailpiece of Cl-A769662. This phenolic oxygen participates in a bifurcated interaction in which it additionally donates a hydrogen bond to the backbone carbonyl of G19 of a1. Together, a network of hydrophobic and polar interactions serves to anchor Cl-A769662 at the CBM-KD interface.

To better understand the structure-activity relationship of the A769662 series and to confirm the assignment and modeling of the allosteric binding site, we solved the structure of AMPK_{xtal} in the presence of a dibromo analog of A769662 (Br_{2}-A769662_{core}) (Figure 4). The presence of two bromines enabled us to collect anomalous diffraction data that allowed for unambiguous placement of the ligand (Figure S2D). Evaluation of the AMPK_{xtal} structures with Br_{2}-A769662_{core} and Cl-A769662 reveals nearly identical poses for the thienopyridone core, confirming our previous interpretation of the electron density.

Comparison to the recent A769662-bound α2β1γ1 structure reveals that in both isoforms, the core of the ligand is positioned with a remarkably similar pose (Figure 3C). These similarities occur even though the α1γ1 structure represents a soaking experiment, while the α2β1γ1 structure was generated by cocrystallization and is in-line with the high level of sequence identity (~93%) between the N-lobes of α1 and α2 including absolute conservation of residues contacting ligand. As this structure represents an improvement in resolution relative to the α2β1γ1 structure with A769662 (3.35 Å versus 3.9 Å), we were able to examine the binding mode in additional detail.

**Ligand Deconstruction Allows Probing of the Allosteric Pocket**

To map the importance of different regions of the allosteric pocket for ligand binding, we investigated binding of four analogs of A769662 differing in the nature of the tailpiece and halogen appended to the core (Figure 4A). The highest binding affinity, as determined by surface plasmon resonance (SPR) in the presence of AMP, is observed for Cl-A769662, which provides a modest (~7-fold) increase in potency relative to parent (A769662). The small potency boost is likely explained by burial of the chlorine within a small hydrophobic pocket comprised primarily of residues F90 from α1 and V81, V113, and I115 of β1. The phenol plays a prominent role in binding affinity through both the bifurcated hydrogen bonds with K31 and G19 of α1 and through van der Waals interactions with the CBM and KD. As such, deletion of the phenol produces an

---

**Table 1. Data Collection and Refinement Statistics**

<table>
<thead>
<tr>
<th>Data Collection</th>
<th>AMPK</th>
<th>AMPK</th>
<th>AMPK</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPK</td>
<td>α1β1γ1</td>
<td>α1β1γ1</td>
<td>Cl-A769662</td>
</tr>
<tr>
<td>Data Collection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spacegroup</td>
<td>P61,22</td>
<td>P61,22</td>
<td>P61,22</td>
</tr>
<tr>
<td>Unit cell dimensions (Å)</td>
<td>a = 124.4</td>
<td>b = 124.4</td>
<td>c = 404.0</td>
</tr>
<tr>
<td></td>
<td>a = 124.6</td>
<td>b = 124.6</td>
<td>c = 402.9</td>
</tr>
<tr>
<td></td>
<td>a = 123.9</td>
<td>b = 123.9</td>
<td>c = 401.6</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>30-3.5</td>
<td>30-3.5</td>
<td>30-3.5</td>
</tr>
<tr>
<td></td>
<td>(3.5-3.45)</td>
<td>(3.47-3.35)</td>
<td>(3.68-3.55)</td>
</tr>
<tr>
<td>No. of molecules in asymmetric unit</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>No. of reflections for the work data set</td>
<td>78,441</td>
<td>143,108</td>
<td>140,643</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>24,464</td>
<td>27,060</td>
<td>20,989</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>3.2 (2.8)</td>
<td>5.3 (3.5)</td>
<td>6.7 (6.8)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.8 (97.4)</td>
<td>96.9 (80.1)</td>
<td>90.6 (92.6)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>10.4 (43.7)</td>
<td>11.5 (48.9)</td>
<td>14.8 (45.0)</td>
</tr>
<tr>
<td>&lt;I&gt;/&lt;I&gt;</td>
<td>9.2 (2.0)</td>
<td>15.1 (2.0)</td>
<td>10.7 (3.2)</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of reflections for the test data set</td>
<td>1,208</td>
<td>1,352</td>
<td>1,047</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>6,491</td>
<td>6,345</td>
<td>6,008</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>22.2/26.7</td>
<td>21.0/24.9</td>
<td>22.6/26.9</td>
</tr>
<tr>
<td>No. of atoms protein/water</td>
<td>1,208</td>
<td>1,352</td>
<td>1,047</td>
</tr>
<tr>
<td>Rmsd bond length (Å)</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Rmsd bond angles (°)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>B factors (Å^2)</td>
<td>85</td>
<td>104</td>
<td>101</td>
</tr>
</tbody>
</table>
Finally, the Br<sub>2</sub>-A769662<sub>core</sub> possesses an intermediate potency. The bromine atom at the para position of the central phenyl ring projects along the same trajectory as the phenol, but is unable to fulfill hydrogen bonding. This defect is partially rescued by the second bromine on the thienopyridone core, which occupies the same hydrophobic pocket as the chlorine.

Solution Studies Validate Crystallographic Observations

Prior mutagenesis studies have revealed that deletion of the CBM or KD abolishes AMPK activation by A769662 (Göransson et al., 2007; Sanders et al., 2007; Scott et al., 2008; Xiao et al., 2013). However, in contrast to a previous report (Scott et al., 2008), we observe interactions between A769662 and isolated β1 CBM in solution, albeit weak. Nuclear magnetic resonance (NMR) TROSY experiments using <sup>15</sup>N β1 CBM reveal a subset of residues that undergo chemical-shift perturbations in the presence of A769662 (Figures 5A and S5). Mapping these onto our structure shows that the residues with the greatest effect cluster in and around the allosteric binding site (Figure 5A).

Close inspection shows that several of the amino acids in this vicinity are not conserved between β1 and β2. Although A769662 has been observed to bind to β2-containing heterotrimers (Landgraf et al., 2013) and provide some protection of pT172 from dephosphorylation (Hawley et al., 2012), it is a weaker activator of β2-isoforms in biochemical assays (Sanders et al., 2007; Scott et al., 2008; Xiao et al., 2013). We reasoned therefore that generating chimeras in which we swapped amino acids from β1 to β2 and vice versa may lead to enzymes with altered functional specificity. Indeed, swapping only three of the nonconserved amino acids (F82I, T85S, and G86E) was sufficient to greatly diminish A769662 potency toward β1, but greatly increase potency toward β2 (Figure 5B). These chimeras do not fundamentally alter intrinsic AMPK activity, as their response to AMP, which binds in the γ subunit, remains largely unchanged. None of these three amino acids directly contacts ligand. Instead, they are proximal to or in contact with ligand-coordinating residues. The effects of these mutations may therefore reflect subtle changes in structure, dynamics, or topology of the CBM-KD interface that differ between β1 and β2.

We next assessed the relative importance of interface electrostatics. First, we generated a triple mutant in which the three interface lysines of the α subunit were converted to glutamines (K29-31-51Q) and assayed this mutant side-by-side with WT AMPK. Heterotrimeric K29-31-51Q retained intrinsic activity comparable to WT α1β1γ1 (data not shown), but failed to interact with A769662 up to the highest concentration tested (1 μM) (Figure 6). This result is similar to mutagenesis data reported for the α2β1γ1 isoform (Xiao et al., 2013). Next, we investigate an earlier report demonstrating that mutation of serine 108 to alanine (S108A) greatly diminishes interaction with A769662 (Sanders et al., 2007). Mapping these onto our structure shows that the residues with the greatest effect cluster in and around the allosteric binding site (Figure 5A).
et al., 2007). We expanded this result by performing a more conservative comparison between serine and phospho-serine at position 108, reflecting the two likely chemical forms of this residue in vivo (Mitchelhill et al., 1997). In short, we performed in vitro phosphorylation reactions of AMPK and quenched the reaction at distinct time points to generate samples of protein that were highly phosphorylated on T172 within the activation loop of the α1 subunit, but varied in the extent of phosphorylation on S108 of β1. In contrast to the S108A mutation, which nearly abolished activation, AMPK reagents with S108 or pS108 appear to respond to A769662 to similar extent (Figure S6).
Lastly, we generated a set of AMPK truncation variants to probe the extent to which activation by AMP and A769662 are dependent on regulatory domains (Figure S6). Deletion of the CBM of the β1 subunit does not alter activation by AMP, consistent with the known location of nucleotide binding sites in the γ subunit. In contrast, A769662 is unable to activate the ΔCBM variant, in agreement with prior reports (Göransson et al., 2007; Sanders et al., 2007; Scott et al., 2008; Xiao et al., 2013) and in support of our crystallographic observations of the allosteric binding site at the CBM-KD interface. We generated an additional variant termed ΔzRIM, in which we deleted a 62 amino acid segment (333–394) in the z subunit comprising zRIM1/2, along with flanking amino acids. The deleted segment was replaced with a nine-amino acid (AAGPGGAA) linker, yielding a well-behaved heterotrimer with intrinsic activity similar to WT (Figure S6). While this variant was fully activated by A769662, it was largely insensitive to activation by AMP, consistent with previous site-directed mutagenesis (Chen et al., 2009).

**A769662 and AMP Exert Distinct Kinetic Effects on AMPK**

To probe the kinetic mechanism, we performed in vitro assays monitoring the phosphorylation of a model peptide substrate (SAMS peptide) derived from acetyl-CoA carboxylase 1 (Davies et al., 1989). Experiments were performed under saturating concentrations of ATP while varying the concentration of SAMS peptide in the presence or absence of AMP or A769662 (Figure 7A). Though effects of AMP are complicated by the presence of multiple binding sites and potential interactions of ATP at these sites, we nevertheless observe ~2-fold increase in reaction V_max with no effect on K_m for SAMS peptide. This result is similar to an observation made by Carling et al. nearly 25 years ago (Carling et al., 1989). In contrast, A769662 mediates its effect primarily through decreasing K_m for SAMS peptide ~4-fold, while exerting only a modest (~1.4-fold) effect on V_max. We also performed the reverse experiment where we used saturating levels of SAMS peptide while varying the ATP concentration. In this case, neither AMP nor A769662 had a significant effect on the V_max. Figure 7B shows the location of the allosteric site relative to the previously mapped substrate binding groove (Scott et al., 2002).

**DISCUSSION**

The importance of the synthetic activator A769662 for the AMPK field is evident from the >300 citations garnered since its initial disclosure in 2006 (Cool et al., 2006). Despite a decade of advances in AMPK structural biology, the binding site for this ligand has remained elusive. Early studies following the A769662 disclosure discovered that the CBM of the β subunit was necessary for its interaction. Furthermore, a preference was noted for β1 over β2, and a hot spot within this domain was identified to lie at or near S108 (Göransson et al., 2007; Sanders et al., 2007; Scott et al., 2008). However, inspection of structures of the isolated CBM motif revealed that S108 lies at a solvent-exposed surface that reveals no obvious pocket (Polekhina et al., 2005). Consistent with this observation, the CBM domain alone is incapable of high affinity binding of A769662 as shown previously (Scott et al., 2008) and as indicated by weak interactions in our current solution NMR studies. A clue to the A769662 binding site emerged from recent work in which we investigated full-length AMPK in solution by hydrogen-deuterium exchange (HDX) methods (Landgraf et al., 2013). Comparison of the HDX profiles in the presence and absence of A769662 revealed that in addition to the β1 CBM, significant perturbations were...
observed for a peptide in the N-lobe of the α1 KD, implicating its role for ligand binding. These HDX studies suggested that A769662 is likely to bind at the CBM-KD interface (Landgraf et al., 2013). This was recently confirmed by the crystal structures of compound 991 and A769662 bound to z2β1γ1 that revealed a novel allosteric pocket (Xiao et al., 2013).

With ligand-bound structures in hand, we can begin to more fully understand historical observations. Although of critical importance, the CBM provides only one half of the binding site. For the allosteric pocket to form, both CBM and KD must be present at the interface between them. Given the architecture of the AMPK heterotrimer, with the KD and CBM emanating separately from the regulatory core, only a full-length structure containing core, KD, and CBM could allow such an interface to assemble. Therefore, despite the important insights gained from many years of structural studies of AMPK fragments, subunits, and subcomplexes, none of these systems contained all the necessary pieces to visualize A769662 binding.

The crystallographically observed binding site is supported by a wealth of solution studies. First, using truncation constructs, we find that the β1-CBM is essential for AMPK activation by A769662 (Figure S6), consistent with prior reports (Göransson et al., 2007; Sanders et al., 2007; Scott et al., 2008; Xiao et al., 2013). Our 2D-NMR experiments show that addition of A769662 alters the chemical shifts of a small subset of CBM residues, which cluster around the allosteric site (Figures 5 and S5). As A769662 preferentially activates β1-containing AMPK complexes (Sanders et al., 2007; Scott et al., 2008), we reasoned that specificity is likely affected by CBM residues proximal to this interface. Indeed, chimeras in which residues from this surface are swapped between β1 and β2 demonstrate altered ligand specificity (Figure 5). On the other side of the interface, we see a collection of electrostatic interactions involving three surface lysines, K29/31/51 with both A769662 and the β1-CBM. These amino acids are conserved between α1 and α2 and are retained in species ranging from worm to man. Finally, SPR binding studies of A769662 analogs reveal structure-activity relationships consistent with the observed binding mode (Figures 3 and 4).

Our apo and holo z1β1γ1 structures complement the recent disclosure of the structure of z2β1γ1 bound to A769662 (Xiao et al., 2013) and begin the process of elucidating the structural basis of subtype-specific functions of different forms of AMPK. Although the previous reported structure of z2β1γ1 with a different AMPK activator (compound 991) was at 3.0 Å resolution, the structure with A769662 was at considerably lower resolution (3.9 Å). Comparison of the two isoforms (and both apo and holo structures) reveals a similar interface between the KD and CBM involving electrostatic interactions between pS/D108 of the β subunit and K31/K29 of the α1 and α2 subunits, as well as a conserved conformation of the recently identified C-interacting helix. As the C-interacting helix forms a bundle with the αB and αC helices, we postulate that formation of this motif is dependent on maintenance of the α/β interface and is unlikely to be structured if the CBM dissociates. Finally, in both isoforms, the thienopyridone core of the ligand is positioned with a similar pose despite the fact that our ligand was modeled independently and prior to publication of the z2β1γ1 structure (Figure 3C).

Despite these similarities, these structures possess several important differences. First, in the z2β1γ1 structure a critical salt bridge in the KD (K45–E64) is broken, while it is maintained in our apo and ligand-bound z1β1γ1 structures. In addition, with respect to the regulatory core, the position of the KD and CBM are significantly offset (Figure 2). A third difference lies in the AID/α-RIM regulatory motif of the α subunit. In the z2β1γ1 structure, this motif adopts a conformation similar to that previously observed in the structure of the regulatory core + KD (Chen et al., 2013; Xiao et al., 2011). In our z1β1γ1 structures, this regulatory segment is disordered, providing further support that it may sample an ensemble of conformations. Nevertheless, the observed conformation of the C-interacting helix allows us to rule out the possibility that the AID adopts the structure seen previously in an inactive homolog from yeast (Chen et al., 2009) (Figure S7).

The observed structural differences could be due to crystal packing or may reflect true diversity in the conformations of the z1β1γ1 versus z2β1γ1 isoforms. In addition, this may simply...
reflect structural plasticity within the many moving parts of this molecular machine such that we observe two states among the multitude of possible conformations. Understanding these differences may provide unique opportunities for the design of isoform-specific activators.

In this work, we determined the first structure of mammalian AMPK with all functional domains in the absence of a synthetic activator (apo). This structure reveals that the CBM is able to dock atop the KD even in the absence of ligand and demonstrates that binding of A769662, or other potent ligands, is not a prerequisite for formation of the allosteric site. Rather, the CBM-KD interface represents one of many naturally occurring conformations of AMPK in solution. This is consistent with recent work establishing that the autophosphorylation of S108 occurs in cis, i.e., phosphorylation occurs within a heterotrimeric complex (Scott et al., 2014). As Ser 108 of the β subunit in the present structure lies outside the substrate binding groove, flexibility and rearrangement must occur for cis auto-phosphorylation to take place.

Our crystallographic and solution studies suggest fundamentally different molecular mechanisms of AMPK activation elicited by A769662 and AMP. The AMP binding sites on the γ subunit are ~50 Å away from pThr172 on the activation loop of the α subunit KD. There are distinct structural changes that are observed in the γ subunit, especially around site 3 between ATP- and AMP-bound forms. These perturbations are likely to result in altered conformation of the α-RIM, which forms direct interaction with the γ subunit near this site (Chen et al., 2013; Xiao et al., 2011, 2013). These perturbations are in turn conveyed to the activation loop of the kinase by long-range allosteric effects via the AID module. Consequently, the pThr172 site becomes less accessible to phosphatases on binding of AMP, which may account for the observed protection from dephosphorylation. In addition, our kinetic studies show that AMP increases the Vmax of the phosphorylation reaction and may help fine-tune the kinase for optimized phosphoryl-transfer.

In contrast, the binding site for A769662 is located only ~20 Å from the pThr172 site within the activation loop. Binding of A769662 at the allosteric site is likely to stabilize the active conformation of the B- and C-helices of the KD via the C-interacting helix. This may also help displace the AID from the KD to relieve a previously described auto-inhibition event (Chen et al., 2009) (Figure S7). Our kinetic data show that A769662 activates AMPK by lowering the Km for SAMS peptide (~4-fold) with only a small effect on the reaction Vmax. Although the limited diffraction resolution of our structures does not allow for a detailed analysis of the structural differences, the kinetic data imply that binding of A769662 leads to subtle structural changes that result in a more optimal binding of the SAMS peptide. Another possible explanation is that binding of A769662 shifts the equilibrium toward the active conformation of AMPK via stabilization of the CBM-KD interface. Both of these explanations are consistent with recent results showing that A769662 protects portions of the substrate binding site from exchange with deuterium (Landgraf et al., 2013). Regardless, the molecular mechanism that underlies AMPK activation by the synthetic activator A769662 is radically different from that employed by the endogenous activator, AMP.

It is tempting to speculate that in addition to A769662, other ligands might take advantage of this site to bind and modulate AMPK activity. Recently, it has been shown that salicylate, an active metabolite of aspirin, binds to and directly activates AMPK in a manner that is competitive with A769662 (Hawley et al., 2012). Although we were unable to obtain a high-resolution crystal structure of the complex, these results suggest that AMPK might be activated by other synthetic activators in a similar manner.

Figure 7. Kinetic Effects of A769662 and AMP on Phosphorylation of SAMS Peptide
(A) AMPK kinase activity was determined by measuring incorporation of 32P-phosphate from [32P]ATP into the SAMS peptide substrate at saturating ATP (200 μM) and varying concentrations of SAMS peptide. Experiments were run in the absence (DMSO) and presence of 10 μM AMP or 10 μM A769662. Errors denote ± SD of the fit. See also Figure S7.

(B) View of substrate binding cleft of AMPK. In yellow are seven residues (D56, E100, D103, L212, D215, D216, D217) from previous mutation analysis used to map substrate binding (Scott et al., 2002).
AMPKxtal was soaked with 2 mM 5-iodosalicylate overnight before flash freezing. The strongest peaks in the asymmetric unit localize to the allosteric site. 2fo-fc density was not sufficient to permit modeling of the compound.

structure of AMPK with salicylate bound, anomalous diffraction data from an iodinated variant (5-iodosalicylate) supports that salicylate binds at the CBM-KD interface (Figure 8). The experimental electron density for the iodine lies just below the ps/D108 loop and would roughly overlay with the position of the phenol of CI-A769662 in our structure. While the identification of a physiological ligand for the CBM-KD allosteric site remains to be revealed, we hypothesize that AMPK may be a broad energy sensor, responding to changes in levels of nucleotides, carbohydrates, and other metabolic mediators.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

AMPK α1β1γ1 was cloned into a tricistronic vector containing a His-tag at the N terminus of the α subunit (Neumann et al., 2003; Rajamohan et al., 2010). Constructs were generated using standard molecular biology techniques and are described as follows: The α chain of AMPKα1 was comprised of a 22-residue N-terminal tag (MPRAHHHHHHAGENLYFQGAT) that included an initiation Met, a hexahistidine-tag, and a tobacco etch virus (TEV) protease cleavage site, followed sequentially by residues 2-469 of GenBank accession code U40819.1, an eight-residue linker (A5GGPGGS), and residues 525-548 of GenBank accession U40819.1. The β1 chain was comprised of an initiator methionine followed by residues 68-270 of UniProt accession P80386, except that S108 was mutated to D. The sequence of the γ chain was identical to the 330-residue sequence of UniProt accession P80385. Biochemical and biophysical experiments used full-length human AMPK α1β1γ1 with an N-terminal His-tag on the α subunit. For SPR binding experiments, an additional biotinylated biotin-acceptor-peptide (BAP) was included at the N terminus of the γ subunit. For expression, plasmids were transformed into E. coli BL21-CodonPlus cells grown at 37° C and induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside at 18° C overnight. Cell pellets were resuspended in 25 mM Tris 7.5, 300 mM NaCl, 10% glycerol, 2 mM phosphatidylthio/propanoic acid (TCEP), 40 mM imidazole, and lysed by three passages through a microfluidizer at 18k pounds per square inch. Lysate was cleared by centrifugation, passed over HiTrap Ni-NTA resin, and eluted with imidazole. Protein was dialyzed overnight in the presence of TEV protease to remove the His-tag (only for crystallization). AMPK was phosphorylated with CaMKKβ in the presence of calmodulin and Mg-ATP and subsequently purified over Ni-NTA (activity assays) or ion exchange and size-exclusion chromatography (crystallography and biophysics). Purity was assessed by SDS-PAGE and mass spectrometry (Figure S1).

Crystallization and Refinement

AMPKα1 at ~20–15 mg/ml in 25 mM Tris 7.5, 150 mM NaCl, 2 mM TCEP, 10% glycerol, was incubated with 400 μM of both AMP and staurosporine for ~10 min on ice. This mixture was then centrifuged and mixed 1:1 with well buffer (~750 mM ammonium sulfate, ~500 mM lithium sulfate, 100 mM trisodium citrate, 1% ethylene glycol) in sitting drops and incubated at 22° C. Crystals appeared after 3–7 days and were frozen in mother liquor plus 30% glycerol.

Data were collected at IMCA-CAT and GMCA-CAT at Advanced Photon Source (APS) as well as beamline 5.0.1 at Advanced Light Source. Data sets were processed using HKL2000 (Otwinowski and Minor, 1997). The structure was determined by molecular replacement using PHASER (McCoy et al., 2007) with domains derived from 2Y94.pdb (Xiao et al., 2011) and 1Z0M.pdb (Polekhina et al., 2005) as search models. Refinement and rebuilding were carried out using autoBUSTER (Blanc et al., 2004), PHENIX (Adams et al., 2010), MOLREP and Coot (Emsley and Cowtan, 2004), and figures were generated using PyMOL (Schrodinger, version 1.2r3pre).

For the structures in the presence of Cl-A769662 and Br2-A769662 core, crystals were soaked overnight in mother liquor with ~500 μM ligand, 400 μM AMP, and 200 μM staurosporine, along with 10% glycerol. Crystals were frozen in this soak solution supplemented with 30% glycerol, and the structure was solved by molecular replacement using the apo structure as a search model.

Kinase Activity Measurements

The kinase activity of recombinant AMPK samples was monitored in vitro by phosphorylation of the SAMS peptide as previously described (Rajamohan et al., 2010). Where described, AC50 values reported for A769662 and AMP were measured in the presence of 0.25 mM AMPK in assay buffer, and all experiments were performed in triplicate with each half maximal activating concentration (AC50) value representing the mean of three experiments.

Steady-state kinetic parameters were determined by measuring the kinase activity of AMPK via AMPK-mediated incorporation of 32P-phosphate from [32P]ATP into the synthetic SAMS peptide (Davies et al., 1989). This peptide has the amino acid sequence HMRSAMSGLHLVKRR and is derived from residues 73–85 of rat acetyl-CoA carboxylase 1 in which Ser77 is mutated to Ala. The phosphorylated product was retained on the filter via charge interaction while [32P]ATP was removed by filtration. Captured product was then measured by coincident liquid scintillation counting using a PerkinElmer MicroBeta TriLux. Standard kinase reactions were performed in assay buffer containing 50 mM HEPES, pH 7.5, 1 mM EGTA, 10 mM MgO4, 0.25 mM dithiothreitol, 0.01% Tween-20, and 0.01% BSA. There were eight concentrations of the SAMS peptide substrate that were tested in a serial one-to-one dilution scheme, while keeping ATP fixed at 10-fold the KM-value (200 μM) with and without 10 μM AMP or 10 μM A769662. The specific activity of [32P]ATP was maintained at 0.25 μCi/μl. These assays were assembled by the addition of 30 μM of both AMP and staurosporine for 10 min on ice. This mixture was then centrifuged and mixed 1:1 with well buffer (500 mM lithium sulfate, 100 mM trisodium citrate, 1% ethylene glycol) in sitting drops and incubated at 22° C. Crystals appeared after 3–7 days and were frozen in mother liquor plus 30% glycerol.

Data were collected at IMCA-CAT and GMCA-CAT at Advanced Photon Source (APS) as well as beamline 5.0.1 at Advanced Light Source. Data sets were processed using HKL2000 (Otwinowski and Minor, 1997). The structure was determined by molecular replacement using PHASER (McCoy et al., 2007) with domains derived from 2Y94.pdb (Xiao et al., 2011) and 1Z0M.pdb (Polekhina et al., 2005) as search models. Refinement and rebuilding were carried out using autoBUSTER (Blanc et al., 2004), PHENIX (Adams et al., 2010), MOLREP and Coot (Emsley and Cowtan, 2004), and figures were generated using PyMOL (Schrodinger, version 1.2r3pre).

Kinase Activity Measurements

The kinase activity of recombinant AMPK samples was monitored in vitro by phosphorylation of the SAMS peptide as previously described (Rajamohan et al., 2010). Where described, AC50 values reported for A769662 and AMP were measured in the presence of 0.25 mM AMPK in assay buffer, and all experiments were performed in triplicate with each half maximal activating concentration (AC50) value representing the mean of three experiments.

Steady-state kinetic parameters were determined by measuring the kinase activity of AMPK via AMPK-mediated incorporation of 32P-phosphate from [32P]ATP into the synthetic SAMS peptide (Davies et al., 1989). This peptide has the amino acid sequence HMRSAMSGLHLVKRR and is derived from residues 73–85 of rat acetyl-CoA carboxylase 1 in which Ser77 is mutated to Ala. The phosphorylated product was retained on the filter via charge interaction while [32P]ATP was removed by filtration. Captured product was then measured by coincident liquid scintillation counting using a PerkinElmer MicroBeta TriLux. Standard kinase reactions were performed in assay buffer containing 50 mM HEPES, pH 7.5, 1 mM EGTA, 10 mM MgO4, 0.25 mM dithiothreitol, 0.01% Tween-20, and 0.01% BSA. There were eight concentrations of the SAMS peptide substrate that were tested in a serial one-to-one dilution scheme, while keeping ATP fixed at 10-fold the KM-value (200 μM) with and without 10 μM AMP or 10 μM A769662. The specific activity of [32P]ATP was maintained at 0.25 μCi/μl. These assays were assembled by the addition of 30 μM of both AMP and staurosporine for 10 min on ice. This mixture was then centrifuged and mixed 1:1 with well buffer (500 mM lithium sulfate, 100 mM trisodium citrate, 1% ethylene glycol) in sitting drops and incubated at 22° C. Crystals appeared after 3–7 days and were frozen in mother liquor plus 30% glycerol.

Data were collected at IMCA-CAT and GMCA-CAT at Advanced Photon Source (APS) as well as beamline 5.0.1 at Advanced Light Source. Data sets were processed using HKL2000 (Otwinowski and Minor, 1997). The structure was determined by molecular replacement using PHASER (McCoy et al., 2007) with domains derived from 2Y94.pdb (Xiao et al., 2011) and 1Z0M.pdb (Polekhina et al., 2005) as search models. Refinement and rebuilding were carried out using autoBUSTER (Blanc et al., 2004), PHENIX (Adams et al., 2010), MOLREP and Coot (Emsley and Cowtan, 2004), and figures were generated using PyMOL (Schrodinger, version 1.2r3pre).

Surface Plasmon Resonance Experiments

Experiments were performed on a Biacore®2000 instrument (GE Healthcare). BAP-tagged AMPK α1β1γ1 was captured onto a streptavidin sensor chip to levels ranging from 4,000–6,000 response units. Binding experiments were...
performed in 25 mM Tris, pH 7.5, 150 mM NaCl, 250 µM TCEP, 0.01% P20, 0.5 mg/ml BSA, 2% DMSO and ± 150 µM AMP at 25 °C (Supplemental Experimental Procedures).

**Nuclear Magnetic Resonance Spectroscopy**

The NMR TROSY spectra were collected on a Bruker spectrometer equipped with a 1.7 mm micro cryoprobe operating at 600.33 MHz. The NMR samples contained 5 mg/ml of 15N uniformly labeled β1 CBM in 25 mM phosphate buffer, pH 7.5, and 0.02% sodium azide (Supplemental Experimental Procedures).

**ACCESSION NUMBERS**

Coordinates and structure factors have been deposited in the Protein Data Bank under ID codes 4QFG, 4QFR, and 4QFS.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with article online at http://dx.doi.org/10.1016/j.str.2014.06.009.

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

We thank Uwe Schlattner, INSERM, University Joseph Fourier, Grenoble, for his valuable help in getting us started with the AMPK structural biology work. Use of the IMCA-CAT beamline 17-ID (or 17-BM) at the Advanced Photon Source was supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with Hauptman-Woodward Medical Research Institute. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357. GM/CA at APS supported by the National Institute of General Medical Sciences and the National Cancer Institute (Y1-GM-1104). The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, U.S. Department of Energy under Contract No. DE-AC02-05CH11231. All authors are current or former employees of Pfizer.

Received: April 10, 2014  
Revised: June 3, 2014  
Accepted: June 6, 2014  
Published: July 24, 2014

**REFERENCES**


Landgraf, R.R., Goswami, D., Rajamohan, F., Harris, M.S., Calabrese, M.F., Hoth, L.R., Magyar, R., Pascal, B.D., Chalmers, M.J., Busby, S.A., et al.


