Supplementary Information

Structure of Mammalian AMPK and its regulation by ADP
Supplementary Figure 1. Model for the regulation of AMPK activity. The left hand panel represents the active form of AMPK seen in our crystal structure and shows the regulatory fragment (R) and the kinase domain (K), including the phosphorylated (P) activation loop coloured in pink, connected by the linker region that contains the α-hook. The regulatory nucleotide binding pocket, site-3, is shown occupied by ADP (Ade-P-P) with the position that would be occupied by a γ-phosphate as an empty grey circle. Exchange of ADP or AMP by Mg.ATP (Ade-P-P-P) leads to a steric clash with the α-hook that leads to the dissociation of the hook and an increase in the flexibility of the linker that promotes the dissociation of the kinase domain. In this form the activation loop is no longer protected by packing against the regulatory domain and is accessible to attach by phosphatases (PP’ase), as shown in the right hand panel where the activation loop has been dephosphorylated and the kinase is inactive.
ADP protects against dephosphorylation of rat liver AMPK by endogenous phosphatases. AMPK was partially purified from rat liver (Sanders et al. 2007, Biochem J) and incubated for 10 min at 37°C in the presence or absence of MgCl₂ (2.5 mM) and in the presence or absence of varying concentrations of AMP or ADP. T172 phosphorylation was determined by Western blotting and total α subunit expression by blotting with a mixture of anti-α₁ and anti-α₂ antibodies. A representative blot is shown and similar results were obtained from 4 independent experiments.
Supplementary Figure 2b

We have previously shown that mutations in the γ1 subunit, corresponding to mutations identified in human γ2 that cause hypertrophic cardiomyopathy associated with Wolff-Parkinson-White syndrome (Arad et al., 2007, Circ Res), ablate the protective effect of AMP against dephosphorylation of Thr-172 (Sanders et al., 2007, Biochem J). From our previous structure we were able to show that most of these mutations affect residues involved in the regulatory adenine nucleotide binding sites. We have now extended this work by looking at the effect of one of the WPW mutations (R298G, equivalent to R531G in γ2 (Daniel et al., 2002, J Biol Chem)) on the protective effect of ADP on dephosphorylation. Our data show that the mutation has the same effect of negating protection against dephosphorylation by ADP as it does with AMP. Recent work also suggests a mechanism for the enhanced basal activity of this mutation (Oakhill et al., 2010, Pro Natl Acad Sci USA).

ADP protection of dephosphorylation is ablated by a Wolff-Parkinson-White syndrome mutation. Wild type AMPK or AMPK harbouring a point mutation in the γ subunit (R298G) were phosphorylated with CaMKKβ and incubated in the presence or absence of PP2C with or without either AMP (100 μM) or ADP (100 μM). A representative blot showing Thr-172 phosphorylation and total α subunit levels is shown. Similar results were obtained from 3 independent experiments.
Supplementary Figure 3. The structure of coumarin-ADP bound to AMPK.

Ribbons representation of the structure of the regulatory fragment of AMPK with ADP (white sticks) and c-ADP (yellow sticks) bound. (a) and (b) show a front and back view of the regulatory fragment from the c-ADP/AMPK complex with the two ADP moieties taken from the ADP/AMPK complex. The c-ADP complex has a permanently bound AMP at site-4 and two exchangeable c-ADPs (yellow sticks). (c) shows an expanded view of the tighter exchangeable site-1, and (d) shows the weaker site-3. For (c) and (d) the electron density is from an Fo-Fc omit map. The crystallography statistics of ADP/AMPK and c-ADP/AMPK complex are presented below (Supplementary Table 1).
### Supplementary Table 1. Crystallographic statistics for truncated AMPK complexes with c-ADP and ADP

|                              | 2ADP/AMPK complex | c-ADP/AMPK complex |
|------------------------------|-------------------|--------------------|
| **Data Collection**          |                   |                    |
| Space group                  | P2_12_2_1         | P2_12_2_1          |
| Unit cell (Å)                | \( a=48.57, \ b=121.46, c=125.92 \) | \( a=48.94, \ b=124.06, c=125.32 \) |
| Resolution (Å) (high res)    | 20-2.5 Å (2.6-2.5 Å) | 30-2.5 Å (2.6-2.5 Å) |
| Rsym %                       | 5.6 (30.8)        | 6.3 (25.6)         |
| Completeness                 | 98.0 (85.8)       | 99.2 (99.9)        |
| Redundancy                   | 5.9 (5.1)         | 3.6 (3.7)          |
| **Refinement**               |                   |                    |
| Number of reflections (work/free) | 24672/1314  | 25630/1386          |
| \( R_{\text{fac}} \) % (work/free) | 22.8/25.5       | 23.3/27.7          |
| Rms bonds (Å) /angles (°)    | 0.01/1.42         | 0.008/1.30         |
| PDB entry code               | 1XYZ              | 2XYZ               |

Values in parentheses refer to the highest resolution shell.

\[ R_{\text{sym}} = \frac{\sum | <I> - I_j |}{\sum | <I> |} \] where \( I_j \) is the intensity of the jth reflection and \( <I> \) is the average intensity.

\[ R_{\text{work}} = \frac{\sum | |F_o| - |F_c| |}{\sum |F_o|} \]  

\[ R_{\text{free}} = \frac{\Sigma_T | |F_o| - |F_c| |}{\Sigma_T |F_o|} \] where T is a test data set of 5% of the total reflections randomly chosen and set aside before refinement.
**Supplementary Figure 4a and b**

**Effect of NADH on protection against dephosphorylation.** Recombinant AMPK was phosphorylated with CaMKKβ and incubated for 20 min at 37°C with or without PP2C and in the absence or presence of either 0.5 mM or 1 mM NADH (a) or in the presence of varying concentrations of AMP in the presence or absence of 1 mM NADH (b). pT172 and total α1 subunit levels were determined by Western blot analysis and a representative blot is shown in each case. Similar results were obtained from at least 3 independent experiments.
Supplementary Figure 4c and d

**Effect of NADH and ADP on allosteric effect of AMP.** (c) Recombinant AMPK (α1β1γ1) was activated by phosphorylation with CaMKKβ and assayed in the presence or absence of AMP (10 μM) and in the presence or absence of NADH (800 μM). Results are plotted as fold-activation and are the average ± S.E.M. for 3 independent experiments. (d) Recombinant AMPK (α1β1γ1) was activated by phosphorylation with CaMKKβ and assayed in the presence or absence of AMP (100 μM) and increasing concentrations of ADP (as indicated on the figure). Results are plotted as fold-activation and are the average ± S.E.M. for 3 independent experiments.
Identification of the tight exchangeable AXP binding site. A 2.8 Å omit electron density map covering the whole of the AMPK regulatory fragment contoured at 3.0 σ. The map was calculated from phases calculated from a model refined without including any AXP moieties bound to the regulatory fragment. The omit map shows clear electron density for the non-exchangeable AMP-4 and one ADP located only at site-1. The crystals were grown under the previous conditions but the protein was incubated with just one molar equivalent of ADP prior to crystallization. The crystallography statistics of one molar ADP/AMPK complex are presented below (Supplementary Table 2).
**Supplementary Table 2. Crystallographic statistics for truncated AMPK complex with 1 ADP.**

| Data Collection | 1ADP/AMPK complex |
|-----------------|-------------------|
| Space group     | P2₁2₁2₁           |
| Unit cell (Å)   | a=49.03, b=119.92, c=130.15 |
| Resolution (Å) (high res) | 20-2.8 Å (2.9/2.8 Å) |
| Rsym %          | 4.4 (35.8)        |
| Completeness    | 93.8 (93.5)       |
| Redundancy      | 2.7 (2.6)         |

| Refinement       |                   |
|------------------|-------------------|
| Number of reflections (work/free) | 17426/952 |
| R_fσc % (work/free)   | 23.2/27.5 |
| Rms bonds (Å)/angles(°) | 0.01/1.64 |
| PDB entry code     | 3XYZ             |

Values in parentheses refer to the highest resolution shell.

\[ R_{\text{sym}} = \frac{\sum |I_j| <I> - I_j}{\sum <I>} \] where \( I_j \) is the intensity of the jth reflection and \( <I> \) is the average intensity.

\[ R_{\text{work}} = \frac{\sum |Fo| - |Fc|}{\sum |Fo|} \]

\[ R_{\text{free}} = \frac{\sum_T |Fo| - |Fc|}{\sum_{T} |Fo|} \], where \( T \) is a test data set of 5% of the total reflections randomly chosen and set aside before refinement.
Design of the construct used for determining the crystal structure of an active form of AMPK. One of the steps towards diffraction quality crystals involved engineering in a pair of protease recognition sites at both ends of a large flexible loop in the C-terminal domain of α, thus removing residues 471 – 523 (Fig. 3a). We had previously observed that this loop was disordered in our crystal structure of the regulatory fragment of AMPK and reasoned that being able to excise this loop, by specific protease treatment after the protein has been expressed and purified, might enhance crystallization as observed in other systems (Xiao et al., 2003 Proc Natl Acad Sci). We applied the same approach to the flexible loop that connects the glycogen-binding domain (GBD) of the β subunit (Hudson et al, 2003, Curr Biol; Polekhina et al., 2003 Cur Biol) to the rest of the complex. In this case, however, following protease treatment the GBD, unlike the C-terminal domain of the α subunit, separates from the rest of the complex on gel filtration. We therefore proceeded with crystallization studies using a truncated β construct (187-end). Other necessary steps for crystallization included phosphorylation of the AMPK complex by CaMKKβ and incubation with the kinase inhibitor staurosporine. Although the dataset is at medium resolution, the molecular replacement solution was robust and yielded initial electron density which revealed the location of many components that were not present in the original model. Moreover, refinement proceeded smoothly, interspersed with cycles of manual rebuilding, to yield a final model with acceptable crystallographic and stereochemical parameters (Supplementary Table 3). Our final model accounts for all of the polypeptide chain present in the crystallization construct except for residues 300-330 of α1, 187-197 at the N-terminus of β2 and the first 22 residues of γ, which are too disordered to be built. Additionally, residues α1 (283-304, 329-367) and β2 (221-226) have been modeled as alanine.
Mass spectrometry analysis of the crystallized AMPK construct. Comparing the mass spectra of the non-phosphorylated (a) and the phosphorylated (b) AMPK α1(1-470, 524-548)β2(187-272)γ1(1-330) complex, a mass shift of 80 Da is evident for the β2 subunit (observed mass = 10026.3 Da, theoretical mass = 10026.8Da) corresponding to the addition of one phosphate moiety (observed mass = 10106.1 Da, theoretical mass = 10106.8 Da). We have mapped this phosphorylation site to β2 Ser198. In addition, there is a mass shift of 240 Da for the major α1 subunit (observed mass = 55268.6 Da, theoretical mass = 55269.6 Da, calculated using the sequence of α1(GP-1-470-LEVLFQ) (GP from cleaved His tag, LEVLFQ from engineered 3C protease site)) corresponding to the addition of three phosphate moieties (observed mass = 55509.1 Da, theoretical mass = 55509.6 Da). We have mapped two phosphorylation sites to α1 Thr172 and Thr377, however, we did not identify the third phosphorylated residue. γ1 was not phosphorylated as shown in shown in (b).
Supplementary Table 3. Crystallographic statistics for active AMPK complex.

| AMPK-staurosporine-complex |
|-----------------------------|
| **Data Collection**         |
| Space group                 | P4₁2₁2 |
| Unit cell (Å)               | a=133.92, b=133.92, c=141.90 |
| Resolution (Å) (high res)   | 20-3.24 Å (3.44-3.24 Å) |
| Rsym %                      | 6.6 (50.6) |
| Completeness                | 93.3 (95.8) |
| Redundancy                  | 4.5 (4.5) |

| **Refinement**              |
| Number of reflections (work/free) | 18578/988 |
| R_{fac} % (work/free)         | 23.5/29.3 |
| Rms bonds/angles(°)          | 0.010/1.56 |
| PDB entry code               | 4XYZ |

Values in parentheses refer to the highest resolution shell.

\[ R_{sym} = \Sigma j |<I> - I_j|/\Sigma |<I>| \] where \(I_j\) is the intensity of the jth reflection and \(<I>\) is the average intensity.

\[ R_{work} = \Sigma |Fo| - |Fc|/\Sigma|Fo|. \]

\[ R_{free} = \Sigma_T |Fo| - |Fc|/\Sigma_T|Fo|, \] where T is a test data set of 5% of the total reflections randomly chosen and set aside before refinement.
The figure shows a stereo pair of a portion of an Fo-Fc omit electron density map covering the activation loop (residues 157 to 183) of the kinase domain taken from the active kinase construct. The position of the phosphorylated Thr-172 residue is indicated. The map is contoured at 2.5σ. The conformation of the loop around the phosphorylated Thr-172 enables the phosphorylated side chain to bind into a pocket created by the tandem Arg-Asp residues from the kinase as seen in other protein kinase structures.
The figure shows a stereo pair of a portion of an Fo-Fc omit electron density map covering the α-hook of the α subunit taken from the active AMPK structure. The view shows the main chain of the loop (residues 366 to 386) in blue. The map is contoured at 2σ. In the middle of the hook structure Thr-377 (shown in stick representation) is seen to be phosphorylated. We think it is an autophosphorylation site, but its mutation has no effect (data not shown) and we therefore conclude that phosphorylation at this site is not important for regulation.
**Supplementary Figure 8.** (a) The stereo view for Figure 3b from the main manuscript, the dotted line between $\alpha$-299 and $\alpha$-331 represent the disordered part of the linker region. The left and middle panels are displayed as wall-eye stereo, and the middle and right panels displayed as cross-eye stereo. (b) The reverse view of (a) with the ends of the two 3C protease sites joined by a black dashed line ($\alpha$-469 and $\alpha$-524).
Supplementary Figure 9

**Space filling representation of the kinase domain** from (a) the isolated, and inactive, human α2 structure (2H6D) and (b) the active kinase domain from our current structure. The small lobe (ca. residues 10 to 100) and large lobe (ca. residues 101 to 278) of the kinase domain are coloured in pale-yellow and yellow respectively. (a) Only part of the activation loop is ordered (residues 157 to 166 and 180 to 183) in the inactive kinase and this is shown in green. (b) The whole of the activation loop is ordered in our structure and is shown in violet, while the superposed activation loop from the inactive kinase is shown in green to show how the more closed conformation of the kinase in our structure would result in steric overlap between the small lobe of the kinase and the superposed loop from the inactive kinase.
Supplementary Figure 10. Sequence conservation in the α-hook region

The electron density for the α-linker is largely continuous over the main chain but has poor definition for many of the side chains. However, in the middle of the α-hook region there is a strong side chain feature that is present in all the datasets we have looked at, which persists at high contour levels. We know that Thr-377 is phosphorylated in our sample and that it is the only residue in this region that is. If we build the α-hook region placing P-Thr-377 into this density feature, the P-Thr is oriented away from the γ subunit, but six flanking residues mediate the interface with the γ subunit. Of these six, four are conserved between α1 and α2 isoforms in all vertebrates (R/K-373, R-375, D-379 and N-382) and two are not (H/C-376 and E/A-380, see below). When we generate a site-directed mutant that changes two of these conserved residues (R-375 & D-379) and two non-conserved ones (T/P-377 & E/A-380) the resulting enzyme is active, and is allosterically activated by AMP, but is no longer subject to protection against dephosphorylation (Fig 4). Taken together, these data support our conclusion that the α-hook region is important in mediating protection against dephosphorylation in vertebrates. Further structural/biochemical studies will be required to understand the molecular mechanisms of AXP readout in AMPK analogues from more distant species although we note that the residues involved in the interface between the kinase domain and the regulatory subunit are highly evolutionarily conserved.

AMPK-related sequences were gathered with NCBI blastp searches using human AMPKα1 as a query and subsequently aligned using MAFT (as implemented at the EBI web site). Partial sequences were excluded and the dataset was rendered non-redundant. The retained sequences were realigned with MAFT and inspected for conservation in the region containing the α-hook.

The α-hook (residues 373-382 in human AMPKα1) resides in a region which shows a large degree of variability across species from mammals to yeasts. The region is, however, well conserved among vertebrate AMPKα1 and AMPKα2 proteins with Tetraodon (pufferfish) and other telecosts as the most extant sequences. The common motif can be expressed as: [RK]-x-R-[HC]-[TP]-L-D-[EA]-L-N (where the first and second residues in the brackets are from AMPKα1 and AMPKα2, respectively). The AMPK-like sequence of Saccoglossus kowalewski (acorn worm, a hemichordate) is the closest homologue that does not show similarity to the vertebrate α-hook sequences. To search for the presence of more divergent, but related, sequences in non-vertebrate AMPKs, we performed PHI-blast searches with the α-hook pattern above as well as several shorter and more generalised patterns including [RK]-[HC]-[TSP]-[LIV]-[DE]. No hits were found and we conclude that the α-hook is a
unique feature of vertebrate AMPKs.
Of the 10 residues in the α-hook, 5 are identical, and 1 is conserved, among the vertebrate AMPKα1 and α2. Of these 6 residues, 4 - R/K-373, R-375, D-379 and N-382 form the interface with the γ subunit. The other 2 non-conserved residues that also contribute to the interface are H/C-376 and E/A-380. On this basis it is reasonable to assume that the α-hook of AMPKα2 could make a similar interaction with the γ subunit as AMPKα1.

Multiple alignment of the α-hook region in AMPKα1 and α2. The alignment was made non-redundant so that only one instance of each different sequence is shown. At the bottom, labelled outgroup, the corresponding sequence from Saccoglossus kowalevskii (acorn worm, a hemichordate), which does not contain the conserved α-hook motif. Above the alignment is shown the extent of the α-hook motif (blue line). The black boxes mark the 4 conserved residues that interface with the γ subunit while the grey boxes mark the two non-conserved gamma-interacting residues. The consensus sequence is shown above. The alignment in colour-coded by the clustalx colouring scheme and uniprot sequence identifiers and sequence ranges are shown to the left. Note that the Tetraodon sequence (Q4SQH4) has a lysine in the position corresponding to T377.
Ribbons representation of selected components of structure close to the α-hook interacting site on the γ subunit. The figure shows the superposition of three structures. The ADP (thin sticks) and Mg.ATP (thicker sticks) complexes from our earlier crystal structure of the regulatory core of AMPK (2V9J) with the α-hook region, and associated omit electron density, from the current active AMPK complex. Comparing the ADP and Mg.ATP complexes of the regulatory fragment shows that the binding of ATP results in the displacement of Arg-69, which would cause a steric clash with the α-hook structure in the context of our active AMPK complex leading us to propose that the binding of Mg.ATP would be incompatible with the observed α-hook structure.
**Supplementary Figure 12**

**Sequence conservation in the AID segment.** Chen et al. suggest that autoinhibition by AID is universal to AMPKs, including the mammalian AMPKs. This argument is based on their alignment of AID sequences across the AMPK superfamily (Chen et al., (2009) Nature, Figure 2a) and by the effect of mutations in rat AMPKα in positions that, according to this alignment, are equivalent to the critical residues in the S. pombe AID. Pang et al. (2007) J Biol Chem and Göransson et al. (2007) J Biol Chem have also provided evidence that the corresponding region in human and rat AMPKα have an autoinhibitory effect. These findings cannot be simply interpreted structurally, however, as an AID domain (also identified as a UBA domain) cannot be identified in mammalian AMPKα (both rat and human) by unbiased bioinformatical approaches. The absence of the UBA (alias AID) domain in mammalian AMPKα1 and AMPKα2 (as well as the two NUAK kinases) has been noted before by Jaleel et al., (2006) Biochem. J. The sequence identity between human AMPKα1 and yeast Snf1p in this region is only 16% (Pang et al., (2007) J Biol Chem). We also failed to identify any statistically significant similarity between the fungal AID sequence and the corresponding region in the mammalian AMPKs using two sensitive sequence comparison methods as follows; First: PSI-Blast searches were performed with the AID sequence of spAMPK (to convergence, using blosum 45 as matrix and 15 as E-value cut off) without hits with any vertebrate AMPKs (UBA-containing MARK kinases were, however, picked up). Reciprocal PSI-Blast searches using the corresponding region (as in Chen et al. Figure 2a), similarly failed to identify the fungal AID sequences. Second: Hidden Markov Models were generated (using the HMMER 3.0 package) from representative alignments of the fungal AID sequences and the corresponding sequences of the vertebrate AMPKs. These HMMs failed to detect any significant hits across the two families of AMPKs. Hence, even these most sensitive bioinformatical methods fail to support the proposition that mammalian AMPKα1 has an evolutionarily conserved AID sequence. We do note, however, that the region of the mammalian AMPKs in question (hAMPKα1 residues 298-348) is highly conserved among chordates and is predicted (by JPred) to contain four helical segments (see Figure). Therefore, it is certainly possible that this region in mammalian AMPKα1 adopts an analogous structure where helical elements may serve a similar function. If so, the molecular mechanism is most likely different from that seen in *S. pombe* AMPK/SNF1-like kinases.
Multiple alignment of the AID region in fungal AMPKα/SNF1 and the corresponding region in metazoan AMPKα. Metazoan and fungal AMPKα sequences were identified by blastp searches (using EXPASY and NCBI servers) and separately aligned with the MAFFT algorithm as implemented in Jalview 2.5 (Waterhouse et al., 2009 Bioinformatics, and colour-coded by the built-in clustalx colour scheme. α-helices determined by crystallography are marked with red for SNF1_SCHPO (pdb:3h4j). Secondary structure predictions (Jpred; Cole et al., 2008 Nucleic Acids Res) are marked with blue (helix) and green (strand) boxes, where the colour intensity reflects prediction reliability. Uniprot...
sequence IDs and sequence ranges are shown to the left. Sequence IDs starting with 'gi' were obtained from GenPept. The human AMPKα1 and AMPKα2 are highlighted with red dots, while fission yeast and bakers yeast SNF1 are highlighted with blue dots. The regions in fungal and metazoan AMPKs suggested by Chen et al., (2009) Nature, to be homologous are indicated by lines above the alignment.

Full Methods

AMPK functional assays
Recombinant AMPK complexes (α1β1γ1) were expressed in E. coli BL21 (DE3) cells and purified by affinity chromatography using nickel-Sepharose. AMPK complexes were phosphorylated by incubation with CaMKKβ in the presence of 200 μM ATP, 2.5 mM MgCl2 and 1 mM dithiothreitol for 20 min at 37°C. For allosteric effects, AMPK activity was determined by phosphorylation of the SAMS peptide in the presence or absence of varying concentrations of nucleotide, as indicated in the appropriate figure legends. Results are plotted as fold activation relative to the activity in the absence of added nucleotide and are the mean ± S.E.M from at least 3 independent experiments. For dephosphorylation studies, an aliquot of phosphorylated AMPK was incubated in 50 mM HEPES, pH 7.4, 100 mM NaCl, 2.5 mM MgCl2, in the presence or absence of recombinant PP2Cα (26 ng) and in the presence or absence of varying concentrations of nucleotide for 20 min at 37°C (or as indicated in the appropriate figure legends). For determination of AMPK activity, the reaction mixture was diluted 1:40 in ice-cold buffer (50 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 10 % glycerol) to prevent further dephosphorylation. The appropriate nucleotides were added to the diluted samples in order to equalize their final concentration in all samples and AMPK activity measured using the SAMS peptide assay. Results are plotted as a percentage of the activity measured in the absence of PP2C and are the mean ± S.E.M from at least 3 independent experiments. For Western blot analysis, the dephosphorylation reaction was terminated by the addition of gel-loading buffer. Samples were resolved by SDS-PAGE and subjected to Western blot analysis. T172 phosphorylation was determined using rabbit anti-pT172 antibody (Cell Signaling). Total AMPK was detected using sheep anti-α1 or anti-α2 antibodies. Primary antibodies were detected using LI-COR IRDye® Infrared Dye secondary antibodies and visualised using an Odyssey Infrared Imager (LI-COR Biotechnology). Quantification of results was performed using Odyssey software and expressed as a ratio of the signal obtained with the phospho-specific antibody relative to the appropriate total antibody. The relative quantitation of
pT172:total α is plotted as a percentage of the value in the absence of PP2C. A representative blot is shown and data displayed as the average ± S.E.M. from at least 3 independent experiments. Rat liver AMPK was purified up to the DEAE-Sepharose step$^{23}$ and dialysed into 50 mM HEPES, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.1 mM phenylmethane sulphonyl fluoride, 4 μg/ml trypsin inhibitor in order to remove the protein phosphatase inhibitors present in the purification buffer. Following dialysis, AMPK was incubated in the presence or absence of 2.5 mM MgCl₂, and the presence or absence of varying concentrations of AMP or ADP for 10 mins at 37°C. Aliquots were removed for Western blot analysis as described above.

**Equations used in the analysis of binding experiments with fluorescent nucleotides**

**1:1 interactions** - The interaction of a protein (P) with a ligand (L) to form a simple 1:1 complex (PL) is represented by the following scheme:

\[
P + L \rightleftharpoons PL
\]

with the dissociation constant, \( K_d \), defined as \( K_d = \frac{[P][L]}{[PL]} \)

For any mixture of P and L the observed fluorescence signal (\( F_{OBS} \)) is given by:

\[
F_{OBS} = F_P[P] + F_L[L] + F_{PL}[PL] = F_P[P_0] + F_L[L_0] + (F_{PL} - F_P - F_L)[PL]
\]

(1)

where \( [X_0] \), \( [X] \), and \( F_X \) represent the total concentration, the equilibrium concentration, and the molar fluorescence coefficient of species X. A non-linear least-squares fit to equation (1) with \( [PL] \) calculated using equation (2) yields the \( K_d \) for the interaction and the \( F_X \) values.

\[
[PL] = \frac{(K_d + [P_0] + [L_0]) - \sqrt{(K_d + [P_0] + [L_0])^2 - 4[P_0][L_0]}}{2}
\]

(2)

**2:1 interactions** - When the ligand (L) binds to two sites on a protein (P) the following completely general scheme is appropriate:
The dissociation constants for the two sites are defined as:

\[ K_{d,I} = \frac{[P][L]}{[PL_I]} \quad K_{d,II} = \frac{[P][L]}{[PL_{II}]} \]

and \( \alpha \) is a ‘cooperativity factor’ which is used to account for the fact that binding at one site may increase \( (\alpha < 1) \) or decrease \( (\alpha > 1) \) the affinity of the ligand for the other site. For any mixture of P and L the observed fluorescence signal \( (F_{OBS}) \) is given by:

\[ F_{OBS} = F_P[P] + F_L[L] + F_{PL_I}[PL_I] + F_{PL_{II}}[PL_{II}] + F_{PL_{II}}[PL_{II}] \quad (3) \]

A non-linear least squares fit to equation (3) yields the \( K_d \) values for the interaction and the \( F_X \) values.

In this case the concentrations are calculated in the following way. The free ligand concentration is given by the root of the following equation:

\[ C_3[L]^3 + C_2[L]^2 + C_1[L] + C_0 = 0 \]

where the coefficients are:

\[ C_3 = \alpha \]

\[ C_2 = -\alpha[L_0] + K_{d,I} + K_{d,II} + 2\alpha[P_0] \]

\[ C_1 = -[L_0]K_{d,I} - [L_0]K_{d,II} + K_{d,I}K_{d,II} + [P_0]K_{d,I} + [P_0]K_{d,II} \]

\[ C_0 = -[L_0]K_{d,I}K_{d,II} \]

\([P]\) can then be obtained from equation (4)

\[ [P] = \frac{K_{d,I}K_{d,II}([L_0] - [L])}{K_{d,I}[L] + K_{d,II}[L] + 2\alpha[L]^2} \quad (4) \]

When \([P]\) and \([L]\) are known \([PL_I]\) and \([PL_{II}]\) can be obtained from the expressions for the \( K_d \)s and \([PL_{II}]\) from equation (5)

\[ [PL_{II}] = \frac{\alpha[L]^2[P]}{K_{d,I}K_{d,II}} \quad (5) \]

**Competition** - When two ligands (L and N) compete for binding to a single site on a protein (P) the following scheme is appropriate:

\[ P + L \rightleftharpoons PL \]

\[ P + N \rightleftharpoons PN \]

The dissociation constants for the two ligands are defined as:
For any mixture of P, L and N the observed fluorescence signal (F_{OBS}) is given by:

\[ F_{OBS} = F_P[P] + F_L[L] + F_N[N] + F_{PL}[PL] + F_{PN}[PN] \] \tag{6} 

A non-linear least squares fit to equation (6) yields the K_d values for the interaction and the F_X values. If the protein and the displacing ligand (N) are non-fluorescent, as is usually the case, then F_N = F_{PN} = F_P = 0. For this system the concentrations are calculated in the following way. The free protein concentration is given by the root of the following equation:

\[ C_3[P]^3 + C_2[P]^2 + C_1[P] + C_0 = 0 \]

where

\[ C_3 = 1 \]
\[ C_2 = -[P_0] + K_{d,L} + K_{d,N} + [L_0] + [N_0] \]
\[ C_1 = -[P_0]K_{d,L} - [P_0]K_{d,N} + K_{d,L}K_{d,N} + [L_0]K_{d,N} + [N_0]K_{d,L} \]
\[ C_0 = -[P_0]K_{d,L}K_{d,N} \]

[PL] and [PN] can then be calculated from equations (7) and (8) and the remaining concentrations from the expressions for the K_d's or from conservation of mass (e.g., [L] = [L_0] - [PL]).

\[ [PL] = \frac{[L_0][P]}{K_{d,L} + [P]} \] \tag{7} 
\[ [PN] = \frac{[N_0][P]}{K_{d,N} + [P]} \] \tag{8} 

**Binding experiments**

Unless otherwise noted all binding measurements were performed at 20°C in 25 mM Tris, 1 mM TCEP (tris(2-carboxyethyl)phosphine), 100 mM NaCl (pH 8). Free NADH has an emission maximum at 465 nm (with excitation at 340 nm). In the presence of excess AMPK the emission maximum is blue shifted to 435 nm and the fluorescence emission intensity is increased. The coumarin analogues of ATP and ADP (C-ATP, C-ADP: 3’-(7-diethylaminocoumarin-3-carbonylamino)-3’-deoxyadenosine 5’ di- and triphosphate) have emission maxima at 479 nm (with excitation at 430 nm). In the presence of excess AMPK the emission maxima are blue shifted to ~ 470 nm and the fluorescence intensity is increased. All fluorescence measurements were made using a Jasco FP-6300 spectrofluorimeter equipped with an ETC-273T Peltier temperature controller.
**NADH binding** - Measurements made under standard buffer conditions showed that the binding of NADH to phosphorylated AMPK is weak (Main text: Fig. 2b inset). Because it is only possible to distinguish between one and two site binding models when a ‘close to stoichiometric’ titration can be performed (i.e., under conditions where essentially all the added AMPK binds to the NADH) we made additional measurements at 1°C in a salt free buffer (Fig. FM1).

![Figure FM1 – Titration of 20 μM NADH with phosphorylated AMPK in a low salt buffer at 1°C. The black and red lines through the data points are the best fits for 1:1 (Kd = 8.2 ± 1.4 μM) and 2:1 models respectively. The 2:1 fit assumed identical non-interacting sites with the same fluorescence signal. The residuals (offset by 20) show that the 1:1 fit is superior.](image)

Although the fit to a 1:1 model is significantly better than that to a 2:1 model with two non-interacting sites with the same affinity and the same fluorescence signal it is not possible to exclude the possibility that NADH also binds with lower affinity to a second site. However, two additional pieces of evidence suggest that a 1:1 model for the binding of NADH is appropriate. First, the experiments in which NADH is displaced by AXPs (Figure 2b main text) show that when a mixture of AMPK (32 μM) and NADH (15 μM) is titrated with AXPs almost complete displacement of NADH is observed when just
over one equivalent of AXP is added. Second, the C-AXPs, which bind to two sites on AMPK (see below), cannot be completely displaced by NADH (see Fig FM3). NADH binding data were therefore analyzed using non-linear least-squares fits to equation (1). In a titration of NADH (L) with AMPK (P) only two parameters need to be determined because $F_P = 0$ and $F_L$ can be determined from the fluorescence signal observed from the NADH solution prior to the addition of AMPK. The dissociation constant for NADH binding ($65 \pm 14 \mu M$ in standard buffer conditions) was unaffected by the presence of 5 mM MgCl$_2$ in the buffer, consistent with fact that NADH binds magnesium very weakly.

**Coumarin-AXP binding** – Initial measurements made under standard buffer conditions showed that C-ADP and C-ATP bound with high affinity to two sites. In order to confirm this we performed titrations at high starting concentrations in order to have titrations that were closer to stoichiometric. Figure FM2 clearly demonstrates that a 1:1 binding model for C-ATP is not appropriate.

![Figure FM 2 – Titration of 24 μM C-ATP with phosphorylated AMPK. The solid red line is the best fit to a 1:1 binding model](image)

C-AXP binding data were therefore analyzed using non-linear least-squares fits to equation (3). When C-AXP (L) is titrated with AMPK (P) there are six parameters to be determined since $F_P = 0$ and $F_L$ can be determined from the fluorescence signal observed from C-AXP alone. In order to simplify the
analysis we assumed that $F_{PL,I} = F_{PL} + F_{PL,II}$ and that $\alpha = 1$ (i.e., that the binding is non-cooperative). It should be noted that cooperativity can only be assessed in experiments in which the free ligand concentration can be determined and this is not generally possible in experiments using optical methods. $K_{d,I}$ and $K_{d,II}$ were estimated to be $0.82 \pm 0.19$ and $4.25 \pm 1.35 \mu M$ for C-ATP, and $1.13 \pm 0.22$ and $4.15 \pm 1.7 \mu M$ for C-ADP. $F_{PL,I} / F_L$ and $F_{PL,II} / F_L$ were estimated to be $9.5 \pm 0.5$ and $4.2 \pm 0.3$ respectively, indicating that occupancy of the high affinity site generates the larger fluorescence change.

The addition of MgCl$_2$ to the buffers weakened the binding of these analogues at both sites by between five- and fifteen-fold. The effect of magnesium appeared to saturate at $[Mg^{2+}] > 0.5$ mM for C-ATP and at $[Mg^{2+}]$ between 2 and 5 mM for C-ADP, consistent with the fact that Mg$^{2+}$ binds significantly more strongly to ATP than to ADP. $K_{d,I}$ and $K_{d,II}$ were estimated to be $8.5 \pm 3.2$ and $18.6 \pm 6.2 \mu M$ for Mg-C-ATP.

**Displacement of NADH by AXPs and NAD** – When AXPs are added to a mixture of AMPK (32 $\mu M$) and NADH (15 $\mu M$) (Figure 2b main text) the NADH is completely displaced when just over one equivalent of AXP is added. This is consistent with there being a single binding site for NADH and with the observation that AXPs bind to two sites with significantly different affinities (see below). NADH displacement data were therefore analyzed using non-linear least-squares fits to equation (6) with the $K_d$ for NADH binding fixed at the value determined in the direct titration (65 $\mu M$). Although this approach ignores any contribution from the binding of AXPs to the weaker of the two sites the dissociation constant determined for binding of AXP to the stronger of the two sites is in good agreement with that determined using competition with the C-AXPs (see Table 1, main text). The dissociation constant for NAD$+$ was estimated using the same approach.

**Displacement of coumarin-AXPs by AXPs and NADH**
Figure FM3 – Displacement of C-ATP from phosphorylated AMPK with ATP and NADH. NADH is not able to completely dissociate the C-ATP, consistent with view that it binds to a single site. Displacement of C-ATPs by AXPs is a more complicated analysis problem because of the multiplicity of species formed. The displacement of C-ATP by ATP clearly occurs in two phases (Figure FM3) and experiments performed at different starting ratios of C-ATP to AMPK indicated that the C-ATP displaced by low concentrations of added AXP was from the stronger of the two sites. Approximate values for the AXP binding constants were estimated by treating the two parts of the curve separately and using equation (6). We then developed a method which permitted analysis of the whole curve. For any mixture of P (AMPK), L (C-AXP) and X (AXP) the observed fluorescence signal (F_{OBS}) is given by:

\[
F_{OBS} = F_L[L] + F_{PL_1}[PL_1] + F_{PL_{II_1}}[PL_{II_1}] + F_{PL_{II_1}L_{II_2}}[PL_{II_1}L_{II_2}] + F_{PL_1}[PL_1X_{II_1}] + F_{PL_{II_1}}[PX_{II_1}L_{II_2}] \quad (9)
\]

Note: Because the AMPK and AXP have no fluorescence contribution we were able to set \( F_P = F_{PX_1} = F_{PX_{II_1}} = F_{PX_{II_1}X_{II_2}} = 0 \) and we also included the assumptions that \( F_{PL_{II_1}X_{II_2}} = F_{PL_{II_1}} \) and \( F_{PX_{II_1}L_{II_2}} = F_{PL_{II_1}} \). The displacement data were then analyzed using non-linear least-squares fits to equation (9) with the \( K_d \)s for C-ATP binding fixed at the value determined in the direct titrations (see above). Although it would in principle be possible to calculate concentrations through the approaches described
above it was simpler in this case to calculate them using the method developed by Storer and Cornish-Bowden (A. C. Storer and A. Cornish-Bowden (1976) Biochem. J. 159: 1-5).

Crystallography

Truncated AMPK, γ1(rat) β2(human; 187–272) His-α1(rat; 1-469, 524–548), with a pair of engineered 3C protease sites at either end of the α loop 470-523, was cloned into a tricistronic vector and subsequently expressed in E. coli BL21 (BL21-CodonPlus-RIL, Stratagene). Proteins were purified using a nickel affinity chromatography (His-Trap, GE Healthcare), anion exchange (Mono Q, GE Healthcare) and gel filtration (Superdex 200, GE Healthcare). The purified protein was phosphorylated using CAMKKβ kinase overnight at 25°C then re-purified by gel filtration. The complex stock solution was prepared at 15 mg ml⁻¹ in 50 mM Tris, pH 7.0, 100 mM NaCl and 1 mM TCEP, mixed with a threefold molar excess of AMP and one fold of staurosporine. Crystals were grown by vapour diffusion technique at 18°C in hanging drops. Drops were prepared by mixing equal volumes of protein complex with reservoir solution containing 8% isopropanol and 5% MPD in 100 mM TRIS at pH 7.5. Crystals were first transferred into mother liquor with an additional 30% glycerol, before plunging into liquid nitrogen. Diffraction data were collected on ADSC detector, Diamond Light Source, Oxford. Data were integrated using Denzo and scaled with Scalepack. The structure was solved by molecular replacement using Amore using 2V8Q.pdb and 2H6D.pdb as the search models. Standard refinement was carried out with Refmac with manual model building with COOT. Figures were created with Pymol (http://pymol.sourceforge.net/)