Characterization of the AMP-activated Protein Kinase Kinase from Rat Liver and Identification of Threonine 172 as the Major Site at Which It Phosphorylates AMP-activated Protein Kinase*

(Received for publication, May 14, 1996, and in revised form, July 29, 1996)

Simon A. Hawley‡§, Matthew Davison¶, Angela Woods¶, Stephen P. Davies¶, Raj K. Beri¶, David Carling, and D. Grahame Hardie**

From the ‡Biochemistry Department, The University, Dundee DD1 4HN, Scotland, United Kingdom, Zeneca Pharmaceuticals, Alderley Edge, Macclesfield SK10 4TG, United Kingdom, and the Medical Research Council Molecular Medicine Group, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, United Kingdom

We have developed a sensitive assay for the AMP-activated protein kinase kinase, the upstream component in the AMP-activated protein kinase cascade. Phosphorylation and activation of the downstream kinase by the upstream kinase absolutely requires AMP and is antagonized by high (millimolar) concentrations of ATP.

We have purified the upstream kinase >1000-fold from rat liver; a variety of evidence indicates that the catalytic subunit may be a polypeptide of 58 kDa. The physical properties of the downstream and upstream kinases, e.g. catalytic subunit masses (63 versus 58 kDa) and native molecular masses (190 versus 195 kDa), are very similar. However, unlike the downstream kinase, the upstream kinase is not inactivated by protein phosphatases. The upstream kinase phosphorylates the downstream kinase at a single major site on the α subunit, i.e. threonine 172, which lies in the “activation segment” between the DFG and APE motifs. This site aligns with activating phosphorylation sites on many other protein kinases, including Thr177 on calmodulin-

AMPKK, AMP-activated protein kinase kinase; PP1, protein phosphatase 1; PP2C, protein phosphatase-2C; PTH, phenylthiohydantoïn; CaMKI, calmodulin-dependent protein kinase I; HPLC, high pressure liquid chromatography.
Characterization of Protein-activated Kinase Kinase

Initially it was unclear whether the effect of AMP on phosphorylation of AMPK by AMPKK was due to binding of the nucleotide to the substrate (AMPK), to the enzyme (AMPKK), or to both. Although we reported that the former mechanism could explain the effect, at least in part (24), more recently we have presented evidence that AMP also activates AMPKK directly, i.e. that the latter is itself an AMP-activated protein kinase (27). An answer to this question was possible because we found that AMPKK would also phosphorylate and activate (at least in vitro) calmodulin-dependent protein kinase I, an enzyme that was not itself sensitive to AMP.

These findings show that the upstream protein kinase AMPKK plays an active role in the overall activation of the cascade by AMP rather than merely being a passive, constitutively active partner. This makes the further characterization of AMPKK an important goal. Here we report on the purification and some physical and regulatory properties of AMPKK, and we also identify the major site at which it phosphorylates and activates AMPK.

Experimental Procedures

Materials—5'-AMP and ATP were from Boehringer (Lewes, UK). Phenylmethylsulfonyl fluoride, mannitol, benzamidine, palmityl-CoA, Brj-35, and n-octyl β-D-glucopyranoside were from Sigma (Poole, UK). Trypsin (modified sequencing grade) was a gift from Dr. P. T. W. Cohen. Protein phosphatases 2A1 (ABC holoenzyme from bovine heart, from the Protein Phosphorylation Unit, Dundee) was purified as for rabbit muscle (31)) were gifts from David Barford (Oxford, UK). Okadaic acid (Na<sub>98</sub>) (98% pure) was from Calbiochem-Novabiochem (Nottingham, UK). Centricon-30 concentrators were from Amersham (Bucks, UK). [14C]FSBA was from NEN, (Stevenage, UK). All other reagents were Anaal grade from BDH (Poole, UK).

Proteins and Peptides—5'-Nucleotidase (from Crotalus adamanteus venom) bound to agarose, soybean trypsin inhibitor (type II-S), SDS-PAGE, and gel filtration markers were from Sigma, Bovine brain calmodulin was from Boehringer. Prestained "rainbow" molecular weight markers were from Amersham. Rabbit protein phosphatase-1<sub>1b</sub>, (28) and human protein phosphatase-2Ca (26) were bacterially expressed proteins and were gifts from Dr. P. T. W. Cohen. Protein phosphatases LAR (29), CI-100 (30), and ZA<sub>1</sub> (ABC holoenzyme from bovine heart, purified as for rabbit muscle (31)) were gifts from Dave Barford (Oxford University), Steve Keyse (Dundee University), and Nick Morriss (MRC Protein Phosphorylation Unit, Dundee). Protein phosphatase 2A<sub>2b</sub> (PP2A<sub>2b</sub>, catalytic subunit) was purified from bovine heart as described previously for rabbit muscle (31). The "SAMS" and "AMARA" peptides (32) were synthesized and purified as described previously. AMPK-α antiseraum was raised against the peptide PGLKPHPERMPPLI (residues 361–374 of AMPK-α) and we also identify the major site at which it phosphorylates and activates AMPK.

Buffers—The protease inhibitors benzamidine (1 mM), soybean trypsin inhibitor (1 μg/ml), and phenylmethylsulfonyl fluoride (1 mM) were added to buffers A, B, C, and D immediately prior to use (buffer A: 9.05 M Tris/HCl, pH 8.4, at 4 °C, 0.25 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 μM diethiothreitol; buffer B: 0.05 M Tris/HCl, pH 7.4 at 4 °C, 0.25 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 0.02% (w/v) Brj-35, 10% (v/v) glycerol, 1 mM diethiothreitol; buffer C: as buffer B but without NaF and sodium pyrophosphate; buffer D: buffer C plus 0.1% (w/v) NaCl, 50 mM MgCl<sub>2</sub>). Buffers were made up in small aliquots in liquid N<sub>2</sub>, and stored at −70 °C. Aliquots of the Q-Sepharose fraction were stored in the same way and used in some experiments (Figs. 1 and 4–10).

Enzyme Assays—PP2A was assayed using phosphorylase a (31). One unit of PP2A dephosphorylates 1 μmol of substrate/min at 30 °C. AMPK was assayed as described (34) except that, unless stated otherwise, the AMARA peptide (200 μM) replaced SAMS. One unit of AMPK phospho- phorylates 1 μmol of substrate/min at 30 °C. AMPKK was assayed by its ability to reactivate dephosphorylated AMPK; a single preparation of AMPKK was added to 1 volume of ATP, MgCl<sub>2</sub>, and AMP in buffer E, and 3 volumes of this mixture was added to 1 volume of AMPKK in buffer E. Final concentrations were 1 mM ATP, 5 mM MgCl<sub>2</sub>, and 100 μM AMP. The reaction was incubated at 30 °C, and 5-μl aliquots were removed at 0 and 25 min for the AMPKK assay as described above. One unit of AMPKK was that amount which increased the activity of dephosphorylated AMPK by 1 unit/ml (calculated at the dilution of the stock AMPK) per min at 30 °C.

Protein Analysis and Electrophoresis—Protein concentration was determined by the method of Bradford (35). SDS-PAGE was on 10% gels using the method of Laemmli (36) in a Bio-Rad Mini-Protein II gel apparatus unless stated otherwise.

Purification of AMPK—AMPKK was purified using modifications of the previous method (11). All procedures were at 4 °C unless stated otherwise. Livers from male Wistar rats were left at room temperature for 1–2 min to become hypoxic (this elevates AMP and activates AMPK (5)), chopped coarsely, and washed in buffer A. A 2.5–6% polyethylene glycol 6000 (PEG) fraction was prepared as described (11), resuspended in buffer B using a ground glass homogenizer, and applied to a DEA-Sepharose 6 Fast Flow column (flow rate 15 cm/min). The column was washed until A<sub>280</sub> < 0.05, and protein was eluted with buffer B plus 0.2 M NaCl. Active fractions were loaded directly onto Blue-Sepharose (5 × 7 cm) and washed extensively, and protein was eluted with buffer B plus 0.8 M NaCl. Active fractions were precipitated with ammonium sulfate, dissolved, and dialyzed as for the previous procedure (11) and then dialyzed against 1 × g, 10 min. Then fractions (200 μl) were pooled and applied to a Q-Sepharose HiLoad 16/10 column at 3 ml/min, the column was washed until A<sub>280</sub> < 0.05, and protein was eluted with a linear gradient from 0 to 450 mM NaCl in buffer B. Active fractions were pooled, concentrated to <0.5 ml in Centricron-30 concentrators, and applied to a high performance Sephacryl S-200 HiLoad column in buffer C at 0.5 ml/min. Active fractions were pooled, concentrated to 10 units/ml in Centricron-30 concentrators, frozen in small aliquots in liquid N<sub>2</sub>, and stored at −70 °C.

Purification of AMPKK—All procedures were at 4 °C unless stated otherwise. The 6% PEG supernatant from the AMPK preparation above was made to 10% (w/v) PEG and centrifuged (300,000 × g, 20 min). The pellet was resuspended in buffer B and applied to a DEA-Sepharose (Fast Flow) column (6 × 15 cm). The column was washed until A<sub>280</sub> < 0.05, and protein was eluted with buffer B plus 0.2 M NaCl. Active fractions were loaded directly onto Blue-Sepharose (5 × 7 cm), and the flow-through was applied to a high performance Q-Sepharose HiLoad 16/10 column in buffer B plus 200 mM NaCl at 3 ml/min. The column was washed until A<sub>280</sub> < 0.05 and then AMPKK was eluted with a 96% linear gradient from 0 to 500 mM NaCl at 3 ml/min. Active fractions were pooled, concentrated to <0.5 ml in Centricron-30 concentrators, and then diluted 3-fold in buffer D. An ATP–γ-Sephacyr (9 column (1.5 ml) was equilibrated in buffer D. AMPKK was applied, stirred into the matrix, and allowed to stand for 20 min. The column was washed at −1 ml/min with 40 ml of buffer D and then 25 ml of buffer D plus 5 mM ATP. Active fractions were pooled, concentrated in Centricron-30 concentrators, frozen in small aliquots in liquid N<sub>2</sub>, and stored at −70 °C.

Labeling of AMPKK Using γ<sup>32</sup>P/ATP or [35S]FSBA—AMPKK (125 μM/μl) ATP–γ-Sephacyr preparation in buffer E) was incubated at 30 °C for 40 min with either (a) 200 μM γ<sup>32</sup>P/ATP (1.5 × 10<sup>5</sup> cpm/μl) or (b) 200 μM of [35S]FSBA (80,000 cpm/μl). Reactions were stopped
by the addition of SDS-PAGE sample buffer. Autoradiography (ATP label was performed on dried gels at −70 °C using Hyperfilm-β max with Kodak X-Omatic intensifying screens. Phosphor imaging (FSBA labeling) was performed using a Molecular Dynamics PhosphorImager.

Protein Phosphatase Treatment of AMPKK—AMPKK (770 units/ml) was incubated with protein phosphatases PPA2α, (50 milliunits/ml), PPA2α (50 milliunits/ml), PP1γ (50 milliunits/ml), PP2C (1.5 milliunits/ml), CL-100 (6 µg/ml), or LAR (6 milliunits/ml) for 20 min at 30 °C in buffer C.

Phosphorylation and Immunoprecipitation of AMPK for Sequencing Studies—AMPK was purified to the gel filtration step as described above, except that NaF and sodium pyrophosphate were omitted from the gel filtration buffer. AMPK (14,000 units) was dephosphorylated using PPA2α (500 milliunits) for 15 min at 30 °C in 8 ml of buffer C. The reaction was stopped by adding okadaic acid to 100 nM, and then incubation was continued for 8 min in the presence of AMP (100 µM), MgCl2 (5 mM), and γ[32P]ATP (200 µCi; 400 cpm/pmol) in the presence or absence of AMPKK (210 units). Reactivation was terminated by the addition of EDTA to 20 mM, and then AMPK was immunoprecipitated by five sequential additions of anti-AMPK-β subunit antibody. In each case, 200 µl of a 50% (v/v) slurry of protein A-Sepharose in buffer B containing 1% (w/v) Triton X-100 was added, and the resulting mixture was incubated in 2 at 4 °C with constant shaking. The immunocomplex was collected by centrifugation and washed extensively with buffer B containing 1% (w/v) Triton X-100. The immunoprecipitates were combined, boiled with SDS-PAGE sample buffer, resolved by SDS-PAGE on 10% gels, and transferred electrophoretically to polyvinylidene difluoride membranes (37).

Tryptic Digestion of AMPK and Purification of 32P-Peptides for Sequencing Studies—Peptides were digested on polyvinylidene difluoride membranes using trypsin (37). The membranes were stained with sulforhodamine (Eastman Kodak Co.) (38). Regions of the membrane containing the α and β subunits were excised, cut into ~4 × 4-mm pieces, and incubated with sufficient trypsin solution (100 µg/ml in 200 mM NH4HCO3 containing 1% (w/v) α-oxyl β-tryptophanamide) to fully wet the membranes. After 24 h at room temperature, 0.1 volume of 100 mM dithiothreitol in buffer A was added, and incubation continued for a further 24 h. The membrane pieces were extracted once with 0.6–1.0 ml of buffer A, followed by transfer of the supernatant to clean polyvinylidene difluoride membranes (37).

Establishment of an Assay for AMP-activated Protein Kinase Kinase—Purification and characterization of AMPKK required the development of a sensitive and quantitative assay. We utilized its ability (24) to catalyze the time- and MgATP-dependent reactivation of purified AMPK, which had been inactivated by pretreatment with the purified catalytic subunit of protein phosphatase-2A (PP2Aα). We found it necessary to perform the protein phosphatase treatment immediately prior to the AMPKK assay, since the dephosphorylated form of AMPK appears to be unstable on storage. After the protein phosphatase treatment, okadaic acid was added to prevent the protein phosphatase interfering with the two subsequent kinase reactions. Fig. 1 shows that this reactivation is suitable for a quantitative assay using a crude postmitochondrial supernatant fraction from rat liver, a PEG precipitate prepared from it, or a much more highly purified fraction (Q-Sepharose). With crude fractions, the assay became nonlinear with protein concentration when the degree of reactivation was >30%. Remarkably, after purification through four steps (Q-Sepharose fraction) the assay was essentially linear right up to 100% reactivation.

In the initial studies, we assayed AMPK after reactivation using as substrate the synthetic SAMS peptide (HMRSAMSGLHLVKKR), which is based on the primary phosphorylation site on rat acetyl-CoA carboxylase (34). However, as we purified AMPKK further this substrate began to give high blanks, i.e. there was significant phosphorylation of the peptide even if dephosphorylated AMPKK was not added. This was eventually traced to the presence of protein kinase(s) that co-purified with AMPKK. The problem of the high blanks could be overcome by replacing SAMS with the peptide AMARAASAAALARRR, which we developed as a basis for specificity studies on AMPK and its homologues (32). Not only is this a better substrate for AMPKK, it is also a better substrate for AMPKK, which was therefore used as the starting point for further purification.

Amino Acid Sequencing—Fractions containing radiolabeled peptides were pooled and dried down onto a Sequelon alyamine membrane disc (Millipore Corp.). The peptides were coupled to the disc according to manufacturer’s instructions, and the discs were washed with 1 ml of 50% (v/v) methanol followed by 1 ml of 0.1% (v/v) trifluoroacetic acid in acetonitrile:water (9:1). Some of the radioactive peaks from these initial runs were pooled, dried by rotary evaporation, and resuspended in 50 µl of buffer F (10 mM ammonium acetate pH 6.5) followed by 10 µl of 100 mM dithiothreitol and 60 µl of 200 mM ammonium acetate, pH 6.5. Peptides were separated by reversed phase HPLC as before except using buffer F in place of 0.1% trifluoroacetic acid.

Recovery of radioactivity from HPLC in either buffer was 50–70%.

RESULTS

Establishment of an Assay for AMP-activated Protein Kinase Kinase—Purification and characterization of AMPKK required the development of a sensitive and quantitative assay. We utilized its ability (24) to catalyze the time- and MgATP-dependent reactivation of purified AMPK, which had been inactivated by pretreatment with the purified catalytic subunit of protein phosphatase-2A (PP2Aα). We found it necessary to perform the protein phosphatase treatment immediately prior to the AMPKK assay, since the dephosphorylated form of AMPK appears to be unstable on storage. After the protein phosphatase treatment, okadaic acid was added to prevent the protein phosphatase interfering with the two subsequent kinase reactions. Fig. 1 shows that this reactivation is suitable for a quantitative assay using a crude postmitochondrial supernatant fraction from rat liver, a PEG precipitate prepared from it, or a much more highly purified fraction (Q-Sepharose). With crude fractions, the assay became nonlinear with protein concentration when the degree of reactivation was >30%. Remarkably, after purification through four steps (Q-Sepharose fraction) the assay was essentially linear right up to 100% reactivation.
by conventional means. Further studies were therefore conducted with this preparation.

Molecular Mass of AMP-activated Protein Kinase Kinase and Its Catalytic Subunit—SDS-PAGE revealed that the preparation still contained several polypeptides, but with a species of apparent molecular mass 58 kDa being a major component (Fig. 3, lane 1). When incubated with \([\gamma-32P]ATP\), this polypeptide was essentially the only labeled species (Fig. 3, lane 2). We also conducted experiments aimed at identification of the catalytic subunit using the reactive ATP analogue FSBA. This reagent inactivates the catalytic subunit of cyclic AMP-dependent protein kinase by reacting with the lysine residue (40) that is conserved in the ATP binding site of all other protein kinases (41). As expected, FSBA caused a time-dependent, exponential decay of AMPKK activity, with half-times of 18 min at 250 \(\mu\)M, and 43 min at 100 \(\mu\)M FSBA (data not shown). Using \([14C]FSBA\), the 58-kDa polypeptide was the only significant radioactive species detectable after an SDS-PAGE gel was analyzed by PhosphorImager analysis (Fig. 3, lane 3). The radioactivity migrating at the dye front was also evident in a control incubated without AMPKK (not shown). Using \([3H]FSBA\), the 58-kDa polypeptide was the only significant radioactive species detectable after an SDS-PAGE gel was analyzed by PhosphorImager analysis (Fig. 3, lane 3). The radioactivity migrating at the dye front was also evident in a control incubated without AMPKK (not shown). Using \([14C]FSBA\), the 58-kDa polypeptide was the only significant radioactive species detectable after an SDS-PAGE gel was analyzed by PhosphorImager analysis (Fig. 3, lane 3). The radioactivity migrating at the dye front was also evident in a control incubated without AMPKK (not shown). Using \([3H]FSBA\), the 58-kDa polypeptide was the only significant radioactive species detectable after an SDS-PAGE gel was analyzed by PhosphorImager analysis (Fig. 3, lane 3). The radioactivity migrating at the dye front was also evident in a control incubated without AMPKK (not shown).

We also estimated the native molecular mass (42) of AMPKK by combining the Stokes radius determined by gel filtration (5.5–5.7 nm; Fig. 4) and the sedimentation coefficient determined by glycerol gradient centrifugation (8.4–8.6 S; Fig. 5), which yielded a value of 198 ± 5 kDa. The frictional ratio \((f/f_0)\) was 1.44–1.47, indicating that AMPKK has a highly asymmetric structure. Prolate or oblate ellipsoids of this frictional ratio would have axial ratios of 8 and 10, respectively. The migration of AMPKK on gel filtration was not affected by the presence or absence of 0.2 M NaCl in the buffer (not shown).

Dependence of AMPKK on AMP and ATP and Lack of Effect of Protein Phosphatases—We have previously reported that reactivation of AMPK by AMPKK is absolutely dependent on the presence of 5′-AMP (24). In the present study we examined whether the effect of AMP on the AMPKK reaction could be antagonized by high concentrations of ATP. Fig. 6 (open circles) shows the effect of varying the ATP concentration, at a constant AMP concentration of 100 \(\mu\)M, during the AMPKK assay. Rather than the simple hyperbolic curve one might expect for a protein kinase reaction, a more complex curve was obtained, with ATP completely inhibiting the reaction at high concentration. The data could be fitted to a model in which ATP bound with positive cooperativity at the catalytic site(s) (half-maximal activation at 16 ± 6 \(\mu\)M; Hill coefficient \((h) = 1.6 ± 0.5\)) but also bound at higher concentration at inhibitory allosteric site(s) (half-maximal inhibition at 360 ± 80 \(\mu\)M; \(h = 1.9 ± 0.4\)). These results show that, as for the allosteric effect of AMP on AMPK itself (11, 25), the activation of AMPK by AMPKK in the presence of AMP is antagonized by high concentrations of ATP. When the experiment was performed at a higher AMP concentration (500 \(\mu\)M; Fig. 6, filled circles), the whole curve was shifted to the right; half-maximal activation was now at 54 ± 4

![Figure 2](image-url)
Characterization of AMP-activated Protein Kinase Kinase

In order to determine the activating site is fully occupied and the inhibitory site is empty, the concentration giving a half-maximal effect at the activating site,

\[ v = \frac{v_{	ext{max}} [\text{ATP}]^n [\text{AMP}]^m}{A_{	ext{o},5} + [\text{ATP}]^n + [\text{AMP}]^m} \]

where \( v \) is the turnover number, \( v_{	ext{max}} \) is the maximum velocity, \([\text{ATP}]\) is the ATP concentration, \([\text{AMP}]\) is the AMP concentration, \( A_{	ext{o},5} \) is the concentration giving a half-maximal effect at the inhibitory site, and \( n \) and \( m \) are Hill coefficients. Estimates, with standard errors, of these parameters are given under “Results.” Curve fitting was carried out using Kaleidagraph (Abelbeck Software).

When AMPK was incubated with AMPKK in the presence of AMP and \( [\gamma-\text{32P}]\text{ATP} \), an additional labeled polypeptide with an apparent molecular mass of ~58 kDa became evident in the anti-\( \beta \) subunit immunoprecipitate (Fig. 7). This corresponded to p58, the major autophosphorylated polypeptide in the AMPKK preparation (Fig. 3). The labeled p58 was not precipitated by the anti-\( \beta \) subunit antibody in the absence of AMPK (not shown). Unfortunately, p58 was not clearly resolved from the immunoglobulin heavy chain on the Coomassie-stained gel, so it was not possible to estimate its abundance relative to the AMPK subunits.

The \( \alpha \) subunit labeled in the presence or absence of AMPKK was purified by SDS-PAGE followed by electrophoretic transfer to polyvinylidene difluoride membrane. The polypeptide was digested on the membrane with trypsin as described under “Experimental Procedures.” Fig. 8 shows the resolution of phosphopeptides obtained by reversed phase HPLC in 0.1% trifluoroacetic acid for the AMPKK-treated sample and for the autophosphorylation control. In addition to a small peak of radioactivity in the column breakthrough, autophosphorylation of AMPKK gave rise to four radiolabeled peptide peaks (labeled 1–4 in Fig. 8, with peak 1 being a shoulder on the leading edge of peak 2). Peaks 1–4 were also seen in very similar amounts when AMPK was incubated with AMPKK, but
Characterization of AMP-activated Protein Kinase Kinase

Some radioactivity was also recovered in cycle 19 and, with one preparation, in cycle 20 (Fig. 9), corresponding to Ser173 and Cys174, respectively. While it is not possible to rule out a slight phosphorylation of Ser173, we think these results are most likely explained by carry over of Thr172 into cycles 19 and 20, due to the repetitive yield being less than 100%. In the run shown in Fig. 9A, the carry over of the PTH-amino acid from cycle 15 into cycle 16 was >30%. Sequencing of peak AA2 (pooled from runs derived from trifluoroacetic acid peaks 6 and 7) yielded the same major sequence as for peak 5. Once again the radioactivity was recovered in cycle 18 during solid phase sequencing (data not shown). There was insufficient material in peak AA3 to allow sequence determination.

Fig. 10 shows separation of phosphorylated tryptic peptides
We have previously reported that both AMPK and AMPKK are directly activated by AMP (27). This study reveals further similarities between the upstream and downstream protein kinases. The molecular masses of the catalytic subunits (AMPKK versus AMPK, 58 versus 63 kDa), the Stokes radii (5.5–5.7 versus 5.4–5.8 nm), the sedimentation coefficients (8.4–8.6 versus 7.9–8.4 S), and the estimated native molecular masses (196 ± 5 kDa versus 190 ± 10 kDa) are all very similar. The frictional ratios (f/f₀ = 1.44–1.47 versus 1.46–1.50) also indicate that both are highly asymmetric molecules. This raises
the possibility that, like AMPK, which consists of \(\alpha (63\text{-kDa}), \beta (38\text{-kDa})\) and \(\gamma (35\text{-kDa})\) subunits, AMPKK might also contain additional subunits as well as the 58-kDa catalytic subunit. No obvious candidates for these were seen in a Coomassie-stained gel of the immunoprecipitate shown in Fig. 7, but they could have been obscured by the subunits of AMPK and/or by the heavy or light chains of the immunoglobulin. Whatever the structure of the AMPKK oligomer, migration on a gel filtration column was not affected by the presence or absence of 0.2 \(\times\) NaCl in buffer B, indicating that the oligomer is stable under these conditions.

In this study we identify threonine 172 as the major site phosphorylated by AMPKK on AMPK-\(\alpha\), leading to activation of AMPKK. We believe that all three radioactive peptide peaks that were labeled only after incubation with AMPKK are derived from this site. Trifluoroacetic acid peaks 5–7 eluted very close together (Fig. 8) and also yielded three closely eluting peaks on HPLC in ammonium acetate pH 6.5 (AA1–AA3, not shown). Although we did not have sufficient AA3 for sequence studies, AA1 and AA2 gave identical sequences, corresponding to the tryptic peptide commencing at Ile\(^{155}\) in AMPK-\(\alpha_2\) or AMPK-\(\alpha_3\), and in both cases the major radioactive amino acid was at cycle 18, corresponding to Thr\(^{172}\). We suspect that trifluoroacetic acid peaks 5–7 (corresponding to AA1–AA3) are generated by differing degrees of oxidation of the two methionines within the peptide.

At least four other \(^{32}\)P-labeled tryptic peptide peaks (Fig. 8, peaks 1–4) were separated from AMPK-\(\alpha\), but these were not affected by the presence of AMPKK and appear to be autophosphorylation sites that are phosphorylated independently of the state of activation of AMPK.

New information to be taken into account was the report (14), published just as we were about to submit this work, that rat liver AMPK catalytic subunit was encoded by not one but at least two genes, termed \(\alpha_1\) and \(\alpha_2\). The \(\alpha_2\) isoform seems to be expressed at a high level in rat liver, but it appears to have a low SAMS peptide activity, with \(\alpha_2\) apparently accounting for at least 90% of the SAMS peptide kinase activity in this tissue (14). The AMPK used for our identification of the phosphorylation site may have been a mixture of both isoforms, although because it had been purified on the basis of activity it presumably contained a substantial proportion of \(\alpha_1\). However, the sequence around Thr\(^{172}\) is perfectly conserved between \(\alpha_1\) and \(\alpha_2\), suggesting that AMPKK is likely to activate both forms. The predicted tryptic peptides containing this site (even allowing for failure to cleave at Arg\(^{171}\)) are also identical for both isoforms. Since the anti-\(\beta\) subunit antibody used for immunoprecipitation depletes all of the AMPK activity from even relatively crude preparations of rat liver (data not shown) it seems likely that it would precipitate both \(\alpha_1\) and \(\alpha_2\). Although we therefore cannot be certain whether the amino acid sequence containing Thr\(^{172}\) was derived only from \(\alpha_1\) or from both \(\alpha_1\) and \(\alpha_2\), our overall conclusions are not affected.

In contrast to the autophosphorylation sites on AMPK-\(\alpha\), the \(\beta\) subunit is more highly phosphorylated in the presence of AMPKK (Fig. 7). More detailed studies on the phosphorylation of the \(\beta\) subunit will be presented elsewhere, but current evidence\(^2\) favors the idea that this is due to autophosphorylation and is not catalyzed by AMPKK.

Although further studies (e.g. site-directed mutagenesis) will be required to conclusively demonstrate that activation of AMPK by AMPKK is accounted for entirely by phosphorylation of Thr\(^{172}\), comparison with other protein kinases lends considerable support to this hypothesis. AMPK joins a long list of protein kinases known to require phosphorylation within the “activation segment” (43) between the “DFG” motif (subdomain VII (41)) and the “APE” motif (subdomain VIII) for activity. The phosphorylation site on protein-serine/threonine kinases is usually a threonine but can also be tyrosine (Gsk3\(^\alpha\)), threonine and tyrosine (mitogen-activated protein kinases), or two serines (mitogen-activated protein kinase kinases). In some cases, such as cyclic AMP-dependent protein kinase, these phosphate groups may be inserted during protein synthesis and appear to be stable \(\textit{in vivo}\). In many other cases such as Cdc2, mitogen-activated protein kinases, mitogen-activated protein kinase kinases, p90 ribosomal protein S6 kinase and AMPK, the phosphorylation can be readily reversed and has important regulatory consequences \(\textit{in vivo}\). In the crystal structure of the C\(\alpha\) subunit of cyclic AMP-dependent protein kinase, the phosphorylated threonine (Thr\(^{197}\)) interacts with a positively charged cluster (His\(^{87}\), Arg\(^{160}\), and Lys\(^{189}\)), thus permitting proper orientation of the pseudosubstrate peptides for phosphate transfer from ATP (43). Two of these three basic residues are conserved in AMPK-\(\alpha_1\) and -\(\alpha_2\) (12, 14).

The yeast (\textit{S. cerevisiae}) homologue of AMPK, Snf1p, is inactivated by treatment with protein phosphatases and can be reactivated by treatment with a crude preparation of mammalian AMPKK (22), indicating that it may be regulated by phosphorylation at the site equivalent to Thr\(^{172}\). A threonine residue (Thr\(^{210}\)) is conserved at this position in Snf1p, and the sequence around it is also highly conserved, with a few conservative replacements on the N-terminal side. Estruch \textit{et al.} (44) have already shown that a T210A mutant is nonfunctional in yeast \(\textit{in vivo}\) with respect to growth on raffinose or expression of invertase. This is entirely consistent with Thr\(^{210}\) being the site on Snf1p phosphorylated by a putative “Snf1p kinase,” which is currently uncharacterized.

Calmodulin-dependent protein kinase I (CaMKI) is activated by phosphorylation by an upstream protein kinase (CaMKII kinase), and CaMKI kinase can also phosphorylate and activate calmodulin-dependent protein kinase IV, at least \(\textit{in vitro}\). The sites of regulatory phosphorylation modified by CaMKI kinase have been shown to be Thr\(^{177}\) (CaMKI (45)) and Thr\(^{396}\) (calmodulin-dependent protein kinase IV (46)), which exactly align with Thr\(^{172}\) on AMPK. We have recently shown that, although AMPKK and CaMKI kinase are distinct enzymes, AMPKK can slowly phosphorylate and reactivate CaMKI, while CaMKI kinase can slowly phosphorylate and reactivate AMPK (27). These observations are entirely consistent with our identification of Thr\(^{172}\) as the regulatory site on AMPK, given the relatively high sequence similarity of AMPK and CaMKI within the activation segment. Whether AMPKK can phosphorylate and activate other protein kinases more distinctly related to AMPK than CaMKI remains to be determined.

Acknowledgments—We thank Fiona Smith for assistance with production of anti-\(\beta\) subunit antibodies; Tricia Cohen for PP1 and PP2C; and David Barford, Steve Keyse, and Nick Morrice for LAR, CL-100, and FP2A, respectively.

REFERENCES
1. Hardie, D. G., Carling, D., and Halford, N. G. (1994) \textit{Semin. Cell Biol.} 5, 409–416
2. Clarke, P. R., and Hardie, D. G. (1990) \textit{EMBO J.} 9, 2439–2446
3. Gillespie, J. G., and Hardie, D. G. (1990) \textit{FEBS Lett.} 296, 59–62
4. Munday, M. R., Campbell, D. G., Carling, D., and Hardie, D. G. (1991) \textit{Eur. J. Biochem.} 175, 331–338
5. Davies, S. F., Carling, D., Munday, M. R., and Hardie, D. G. (1991) \textit{Eur. J. Biochem.} 203, 615–623
6. Sim, A. T. R., and Hardie, D. G. (1988) \textit{FEBS Lett.} 233, 294–298
7. Carling, D., and Hardie, D. G. (1989) \textit{Biochem. Biophys. Acta.} 1012, 81–86
8. Corton, J. M., Gillespie, J. G., and Hardie, D. G. (1994) \textit{Curr. Biol.} 4, 315–324
9. Davies, S. P., Hawley, S. A., Woods, A., Carling, D., Haystead, T. A. J., and Hardie, D. G. (1994) \textit{Eur. J. Biochem.} 223, 351–357
10. Stapleton, D., Guo, G., Michell, B. J., Widmer, J., Mitchellall, K., Teh, T., S. A. Hawley, M. Davison, A. Woods, S. P. Davies, R. K. Beri, D. Carling, and D. G. Hardie, unpublished observations.
Characterization of AMP-activated Protein Kinase Kinase

27887

House, C. M., Witters, L. A., and Kemp, B. E. (1994) *J. Biol. Chem.* 269, 29343–29346

Carling, D., Clarke, P. R., Zammit, V. A., and Hardie, D. G. (1989) *Eur. J. Biochem.* 186, 129–136

Carling, D., Aguan, K., Woods, A., Verhoeven, A. J. M., Beri, R. K., Brennan, C. H., Sidebottom, C., Davison, M. D., and Scott, J. (1994) *J. Biol. Chem.* 269, 11442–11448

Mitchelhill, K. I., Stapleton, D., Gao, G., House, C., Michell, B., Katsis, F., Witters, L. A., and Kemp, B. E. (1994) *J. Biol. Chem.* 269, 2361–2364

Stapleton, D., Mitchelhill, K. I., Gao, G., Widmer, J., Michell, B. J., Teh, T., House, C. M., Fernandez, C. S., Cox, T., Witters, L. A., and Kemp, B. E. (1996) *J. Biol. Chem.* 271, 611–614

Gao, G., Widmer, J., Stapleton, D., Teh, T., Cox, T., Kemp, B. E., and Witters, L. A. (1995) *Biochim. Biophys. Acta.* 1266, 73–82

Beri, R. K., Marley, A. E., See, C. G., Sopwith, W. F., Aguan, K., Carling, D., Scott, J., and Carey, F. (1994) *FEBS Lett.* 356, 117–121

Celenza, J. L., Eng, F. J., and Carlson, M. (1989) *Mol. Cell. Biol.* 9, 5045–5054

Fields, S., and Song, O. K. (1989) *Nature* 340, 245–246

Yang, X., Jiang, R., and Carlson, M. (1994) *EMBO J.* 13, 5878–5886

Yang, X., Hubbard, E. J., and Carlson, M. (1992) *Science* 257, 680–682

Celenza, J. L., and Carlson, M. (1986) *Science* 233, 1175–1180

Woods, A., Munday, M. R., Scott, J., Yang, X., Carlson, M., and Carling, D. (1994) *J. Biol. Chem.* 269, 19509–19515

Alessi, D. R., Street, A. J., Cohen, P., and Cohen, P. T. W. (1993) *Eur. J. Biochem.* 213, 1055–1066

Streuli, M., Krieger, N. X., Hall, L. R., Schlossmann, S. F., and Saito, H. (1988) *J. Exp. Med.* 168, 1553–1562

Keyse, S. M., and Emstein, E. A. (1992) *Nature* 359, 644–647

Cohen, P., Alemany, S., Hemmings, B. A., Resink, T. J., Stralfors, P., and Lim, T. H. (1988) *Methods Enzymol.* 159, 390–408

Dale, S., Wilson, W. A., Edelman, A. M., and Hardie, D. G. (1994) *FEBS Lett.* 361, 191–195

Mitchelhill, K. I., Gao, G., Carling, D., Aguan, K., Woods, A., Verhoeven, A. J. M., Beri, R. K., Brennan, C. H., Sidebottom, C., Davison, M. D., and Scott, J. (1994) *J. Biol. Chem.* 269, 11442–11448