

AMPK/SNF1 structure: a menage a trois of energy-sensing

John W Scott, Jonathan S Oakhill, Bryce JW van Denderen¹

¹St Vincent's Institute and Dept of Medicine, University of Melbourne, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Domain organisation and regulation of AMPK
4. Alpha subunit
 - 4.1. Alpha-2 and Snf1 kinase domain structure
 - 4.2. Substrate binding site
 - 4.3. Autoinhibitory sequence
5. Beta subunit
 - 5.1. Carbohydrate-binding module
 - 5.2. Alpha/gamma subunit binding sequence
6. Gamma subunit
 - 6.1. AMP and ATP binding sites
 - 6.2. Mechanism of AMP activation
7. AMPK as a drug target
8. Perspectives
9. Acknowledgements
10. References

1. ABSTRACT

The AMP-activated protein kinase (AMPK) is the critical component of a highly conserved signalling pathway found in all eukaryotes that plays a key role in regulating metabolic processes in response to variations in energy supply and demand. AMPK protects cells from stresses that decrease cellular energy charge (i.e increase the AMP:ATP ratio) by initiating a shift in metabolism towards the generation of ATP while simultaneously down regulating pathways that consume ATP. The role of AMPK as an energy sensor extends beyond the cell and it is now apparent that it is a key regulator of whole-body energy homeostasis. These functions have stimulated considerable interest in AMPK as a promising target to treat metabolic disorders such as obesity and Type 2 diabetes. Recently, crystal structures of heterotrimeric core fragments and individual domains of AMPK from mammals, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* have been solved. Together they provide an impressive insight into the molecular interactions involved in regulating kinase activity, heterotrimeric assembly, glycogen binding, and binding of the regulatory nucleotides AMP and ATP.

2. INTRODUCTION

A fundamental property of every living organism is the ability to harmonise nutrient supply with energy expenditure, therefore it is not surprising that a sophisticated mechanism designed to sense and maintain energy balance, the AMP-activated protein kinase (AMPK) system, has remained evolutionary conserved from single celled eukaryotes to multicellular organisms. In mammals, AMPK is activated by stresses that increase the cellular AMP:ATP ratio such as nutrient deprivation, metabolic poisons, hypoxia, and exercise (1). AMPK conserves energy by stimulating catabolic pathways that generate ATP, while inhibiting anabolic pathways that consume ATP. AMPK is an orthologue of the SNF1 (sucrose non-fermenting 1) protein kinase, which is required for adaptation of the budding yeast *S. cerevisiae* to glucose starvation and other nutritional stresses (2-4). AMPK regulates virtually all aspects of metabolism including fatty acid synthesis and oxidation, cholesterol synthesis, protein synthesis, glucose uptake, gluconeogenesis and glycogen metabolism. Many of the downstream effects of AMPK are achieved by direct phosphorylation of rate-limiting enzymes in these metabolic pathways. Chronic activation

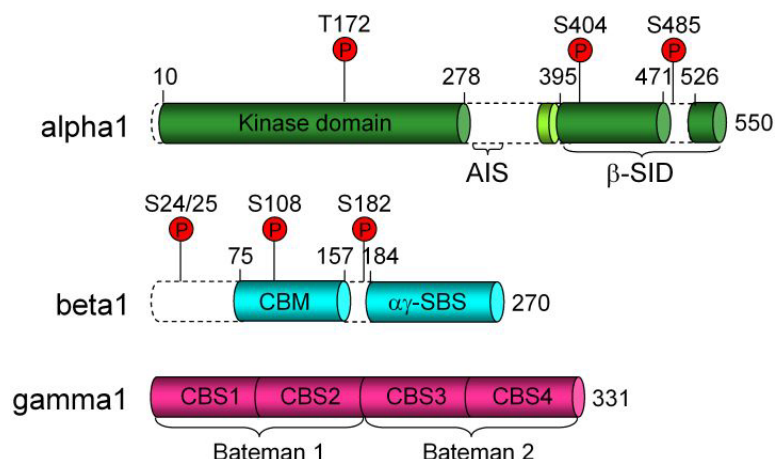


Figure 1. Domain structure of AMPK subunits. Linear representation showing regions for which structural information is either known or can be inferred from yeast orthologue structures (coloured cylinders). Light green cylinder in alpha sequence denotes insert from *S. cerevisiae* structure and may not be represented in mammalian AMPK. Phosphorylation sites are shown in orange circles. AIS: autoinhibitory sequence, SBS: subunit binding sequence.

of AMPK results in metabolic adaptations via regulation of gene transcription such as increased mitochondrial biogenesis as well as enhanced expression of genes involved in glucose metabolism (5-7). Similar metabolic changes are observed with exercise training suggesting that activation of AMPK may be pivotal in mediating many of the beneficial effects associated with regular physical activity (8). There is now compelling evidence showing that AMPK is not only a regulator of cellular energy homeostasis but is also a key player in the control of whole-body energy balance. AMPK is regulated by a number of hormones including leptin, adiponectin, ghrelin, ciliary neurotrophic factor and the cytokine interleukin-6 in a variety of tissues including the hypothalamus (9-14). These discoveries have propelled AMPK into the spotlight as a target for new therapies to tackle the growing epidemic of metabolic disorders including obesity and Type 2 diabetes. Consistent with this concept, AMPK is activated by two classes of glucose lowering drugs, the biguanides and thiazolidinediones, both of which are widely used clinically to treat hyperglycaemia (15, 16).

3. DOMAIN ORGANISATION AND REGULATION OF AMPK

AMPK exists as a heterotrimer composed of an alpha catalytic subunit and two regulatory beta and gamma subunits (Figure 1). In mammals, each subunit is encoded by multiple genes that can also be subject to alternative splicing, yielding at least twelve heterotrimeric combinations that exhibit differences in subcellular localisation and regulation (1). There are two alpha subunit isoforms (alpha-1, alpha-2), both of which have N-terminal kinase domains that are immediately followed by an autoinhibitory region and then a C-terminal domain involved in complex formation (17). There are single orthologues of the alpha subunit in *S. cerevisiae* (Snf1) and *S. pombe* and both have a similar domain structure to the mammalian enzyme. There are two beta subunit isoforms in mammals (beta-1, beta-2), three in *S. cerevisiae* (Sip1,

Sip2, Gal83) and one in *S. pombe*. The beta subunits have N-terminal sequences of poorly defined function although in *S. cerevisiae* they play an important role in subcellular localisation (18). The beta subunits have an internal glycogen-binding domain (GBD) and a highly conserved region at the extreme C-terminus that functions as a scaffold, tethering the alpha and gamma subunits (19-21). The GBD has recently been reclassified as belonging to the Carbohydrate-Binding Module 48 (CBM) family of protein domains that are found in other carbohydrate-binding proteins such as isoamylase and glycogen branching enzyme. There are three mammalian gamma subunit isoforms (gamma-1, gamma-2, gamma-3), one in *S. cerevisiae* (Snf4) and one in *S. pombe*. The gamma subunits have unrelated N-terminal extensions of varying length followed by four copies of a CBS motif (22). These motifs function in pairs to form a discrete structural unit called a Bateman domain and are the regulatory binding sites for AMP and ATP (23).

Regulation of AMPK is complex involving allosteric regulation by AMP and ATP as well as reversible phosphorylation. AMPK is activated by phosphorylation of the critical residue Thr-172 within the activation loop of the kinase domain (24) by several protein kinases including LKB1 and the Ca^{2+} /calmodulin-dependent protein kinase kinases (CaMKK) (25-30). The transforming growth factor-beta-activated protein kinase (TAK1) has also been implicated as a potential upstream kinase in the AMPK signalling pathway (31). Activation of the AMPK complex by AMP occurs by two distinct mechanisms involving direct allosteric activation and inhibition of dephosphorylation of Thr-172 by protein phosphatases (32, 33). The SNF1 complex in *S. cerevisiae* is also activated by phosphorylation of Thr-210 (equivalent to Thr-172) by three upstream kinases Sak1, Elm1 and Tos3, however the SNF1 complex does not appear to be regulated by AMP (34, 35). All the effects of AMP are antagonised by high concentrations of ATP therefore AMPK is a sensor of the AMP/ATP ratio rather than AMP *per se* (36, 37). This

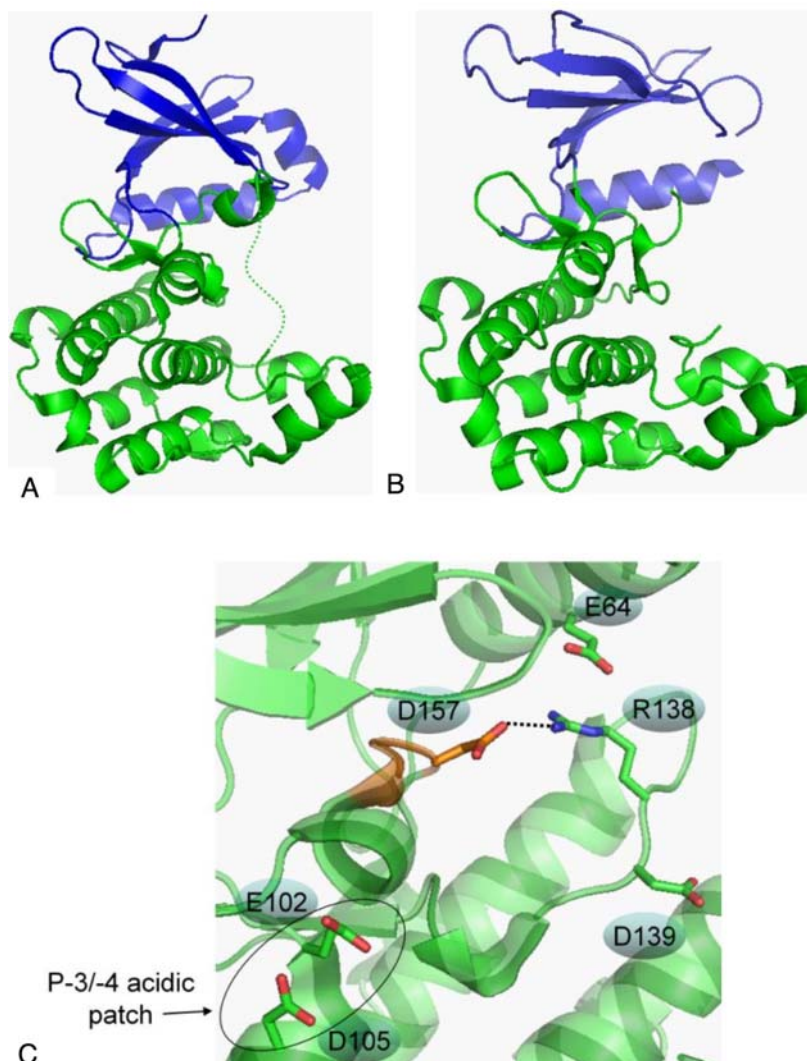


Figure 2. Crystal structure of human AMPK alpha-2 and Snf1 kinase domains. (A) Bilobal organisation of the AMPK alpha-2 kinase domain with N- and C-terminal lobes in blue and green, respectively. (B) Same as (A) except for Snf1 kinase domain. (C) View of active site of human AMPK alpha-2 showing residues involved in substrate recognition (P-3/-4 acidic patch as red spheres) and residues involved in Mg²⁺ co-ordination (Glu-64 and Asp-157), and catalysis (Asp-139 and Arg-138).

sophisticated mechanism allows the AMPK system to respond to subtle changes in the cellular energy charge in an ultra-sensitive manner (38). It is becoming clear that AMPK is a convergence point for multiple signalling pathways, one involving LKB1 and triggered by changes in cellular AMP:ATP, one mediated by CaMKK and elicited by increasing intracellular Ca²⁺, and one possibly mediated by TAK1 and regulated by cytokines.

4. ALPHA SUBUNIT

The two alpha subunit isoforms share approximately 90% sequence identity within their kinase domains but only 61% in their C-terminal regions (2). The alpha subunits exhibit differences in subcellular localisation and AMP-dependence (39), for example, AMPK complexes containing the alpha-2 subunit have been shown

to preferentially localise to the nucleus. In fact, several studies show that nuclear translocation of alpha-2 increases under conditions that stimulate AMPK activity and is important for regulation of downstream processes (40, 41). Alpha-2 complexes are also more AMP-dependent than alpha-1 complexes, however the molecular basis for these differences has yet to be elucidated. The alpha subunits share about 60% sequence identity with the kinase domain of Snf1 and approximately 46% identity with the entire subunit.

4.1. Alpha-2 and Snf1 kinase domain structure

The crystal structure of the kinase domain (KD) from human alpha-2 shows that it adopts a bilobal structure (Figure 2A) characteristic of the eukaryotic protein kinase superfamily (PDB ID: 2H6D). The smaller N-terminal lobe is comprised mainly of five stranded beta-sheets and

two alpha-helices, while the C-terminal lobe is predominantly alpha-helical. The structure was solved for the kinase in the non-phosphorylated state and therefore represents the inactive conformation of the enzyme, providing an insight into the intramolecular interactions that maintain the kinase in the inhibited state. All protein kinase family members have an invariant aspartate residue (Asp-139 in alpha-1/alpha-2 and Asp-177 in Snf1) in their catalytic loops that functions as the base for catalysis and mutation of Asp-139 to alanine renders AMPK catalytically inactive (19, 42). Protein kinases regulated by phosphorylation in the activation loop have an arginine immediately preceding this catalytic aspartate, which is known as the RD motif, and include kinases such as PKA, CaMKI and AMPK (43). Several structural examples show that these kinases require the arginine in the RD motif to be neutralised by the presence of a phosphate on the activation loop in order to promote the correct folding and orientation of the ATP-binding site and active site residues. The activation loop is disordered in the alpha-2 KD structure however the highly conserved DFG and APE motifs that flank the loop are visible. Furthermore, Asp-157 in the DFG motif (which is normally involved in binding the Mg^{2+} ion that co-ordinates the beta and gamma phosphates of MgATP) forms an ionic interaction with Arg-138 in the RD motif, disrupting the ATP-binding pocket (Figure 2B). Arg-138 also sequesters Glu-64 on the C-helix, which normally aligns the phosphates of ATP in the correct orientation for catalysis, causing further disruptions to the active site. These findings suggest a model for activation whereby the phosphate on Thr-172 forms a competing electrostatic interaction with Arg-138, thus freeing Asp-157 and Glu-64 to interact with the Mg^{2+} ion and phosphates of ATP and allow the correct alignment of the catalytic loop. This idea is supported by kinetic studies using an alpha-1 KD Thr172Asp mutant, which partially mimics the effects of phosphorylation. Although the Thr172Asp mutant is active, it has lower specific activity than maximally phosphorylated kinase and also has a weakened affinity for ATP indicating that the single negative charge from the aspartate is unable to fulfill the electrostatic interactions mediated by the phosphate in order to rearrange the active site (44).

There are two crystal structures of the Snf1 KD and in both cases the structure was solved for the enzyme in the dephosphorylated state (Figure 2B) (45, 46). The structures show that the ATP-binding site is disrupted with the C-helix in the small lobe rotated away from the active site. The side chains of Lys-84 and Glu-103, both of which are involved in aligning the phosphates of ATP are disordered. The activation loop of Snf1 is visible in one of the structures however Thr-210 is buried deep within the dimer interface formed by hydrophobic interactions between residues within the activation loop and the G-helix from both protomers. It is proposed that dimerisation of Snf1 is an important regulatory mechanism and that disrupting the dimer may be part of the activation mechanism (45). This is based on the observation that Thr-210 is inaccessible in the dimer and would require a conformational change to allow access to activating upstream kinases. However, there is conflicting evidence as to whether Snf1 KD forms dimers in solution, leading to

the possibility that dimerisation is merely a crystallographic artefact (45, 46). Co-immunoprecipitation experiments show that full-length Snf1 self-associates *in vivo* however the functional implications of this interaction has yet to be determined (45).

4.2. Substrate binding site

The substrate binding site of AMPK has been extensively studied with variant peptide substrates and by site-directed mutagenesis (44, 47). AMPK belongs to the basophilic class of protein kinases, requiring the presence of basic residues in the sequence N-terminal to the site of phosphorylation on target proteins (48). Substrate binding is not dependent on phosphorylation of Thr-172 or the presence of the beta and gamma subunits as an alpha-1 KD Thr172Asp mutant has the same affinity for peptide and protein substrates as phosphorylated heterotrimeric AMPK (44). The core consensus motif for AMPK substrate recognition is H (X,B)XXS/TXXXH where H is a hydrophobic residue, B is a basic residue and S/T serine or threonine residues. AMPK requires a basic residue at either P-3 or P-4 (the P-/P+ nomenclature denotes the residue position N or C-terminal to the phosphorylated residue, respectively) (44, 47, 49, 50). The P-3/P-4 basic residue interacts with an acidic patch (Glu-100, Asp-103 and Glu-144 in alpha-1) located at the interface between the small and large lobes (Figure 2C). Unlike AMPK, SNF1 has a strict requirement for the basic residue to be fixed at the P-3 position in its substrates (45, 46). The P-5 hydrophobic residue binds in a hydrophobic pocket on the C-terminal lobe and is anchored in this pocket primarily by interacting with Leu-212. The substrate-binding site extends across a region much longer than that suggested by variant peptide studies, and includes a hydrophobic groove located on the large C-terminal lobe. Mutagenesis and homology modeling support the view that amphipathic helices occurring in sequences N-terminal to sites phosphorylated by AMPK bind in this groove, which serves to anchor the substrate in an optimal orientation for phosphorylation (44). Indeed, an amphipathic helix is found in the crystal structure of the AMPK substrate HMG-CoA reductase in the sequence preceding the Ser-872 phosphorylation site (51). Unusually, the hydrophobic face of this helix points outward towards the solvent making it accessible to bind the hydrophobic groove on the kinase domain. There are a number of additional determinants that, although not essential, significantly enhance substrate recognition by AMPK and include a basic residue at the P-6 and the P+3 positions. Mutagenesis studies show that the P-6 basic residue interacts with an acidic patch composed of Asp-215 and Asp-217 (44). This patch is located on the large lobe and is conserved between AMPK and Snf1 however it is divergent among the other basophilic protein kinases suggesting that utilisation of this structural feature for substrate binding is restricted to members of the AMPK family. Incidentally, the orthologue of AMPK in the higher plant *Brassica oleracea* shows a strong preference towards substrates with a basic residue at P-6 (52).

4.3. Autoinhibitory Sequence

Both alpha subunits contain an autoinhibitory sequence immediately C-terminal to the kinase domain that

was identified by deletion mutation (17). The autoinhibitory sequence (AIS) was originally thought to span residues 313-394 however further studies have determined that the AIS is much shorter and extends from residues 313-335 (53). Other members of the AMPK-related protein kinase subfamily have ubiquitin-associated domains (UBA) in an equivalent position to the AIS. The UBA domain of the MARK2 kinase (MARK2 is a member of the AMPK-related protein kinase subfamily) has been shown to play an important role in regulation of phosphorylation by LKB1, however the AIS from the alpha subunit of AMPK has weak sequence similarity with these domains (54). Interestingly, the AIS does not inhibit AMPK by blocking the active site since an isolated peptide corresponding to the AIS from alpha-1 does not inhibit AMPK activity *in vitro* (17). Moreover, homology modelling based on the MARK2 kinase domain structure predicts that the AIS adopts an alpha-helical structure, interacting with the kinase domain on the opposite face from the substrate-binding and active sites (53). Mutation of the highly conserved Leu-328 within the AIS strongly increased AMPK activity, indicating that a hydrophobic interaction is crucial to maintain the kinase in an inactive conformation. Several potential binding partners for Leu-328 were identified from the model and mutation of Val-298 also caused an increase in AMPK activity. It is unclear at present how the arrangement of the AIS represses kinase activity although the effect appears to be allosteric in nature since repression persists even when Thr-172 is maximally phosphorylated.

The alpha subunit AIS shares some sequence identity with the autoinhibitory sequence of Snf1, which has been extensively studied by yeast two-hybrid and mutational analysis (17, 55). The AIS of Snf1 binds to the kinase domain as well as Snf4 and deletion analysis demonstrated that residues 392-495 were sufficient to bind Snf4 whereas residues 392-518 were required for interaction with the kinase domain. The current model for Snf1 regulation is that Snf4 activates the enzyme by binding to the AIS thus relieving inhibition of the kinase (56). In the crystal structure of the *S. cerevisiae* heterotrimeric core, residues 460-495 of Snf1 forms an extensive interaction with the second Bateman domain of Snf4 confirming that Snf1 and Snf4 interact directly (57). Mutation of Leu-470 within the AIS to serine renders the kinase independent of Snf4 highlighting the importance of this region in regulating enzyme activity (56). Unfortunately, the fragment corresponding to the AIS in the *S. cerevisiae* structure does not align with the sequence defined as autoinhibitory in AMPK therefore the position of the AIS in the mammalian kinase may differ. Two point mutants within the kinase domain of Snf1 have been identified that render the kinase independent of Snf4 (56). According to the Snf1 KD structure, one of these mutants (Gly53Arg) is located on the small amino-terminal lobe on a beta-hairpin turn while the other (Leu183Ile) is in the active site on the catalytic loop. The simplest interpretation of these findings is that the mutants prevent the AIS from inhibiting the kinase, therefore by-passing the requirement for Snf4 for activation, however, another possibility is that the mutants prevent a direct interaction between the kinase

domain and Snf4. Gly-53 is conserved in both mammalian alpha subunit isoforms (Gly-14) while valine is substituted in the position equivalent to Leu-183 (Val-145). Further studies will be required to elucidate how these mutants affect Snf4 interaction, however these findings demonstrate, at least for the SNF1 kinase, the complex interplay between the kinase domain, the AIS and the Bateman domains of Snf4.

5. BETA SUBUNIT

The beta subunits play a key role in complex formation and are also important in mediating subcellular localisation of AMPK (20, 58). Both mammalian isoforms have N-terminal sequences of unknown function, however the beta-1 isoform is myristylated at the Gly-2 position (59). A Gly2Ala mutant that is unmodified has a four-fold increase in basal activity, suggesting that myristylation plays a direct role in regulating AMPK complexes containing the beta-1 subunit. Furthermore, complexes harbouring the Gly2Ala show a marked relocalisation from an extranuclear to a more homogenous cell distribution, however it has yet to be established whether the myristyl group mediates binding of AMPK to lipid membranes (58). The beta subunits are also autophosphorylated at multiple sites however, with the exception of Ser-108, no function has been ascribed to the other sites (58, 59).

5.1. Carbohydrate-binding module

The beta subunits have an internal carbohydrate-binding module 48 (CBM) that is related to domains found in enzymes involved in glycogen and starch metabolism (19, 21). These domains are non-catalytic and are presumed to localise the enzymes in which they are found close to their substrates. The beta subunit CBM mediates the association of AMPK with glycogen in intact cells, although glycogen has no direct effect on AMPK activity *in vitro*. The crystal structure of the beta-1 CBM from rat has been solved in complex with the cyclic sugar beta-cyclodextrin, a molecule previously used to identify glycogen and starch binding domains in other protein structures (60). In the beta-1 CBM domain structure, five of the seven glucose units of beta-cyclodextrin are in close association with the sugar binding pocket, notably the aromatic side-chains of Trp-100 and Trp-133 forming hydrophobic stacking interactions with three sugars of beta-cyclodextrin. The sugar ring is also held in position by a beta-hairpin structure, which protrudes the ring with Leu-146 at its centre and forms hydrophobic interactions with a number of the glucose units. Although Leu-146 is prominent in the beta-hairpin structure and interacts extensively with beta-cyclodextrin, it is not essential for glycogen binding and perhaps reflects the fact that beta-cyclodextrin is not the natural ligand for the CBM. The nature of the carbohydrate-binding site has also been studied by nuclear magnetic resonance using various linear and branched oligosaccharides. These studies revealed that the CBM has a preference for oligosaccharides longer than five glucose units connected through an alpha1-4 linkage with a single glucose sugar in an alpha1-6 branch (61). Oligosaccharides of this type are more commonly found in glycogen particles undergoing degradation suggesting that

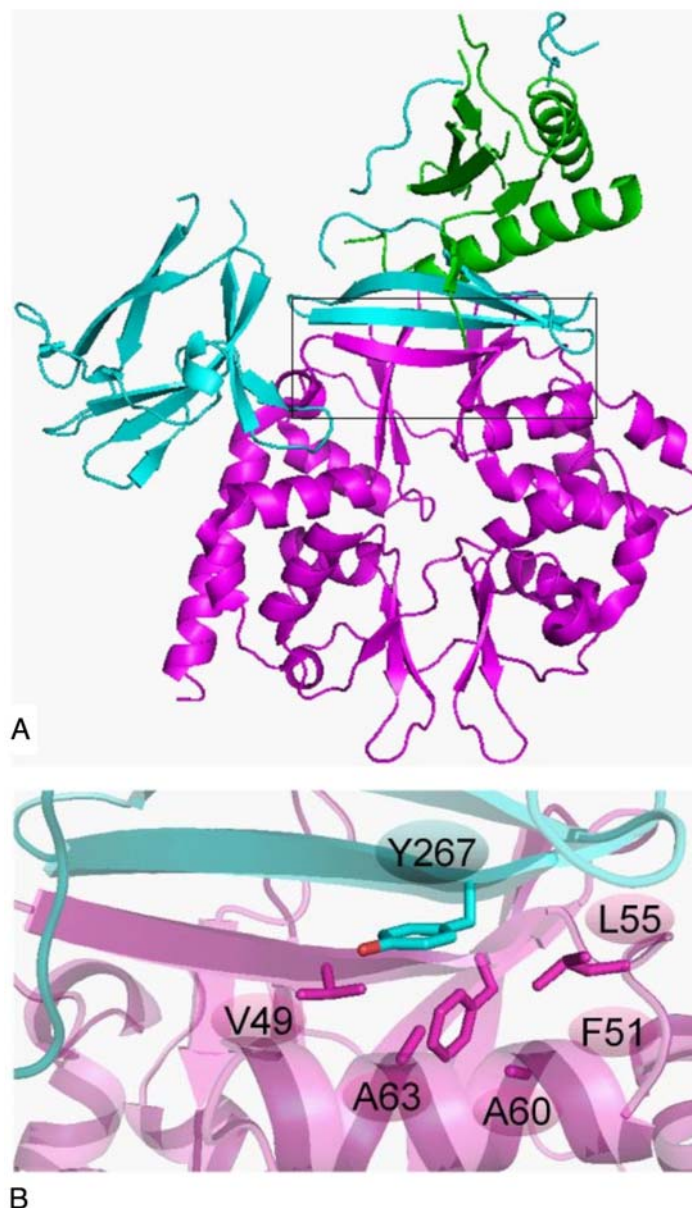


Figure 3. (A) Representation of the regulatory fragment of the *S. cerevisiae* AMPK orthologue. C-terminus of the Snf1 subunit in green, β (Sip2) subunit structures (CBM and SBS) in cyan, Snf4 subunit structure in magenta. (B) Magnified view of the beta subunit SBS with important residues highlighted.

depleting glycogen levels may act as a recruiting signal for AMPK. Another study showed that there is no association between AMPK and mature alpha-glycogen particles purified from rat liver, which was based on the observation that AMPK was absent in a highly purified alpha-glycogen preparation despite the presence of other glycogen-binding proteins such as glycogen phosphorylase and glycogen debranching enzyme (62). This is not entirely surprising as the CBM binds oligosaccharides with dissociation constants in the millimolar range therefore AMPK would be expected to readily dissociate from glycogen (61). Indeed, there may not be a necessity for AMPK to have a tight interaction with glycogen as it is involved in many

other aspects of energy metabolism and may only be required to bind glycogen under particular conditions. In the *S. cerevisiae* regulatory fragment structure the CBM associates closely with the gamma subunit, particularly the N-terminal region and the second CBS motif, and this architecture is likely to be common to other AMPK complexes (Figure 3A) (57). Although no oligosaccharide is present in the *S. cerevisiae* structure, the glycogen-binding site is exposed to the solvent and virtual docking with beta-cyclodextrin shows that there are no apparent obstructions that would prevent glycogen-binding in this conformation. Despite a detailed understanding of the association between AMPK and glycogen at the molecular

level, the functional significance of this relationship remains poorly understood. There is evidence that high cellular glycogen in skeletal muscle represses activation of AMPK however it is not known whether this effect is dependent on the CBM (63). Studies in *S. cerevisiae* with Gal83 raise the possibility that the CBM regulates SNF1 kinase function by binding an unidentified molecule perhaps related to glycogen (64). The evidence supporting this concept is that mutation of Gal83 at residues important for binding glycogen in AMPK alters Snf1/Gal83 kinase activity *in vivo* by a mechanism that is independent of glycogen binding. It is proposed that mutation of Gal83 perhaps reduces or abolishes binding of an unknown molecule, thereby providing an explanation for the fact that Snf1/Gal83 kinase activity of these mutants is unaltered *in vitro*, as the molecule likely dissociates from the kinase during purification.

5.2. Alpha/Gamma subunit binding sequence

The function of the C-terminal region of the beta subunits was first characterised by studies in *S. cerevisiae* which showed its importance in mediating association of the SNF1 complex, which gave rise to the acronym ASC domain (Association with SNF1 kinase Complex). Subsequent studies with mammalian AMPK defined the minimal region required for complex formation and demonstrated that the conserved C-terminus of the beta subunit serves as an anchoring point for the alpha and gamma subunits and is termed the subunit-binding sequence (SBS) (20). This model was recently challenged by a study suggesting that there is no direct interaction between beta and gamma subunits and that the C-terminus of the alpha subunit is responsible for scaffolding the other subunits (65). There is also evidence from yeast two-hybrid studies showing a direct interaction between alpha-2 and gamma-1 isoforms in the absence of beta but only in low glucose conditions, suggesting that the observed interaction between alpha and gamma is transient (66). A more recent study demonstrated that a C-terminal fragment of the beta-1 subunit forms a stable interaction with the gamma-1 subunit in the absence of alpha (67). Furthermore, the crystal structures of the various heterotrimeric core fragments also support the view that the beta subunit SBS is responsible for tethering the alpha and gamma subunits (57, 68, 69). In all three structures, the beta subunit C-terminal sequence adopts an anti-parallel beta-sheet configuration with the first beta-strand at the N-terminus of the gamma subunit (Figure 3B). Mutational analysis showed that Thr-263 and Tyr-267 in this region of the beta-1 subunit play a critical role in binding the gamma subunit, but not the alpha subunit (67). Furthermore, deletion studies of the gamma subunit have confirmed that the N-terminal region preceding the first Bateman domain is important for interaction with the beta subunit (70). The C-terminus of the alpha subunit forms a well-ordered structure comprising four beta-sheets in an anti-parallel conformation and two alpha-helices at its core. The beta subunit region preceding the extreme C-terminus forms an extensive interaction across the alpha subunit C-terminal core, however there is very little interaction between the alpha and gamma subunits. These interactions are conserved in all three trimeric core structures and provide

strong evidence that the C-terminus of the beta subunit binds both alpha and gamma subunits, and that a stable interaction between alpha and gamma is unlikely in the absence of beta.

6. GAMMA SUBUNIT

The presence of a particular gamma subunit isoform in the AMPK complex was found to effect its dependence on AMP and these findings provided the first clues that the allosteric binding sites are located on the gamma subunit (22). The gamma subunits may also be involved in targeting AMPK to specific subcellular compartments as both gamma-2 and gamma-3 isoforms have divergent N-terminal extensions (71, 72). Recently, a large scale analysis of phosphoproteins from mouse liver revealed that the N-terminal extension of the gamma-2 subunit is phosphorylated at a number of sites *in vivo*, suggesting that AMPK complexes containing the gamma-2 subunit may be specifically regulated by other signalling pathways (73).

6.1. AMP and ATP Binding Sites

All three heterotrimeric core fragment structures contain complete gamma subunits in complex with various nucleotides and together provide a striking insight into the nature of nucleotide binding. The Bateman domains are arranged in a head-to-head manner, resembling a flattened disk with a solvent-accessible channel through the centre (Figure 4A). The nucleotide binding sites are located in a cleft formed at the interface between the CBS motifs and the symmetry is such that there are four potential binding sites, two within each Bateman domain. In the mammalian structure, AMP is bound at three of these sites (69) whereas only one site is occupied in the *S. pombe* (68) and none in the *S. cerevisiae* structure (57). Previous biochemical studies showed that the mammalian gamma subunits bind only two molecules of AMP, although further studies demonstrated that of the four potential sites only two readily exchange with AMP and ATP and are responsible for the energy-sensing properties of AMPK (23, 69). The third AMP molecule present in the mammalian structure is tightly bound and does not exchange with free AMP or ATP. Interestingly, the single molecule of AMP or ATP bound in the *S. pombe* structures occupies the site equivalent to the non-exchangeable site in the mammalian gamma subunit. As no biochemical studies have been carried out on the *S. pombe* kinase, it remains to be determined whether nucleotide binding regulates enzyme activity. There are a number of contacts common to each binding site that feature in both the mammalian and *S. pombe* structures. The adenine ring in each site occupies a hydrophobic pocket forming hydrogen bonds with main-chain groups from nearby beta-strands. The 2' and 3' hydroxyl groups of the ribose sugar are held in position by an aspartate residue that is conserved in all sites containing bound nucleotide. The fourth potential site in the mammalian structure is unoccupied due to the presence of an arginine residue in the position normally occupied by an aspartate residue (69). Intriguingly, this fourth site is occupied with ADP in the most recent *S. pombe* structure and surprisingly the 2' and 3' hydroxyl groups of the ribose

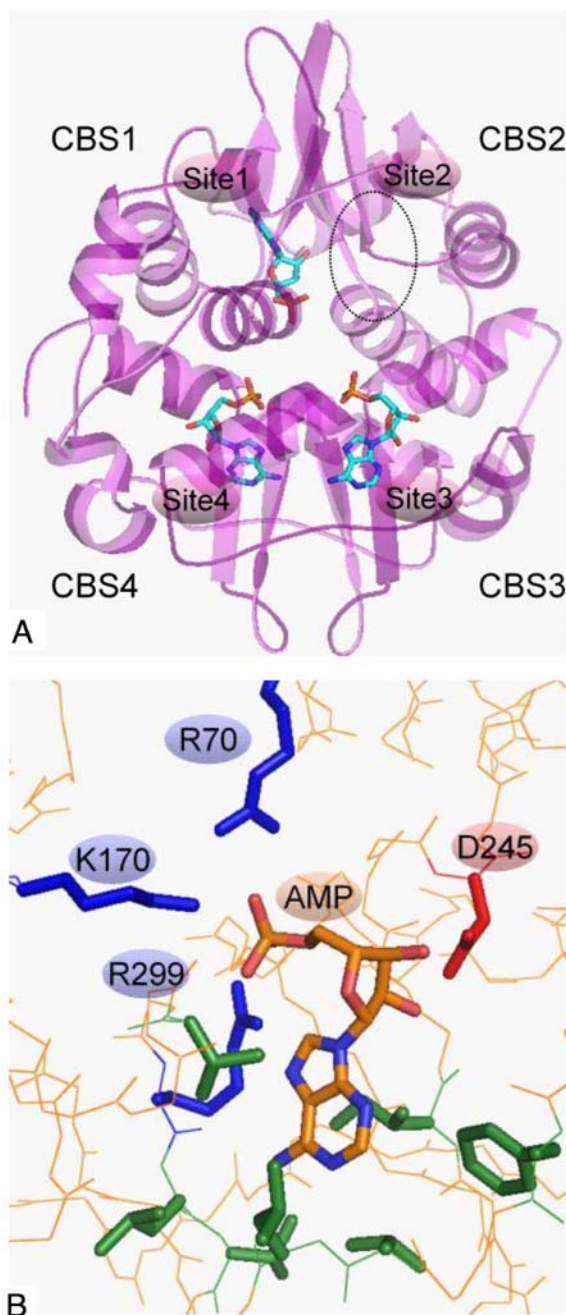


Figure 4. (A) Crystal structure of the gamma-1 subunit from mammalian AMPK showing the orientation of the CBS motifs and AMP-binding sites. The location of a putative fourth site that binds ADP in the *S. pombe* structure is circled. (B) Detailed views of AMP-binding site 1, showing the network of basic residues involved in binding the phosphate groups.

are held in position by Asp-250 from the beta subunit (74). There is an aspartic acid in the corresponding position in the sequence of the beta subunit in mammals raising the intriguing possibility that this site may become occupied by a similar mechanism under certain conditions. The negatively charged phosphate groups from the bound nucleotides are neutralised by a cluster of positively charged residues (Arg-69, His-150, Arg-151, Lys-169, His-297, Arg-299) most of which are located at the mouth of

the AMP-binding pocket (Figure 4B). Interestingly, the residues corresponding to Arg-69 and His-150 are not conserved in *S. cerevisiae* and could potentially explain why the SNF1 complex is not activated by AMP (57).

There are a number of point mutations in the human gamma-2 subunit isoform that are associated with a glycogen storage cardiomyopathy and ventricular pre-excitation (75-78), and nine of the ten residues substituted

are in close proximity to the AMP/ATP binding sites. Six of these amino acids are directly involved in nucleotide binding, including four basic residues (equivalent to Arg-69, His-150, Arg-151, Arg-298 in gamma-1) that co-ordinate binding of the nucleotide phosphate groups (69). The gamma-2 mutations effect both binding and activation by AMP (23, 79). The mutations also increase the basal activity of AMPK by increasing Thr-172 phosphorylation, which likely arises because binding of the inhibitory nucleotide ATP is also reduced, which would explain the dominant nature of the mutations (80, 81).

6.2. Mechanism of AMP activation

The mechanism of how AMP transduces its activating signal to the alpha subunit kinase domain is poorly understood, however a number of models have been proposed based on information provided by the gamma subunit structures. The absence of any significant change in the AMP and ATP bound structures has led to suggestions that the activating signal possibly involves differences in charge and interaction with residues from the other subunits, possibly a phosphorylated residue, rather than conformational shifts in the structure (68, 69). Indeed, this idea is supported by affinity labelling studies using the reactive ATP analogue, fluorosulphonylbenzoyl adenosine (FSBA), which contains a reactive group in the position equivalent to the terminal γ phosphate of ATP (82). FSBA was shown to label the alpha subunit of AMPK at the kinase domain and at a second site, however only labelling of the second site was prevented by AMP. This indicates that FSBA bound at the allosteric site on the gamma subunit interacts with the alpha subunit, raising the possibility that the γ phosphate of ATP also makes a similar contact when bound at the allosteric site. Activation of AMPK by AMP is also influenced by autophosphorylation of Ser-108 on the beta-1 subunit, as mutation of this residue to alanine increased the activation constant for AMP (58). However, it is unlikely that phosphoserine-108 interacts directly with the AMP/ATP binding sites as, according to the SNF1 core structure, the residue occupying the position equivalent to Ser-108 is located some distance from the gamma subunit (57).

There is conflicting evidence regarding the binding affinities of AMP and ATP as one study found that both nucleotides bind with similar affinities (24 μ M and 12 μ M, respectively) (69) whereas an earlier study showed that AMP binds with an affinity at least six-fold higher than ATP (20 μ M and 120 μ M, respectively) (23). If AMP and ATP do bind with similar affinities, then this would suggest that only a small proportion of AMPK in the cell becomes activated because the prevailing ATP concentration is considerably higher than that of AMP. A key prediction of this model is that low concentrations of ATP would inhibit AMPK activity, however inhibition is only observed at millimolar concentrations close to the physiological range for ATP (37). Importantly, both studies found that binding of Mg^{2+} ions to ATP is not required for binding to the gamma subunit, although in the mammalian structure it is clearly evident that the Mg^{2+} ions are still complexed with ATP and are not stripped prior to binding as is the case for ATP bound in the *S. pombe* structures.

Another model to explain activation by AMP has been proposed based on the observation that a number of ligand-regulated protein kinases are autoinhibited by sequences that mimic sites phosphorylated on target proteins, except that they have non-phosphorylatable residues in the position normally occupied by a phosphoacceptor (83). These sequences are known as pseudosubstrates and act as competitive inhibitors, blocking access of protein substrates to the active site in the absence of activating ligand. Pseudosubstrate sequences are usually found adjacent to or overlapping with regulatory ligand-binding sites. Recently, a pseudosubstrate sequence was identified within the second CBS motif in the first Bateman domain of all eukaryotic gamma subunits (84). Positively charged residues within the pseudosubstrate predicted to be involved in interacting with the substrate-binding site on the kinase domain are critical in binding the phosphates of AMP (69, 84). Since these two interactions would be unlikely to occur simultaneously, this suggests a simple mechanism whereby in the absence of AMP, the pseudosubstrate would occupy the substrate-binding site on the kinase domain inhibiting kinase activity. This model is also supported by kinetic observations made using ligand-regulated peptide aptamers isolated from a peptide library screen. Recently, a series of peptide aptamers were characterised that inhibit AMPK by competing for the peptide substrate-binding site (85). Inhibition of AMPK by these peptides reduced the AMP stimulation of the enzyme and caused the inhibited state of the kinase in the presence of AMP to kinetically resemble the basal unstimulated state, which is consistent with pseudosubstrate inhibition. Despite kinetic evidence in favour of the pseudosubstrate model, the current gamma subunit structures show that much of the pseudosubstrate sequence is rigidly bound so it is not yet evident how AMP binding could induce the structural rearrangements predicted by this hypothesis. However, it is almost certain that in the intact structure there will be unforeseen steric influences and post-translational modifications that will impact on the flexibility of the gamma subunit and further structures will be required to reveal the exact mechanism of allosteric regulation of AMPK.

7. AMPK AS A DRUG TARGET

The pivotal role AMPK plays in switching metabolism in favour of glucose and lipid oxidation as opposed to storage and synthesis has stimulated considerable interest in AMPK as a target for treatment of metabolic disorders, particularly Type 2 diabetes and obesity. Several existing drugs have been shown to activate AMPK *in vivo* including metformin, AICAR, rosiglitazone and the newly discovered thienopyridone A-769662 (86, 87). With the exception of A-769662, all the other drugs activate AMPK by indirect mechanisms. Both metformin and rosiglitazone are respiratory chain uncouplers that activate AMPK by inhibiting ATP production (88, 89). AICAR on the other hand is converted in the cell to the AMP-mimetic ZMP by adenosine kinase (36). Although ZMP mimics all the effects of AMP on the AMPK system, it also effects other AMP-sensitive enzymes such as fructose-1,6-bisphosphatase and glycogen

phosphorylase, which likely explains some of its undesirable side effects. Nevertheless, administration of AICAR to various animal models of insulin resistance caused marked improvements in the metabolic abnormalities collectively termed the 'deadly quartet', such as lower circulating triglycerides, decreased blood pressure, enhanced glucose tolerance and insulin sensitivity and decreased abdominal fat (90-93). These findings highlight the benefit of pharmacologically activating AMPK and suggest that models of the AMP-binding domains complexed with various activating ligands may have some utility in driving structure-based design of new AMPK-activators. Crystal structures of the gamma subunit from *S. pombe* and a fragment containing the second Bateman domain from human gamma-1 have been solved in complex with ZMP (74, 94). Both structures show that ZMP binds in a similar manner to AMP but with a few notable differences. In the gamma-1 Bateman domain fragment, the open ring of ZMP prevents it from occupying a deeper position in the binding pocket than the adenine ring of AMP. This conformation allows an additional water molecule to enter the pocket and weaken the interaction between the backbone amide of Ala-227 and the N3 nitrogen of ZMP, potentially explaining the lower affinity of ZMP for AMPK. In both structures ZMP occupies the site equivalent to the non-exchangeable AMP site observed in the mammalian gamma subunit structure so it remains to be seen how ZMP interacts with the energy-sensing exchangeable AMP/ATP binding sites. Recently, compounds belonging to the thienopyridone family were identified as a new class of AMPK activator. The optimised compound A-769662 mimics both effects of AMP on the AMPK system but does not compete for the AMP/ATP-binding sites on the gamma subunit, strongly suggesting the presence of an alternate allosteric site (95, 96). Unlike ZMP, A-769662 has no effect on fructose-1,6-bisphosphatase or glycogen phosphorylase therefore activating AMPK by targeting drugs to the A-769662 binding site may offer a higher degree of specificity compared with the AMP-binding sites.

8. PERSPECTIVES

Regulation of AMPK is exquisitely complex and with the growing number of crystal structures the mechanisms controlling this important energy sensor are beginning to unfold, however many key questions remain unanswered. It is not yet evident how AMP activates AMPK while ATP inhibits it, as there is no obvious change in conformation between AMP and ATP bound structures. Furthermore, unravelling the mechanism of how AMP regulates phosphorylation of Thr-172 is also an important task and will undoubtedly require extended heterotrimeric structures that include the kinase domain. It is now clear that there are four nucleotide binding sites on the gamma subunit, however, as only two of the sites are involved in energy-sensing, do the remaining sites play a role in regulating AMPK? An intriguing possibility is that other metabolic intermediates can regulate AMPK, an idea that is supported by observations from the *S. pombe* structure that ADP is able to occupy the pocket equivalent to the unoccupied site in the mammalian structure. Future studies

should revisit the role of ADP and biochemical characterisation of the *S. pombe* kinase ought to be a priority if further conclusions are to be drawn from structural studies of this enzyme. A further twist is the discovery that the thienopyridone A-769662 mimics the effects of AMP by a mechanism that is independent of the AMP-binding sites. Identification of this novel allosteric site may reveal a promising drug target and allow drugs with increased specificity towards AMPK to be developed.

Sir William Henry Bragg, Nobel laureate and pioneer of X-ray crystallography once remarked '*the important thing in science is not so much to obtain new facts as to discover new ways of thinking about them*'. Such is the complexity of AMPK regulation, these words of wisdom are worth keeping in mind.

9. ACKNOWLEDGEMENTS

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AMPK structure and function

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Abbreviations: AMPK: AMP-activated protein kinase, SNF1: sucrose non-fermenting 1, ATP: adenosine triphosphate, AMP: adenosine monophosphate, AIS: autoinhibitory sequence, CBM: carbohydrate-binding module, SBS: subunit-binding sequence, CaMKK: Ca²⁺/calmodulin dependent protein kinase kinase, TAK1: transforming growth factor beta-activated protein kinase, UBA: ubiquitin-associated domain, CBS: cystathionine-beta-synthase, MARK2: microtubule-associated regulating kinase 2, AICAR: 5-aminoimidazole-4-carboxamide ribotide.

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Send correspondence to: John W Scott, St Vincents Institute and Dept of Medicine, University of Melbourne, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia, Tel: 61-39288-2480, Fax: 61-39416-2676, E-mail: jscott@svi.edu.au

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