Isoform-specific Purification and Substrate Specificity of the 5'-AMP-activated Protein Kinase*

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The 5'-AMP-activated protein kinase (AMPK) mediates several cellular responses to metabolic stress. Rat liver contains at least two isoforms of this enzyme, either α1 or α2 catalytic subunits together with a β and γ noncatalytic subunits in a trimeric complex. The α1 isoform is purified using a peptide substrate affinity chromatography column with ADR1 (222–234)A77R86–87 (LKRSTRPSFSAQ), corresponding to the cAMP-dependent protein kinase phosphorylation site in the yeast transcriptional activator of the ADH2 gene, ADR1. This peptide is phosphorylated at Ser281 by AMPK-α1 with a Km of 3.8 μM and a Vmax of 4.8 μmol/min/mg compared to the commonly used rat acetyl-CoA carboxylase (73–87)A77R86–87 peptide substrate, HMRSAMGLHLVKRR, with a Km of 33.3 μM and a Vmax of 8.1 μmol/min/mg. Thus, the AMPK exhibits some overlapping specificity with the cAMP-dependent protein kinase. The rat liver AMPK α1 isoform has a Kcat 250-fold higher than the AMPK α2 isoform isolated from rat liver. The AMPK α1 isoform readily phosphorylates peptides corresponding to the reported AMPK phosphorylation sites in rat, chicken, and yeast acetyl-CoA carboxylase and rat hydroxymethylglutaryl-CoA reductase but not phosphorylation kinase. Based on previous peptide substrate specificity studies (Dale, S., Wilson, W. A., Edelman, A. M., and Hardie, G. (1995) FEBS Lett. 361, 191–195) using partially purified enzyme and variants of the peptide AMARAASAAALARRR, it was proposed that the AMPK prefers the phosphorylation site motif P(X, βXXS/TXXXF) (Φ, hydrophobic; β, basic). In good AMPK α1 peptide substrates, a hydrophobic residue at the P-4 position is conserved but not at the P-4 position. Oxidation of the Met residues in the rat acetyl-CoA carboxylase (73–87)A77R86–87 peptide increased the Km 6-fold and reduced the Vmax to 4% of the reduced peptide.

The 5'-AMP-activated protein kinase (AMPK) is related to the yeast snf1p subfamily of protein kinases (1) and consists of a catalytic α subunit (63 kDa) and noncatalytic β (40 kDa) and γ (38 kDa) subunits. The AMPK phosphorylates a number of key enzymes involved in the control of lipid metabolism, acetyl-CoA carboxylase, hydroxymethylglutaryl (HMG)-CoA reductase, and hormone-sensitive lipase (2). Because it is activated by elevation of intracellular AMP caused by arsenite and heat shock, it is thought to function primarily in stress responses (3, 4). The activation of the AMPK by AMP results from three contributing mechanisms: direct allosteric activation of the enzyme, AMP activation of an upstream kinase kinase (5, 6); and AMP inhibition of AMPK diphosphorylation (7). Studies on HMG-CoA reductase regulation have reinforced the concept that the AMPK plays a role in stress responses, since mutation of Ser271, the AMPK phosphorylation site in HMG-CoA reductase, to Ala blocked phosphorylation by the AMPK and reduction in HMG-CoA reductase activity caused by ATP depletion but did not affect the regulation of HMG-CoA reductase at the transcriptional level (8). In yeast, the snf1p kinase controls invertebrate transcription in the adaptive response to growth on non-glucose sugars (9) as well as the regulation of acetyl-CoA carboxylase (1, 10), but it is not known whether the AMPK has a similar function.

Recently, we found that multiple isoforms of the AMPK are present in liver (11, 12). A distinct gene accounts for ~90% of the activity in liver extracts (11). The two isoforms of the AMPK are 90% identical in the catalytic core region and 60% identical in their COOH-terminal tails. Since the AMPK is a multisubstrate protein kinase, its substrate specificity has been of particular interest. Previous studies have shown that an analog of the major AMPK phosphorylation site in rat acetyl-CoA carboxylase, or SAMS peptide, H2″MRSaMS7GLHLVKRR, is a relatively specific substrate for the AMPK. Previous specificity studies performed on the AMPK have used partially purified AMPK likely to have contained a mixture of the α1 and α2 isoforms (13–15). Using synthetic peptides based on the SAMS peptide sequence, it was reported that the enzyme has a requirement for two hydrophobic residues at the P-4 and P-5 positions and a basic residue at either the P-2 and/or -4 site (14). These findings have recently been extended using substitutions in the SAMS peptide variant, AMARAASAAALARRR, with partially purified enzyme (15).

We have reexamined the specificity of the AMPK in the light of the presence of multiple isoforms of the enzyme. In this paper, we report the purification of the AMPK isoforms from rat liver and compare their substrate specificities. Using a wide range of peptide substrates, we confirm the importance of a hydrophobic residue at the P-5 position, but not at the P-4 position. Surprisingly, synthetic peptides corresponding to the cAMP-dependent protein kinase phosphorylation site, Ser281, in the yeast transcriptional factor ADR1 are phosphorylated with Km values 10-fold lower than that of the SAMS peptide and can be used for selective substrate affinity purification of the liver α1 isoform. The AMPK α2 isoform isolated from rat...
liver has a very low $K_{m}$ compared to $\alpha_1$ using protein or peptide substrates.

**EXPERIMENTAL PROCEDURES**

**Enzyme Purification from Porcine and Rat Liver**—The porcine liver AMPK was purified by a modification of the procedure reported for rat liver (14). The corned liver (1 kg) was homogenized in a Waring blender with 4 volumes of buffer. A 2.5–7.0% (v/v) polyethylene glycol 6000 fraction was prepared, and the resultant fraction was batched onto 1500 ml of DEAE-cellulose (Whatman) and eluted with buffer containing 0.25 M NaCl (2000 ml). The eluate was chromatographed on 150 ml of Blue Sepharose (Pharmacia Biotech Inc.) and eluted with buffer containing 1 M NaCl. The enzyme fraction was concentrated and desalted by 10% (w/v) polyethylene glycol 6000 prior to affinity chromatography. The peptide substrate affinity column was washed with the same buffer containing 0.1% (v/v) Triton X-100 and 0.5 M NaCl, and the AMPK was eluted with buffer containing 2 M NaCl and 30% (v/v) ethylene glycol. Protein concentration was assayed by the method of Lowry et al. (17) using detergent-compatible protein assay reagents (Bio-Rad).

The rat AMPK was purified (250 g of liver) by a modification of the above procedure used for porcine liver preparations. DEAE-cellulose (500 ml) was used, the eluate was precipitated with 40% saturated (NH$_4$)$_2$SO$_4$, and the resultant pellet was resuspended in 400 ml of buffer and chromatographed on 100 ml of Blue Sepharose. A further wash was introduced to the peptide substrate affinity chromatography step using buffer containing 0.1% (v/v) Triton X-100 and 2 M NaCl immediately prior to elution with 2 M NaCl and 30% (v/v) ethylene glycol.

**AMPK Assay**—AMPK assays were performed using the SAMS peptide substrate, HMRAMSGLHLVKKR-amide (13). 5'-AMP was included at either 20 or 70 $\mu$M as indicated. The enzyme was diluted in buffer (50 mM Tris-HCl (pH 7.5), 0.05% (v/v) Triton X-100) prior to assay, and reactions were initiated by adding 10 ml of enzyme to the reaction mixture. The phosphopeptide was isolated by withdrawing 30-ml aliquots and applying them to Whatman P-81 papers (18).

**Mass Spectrometry Analysis**—Matrix-assisted laser desorption ionization mass spectrometry was performed (Voyager DE mass spectrometer; Perkin-Elmer). For peptides, Perceptive Biosystems Inc.) utilizing delayed extraction technology. Fragment ions were introduced to the mass spectrometer by electrospray ionization. Peptide substrates were affinity chromatography (22), we screened a selected library of synthetic peptides, including analogs of proteins not known to be substrates for the AMPK. Initially, only partially purified enzyme (purified to the Blue Sepharose column) was used so that we could assess the specificity of the enzymes for the AMPK. The peptide sequences tested included the SAMS peptide and peptides derived from the myosin light chains, ADR1, glycogen synthase, and phospholemman (23), a myocardial protein substrate for protein kinase C and cAMP-dependent protein kinase (Table I). The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK 4 was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycosyl synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further.

**RESULTS AND DISCUSSION**

**AMPK Purification**—We were unsuccessful in obtaining purified AMPK from rat liver using the procedure reported previously (16). Since protein kinases can sometimes be purified by peptide substrate affinity chromatography (22), we screened a selected library of synthetic peptides, including analogs of proteins not known to be substrates for the AMPK. Initially, only partially purified enzyme (purified to the Blue Sepharose column) was used so that we could assess the specificity of the enzymes for the AMPK. The peptide sequences tested included the SAMS peptide and peptides derived from the myosin light chains, ADR1, glycogen synthase, and phospholemman (23), a myocardial protein substrate for protein kinase C and cAMP-dependent protein kinase (Table I). The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further. The glycogen synthase peptide PLSRTLSVAAKK was phosphorylated in an AMP-dependent manner at ~40% of the rate of the SAMS peptide, but this peptide is an excellent substrate for a number of protein kinases 24 and was not investigated further. The phospholemman peptides tested were poor substrates and were not investigated further.

When the ADR1(222–234)P229 peptide was coupled to a Pharmacia HiTrap column, the AMPK was bound avidly and required 2 M NaCl plus 30% (v/v) ethylene glycol for elution. Because the enzyme bound so tightly, it was possible to load the enzyme in buffer containing 0.5 M NaCl, an important step in the purification. A balance sheet of the AMPK purification is shown in Table I. The resultant purified AMPK consisted of a 63-kDa $\alpha$-catalytic subunit (which proved to be the $\alpha_2$ subunit, see below) and 40-kDa (8) and 38-kDa (y) noncatalytic subunits.

L. A. Witters, unpublished data.
5′-AMP-activated Protein Kinase

Table I
Peptide library screen with AMPK

| Peptide   | Sequence                | +AMP     | +AMP | Relative activity |
|-----------|-------------------------|----------|------|-------------------|
| SAMS      | HMRSSAMGSGLHVKRR        | 12,314   | 44,163| 100               |
| smMLC(6–23) | KAKTTKRPQRTSNVFS        | 1,959    | 7,911 | 18                |
| smMLC(1–23)Y | SSKRKAYTTKRPQRTSNVFA    | 1,831    | 6,612 | 15                |
| ADR1(225–234) | LTRRASFSQA            | 7,218    | 19,021| 43                |
| ADR1(222–234)P229 | LKTLTRPSFSQA         | 8,645    | 22,805| 52                |
| GS(1–12)/AK11,12 | FLRHTLSVAARK       | 4,174    | 17,428| 40                |
| PLM(58–72) | GTFRSSIRLSTRRR         | 550      | 476   | 1.1               |

*smMLC, smooth muscle myosin light chain (24); GS, glycogen synthase (24); PLM, phospholemman; cAPK, plasma membrane substrate (23).

Table II
Purification balance sheet for rat liver AMPK α₁

| Sample          | Volume | Activity | Protein | Specific activity | Yield | Purification |
|-----------------|--------|----------|---------|-------------------|-------|--------------|
|                 | ml     | µmol/min | mg      | nmol/min/mg       | %     |             |
| Pre-BS*         | 400    | 10.46    | 7750    | 1.35              | 100   | 1            |
| BS eluate       | 120    | 3.839    | 60      | 64.00             | 36.7  | 47           |
| Pre-ADR1 AC     | 8      | 3.177    | 38.4    | 82.70             | 30.4  | 61           |
| ADR1 AC eluate  | 0.75   | 1.822    | 0.124   | 14.697            | 17.4  | 10,887       |

*Pre-BS, active DEAE-cellulose fraction that had been concentrated by 40% saturated (NH₄)₂SO₄ precipitation; ADR1 AC, peptide affinity column; Pre-BS, active DEAE-cellulose fraction that had been concentrated by 40% saturated (NH₄)₂SO₄ precipitation. ADR1 AC, peptide affinity column.

(26) (Fig. 1A). The AMPK was not evident on SDS-PAGE by protein stain until the final step of purification (Fig. 1A). Western blot analysis with anti-α₁ antibody suggested the presence of some minor proteolytic fragments in the purified enzyme (Fig. 1B). In some preparations, the AMPK was associated with high molecular mass material that corresponded to non-muscle myosin, as assayed by tryptic peptide sequencing (data not shown). Typically, an apparent purification of ~11,000-fold with a yield of 15% and a recovery of at least 90 µg (range, 90–200 µg) of enzyme was obtained from 250 g of rat liver (Table II). Approximately 1 kg of porcine liver was required to yield a similar amount of purified enzyme. The fold purification may be an overestimate due to the presence of uncharacterized inhibitory material in the early fractions. Only the α₁ isoform of the AMPK and not the α₂ isoform binds to the peptide affinity column. The reason for this selectivity is not known, but it allowed us to prepare a partially purified fraction of AMPK α₂ using the peptide affinity column as the first step in purification. The specific activity of the purified rat AMPK (α₁ isoform) ranged from 8 to 27 µmol/min/mg without any obvious difference in the SDS-PAGE profile. This may reflect differences in the state of activation of the isolated enzyme due to phosphorylation by the upstream kinase kinase (3, 5). Using electro spray mass spectrometry, we have observed ions corresponding to the β and γ subunits but not the α subunit; therefore, we have been unable to assess the state of endogenous phosphorylation of the α subunit directly for different preparations.

The avidity of the enzyme for the peptide bound to the Pharmacia HiTrap resin was greater than could be expected from the free peptide binding to the enzyme (K_m ~ 3 µM). It seems reasonable that the enhanced binding is due in part to the aminohexanoic acid linker between the peptide and the resin. In the case of the cAMP-dependent protein kinase, there is a hydrophobic pocket between the D and G helices that is responsible for high affinity binding of the peptide inhibitor PKI (5–22) at the P^11 position. Since the ADR1(222–234)P229 peptide, LKTLTRPSFSQA, is linked through the amide residues on its NH₂ terminus or Lys residues, it is possible that the hydrophobic linker group has been fortuitously juxtaposed to a related hydrophobic pocket on the AMPK.

Peptide Substrate Specificity of AMPK Isoforms—Initially, we assessed whether purified α₁ isoform-phosphorylated synthetic peptides corresponded to reported phosphorylation sites for the AMPK in acetyl-CoA carboxylase, HMG-CoA reductase, and phosphorylase kinase. The SAMS peptide rat acetyl-CoA carboxylase (73–87)/R_66–87 was phosphorylated with a K_m of 33 µM and a V_max of 8.1 µmol/min/mg compared with a K_m of 59 µM reported previously (14) (Table III). The corresponding chicken acetyl-CoA carboxylase peptide cACoAC (74–88)/R_87–88 was phosphorylated with a similar K_m of 43 µM but a higher V_max of 23 µmol/min/mg. Increasing the length of the cACoAC (74–88) peptide to 25 residues, cACoAC (74–98) reduced the K_m from 43 µM to 12 µM but also decreased the V_max from 23 to 4 µmol/min/mg (Table III). The other chicken and yeast acetyl-CoA carboxylase peptides were also tested as substrates, with yeast acetyl-CoA carboxylase (1151–1165)/R_1164–1165 being the better substrate, with a K_m of ~10 µM and a V_max comparable with that of the SAMS peptide. Interestingly, the phosphorylase kinase α-subunit peptide (1014–1023), FRRLSISES, was not a substrate, nor was the analog FRRLSISE, for either AMPK α₁ or AMPK α₂. Despite an earlier report that phosphorylase kinase was a substrate for the AMPK (27), albeit weaker than for the α-subunit-dependent protein kinase, the phosphorylase kinase peptide was not a substrate. Thus, the reported phosphorylation of the phosphorylase kinase α-subunit may have been due to a contaminating protein kinase or phosphorylation of an alternative site on phosphorylase kinase. The peptides cACoAC (1209–1229)/R_1222–23 and cACoAC (1194–1208)/R_1207–8 were phosphorylated with K_m values somewhat higher than those of the SAMS peptide and with similar V_max values (Table III); however, yeast acetyl-CoA carboxylase (1136–1150)/R_1149–50 was not a substrate for the AMPK. These results indicate that the AMPK is capable of phosphorylating a range of substrates.
of peptides corresponding to its reported phosphorylation site sequences, but not phosphorylase kinase.

**ADR1 Peptide Phosphorylation**—The ADR1 peptide ADR1(222–234)P, LKKLTRRPSFSQA, was phosphorylated with an apparent $K_m$ of 3.7 μM. This is 8-fold lower than the $K_m$ for the SAMS peptide and 3-fold lower than the $K_m$ for the AMARA peptide (AMARAASAAALARR) recently reported (15). The parent ADR1(225–234) peptide was phosphorylated with an apparent $K_m$ of 5 μM, indicating that the Pro substitution at residue 229 was not important. Since the ADR1 peptide contained three potential phosphorylation sites, we determined the site of phosphorylation. Following cleavage of the phospho-ADR1 peptide with chymotrypsin, the phosphopeptide mass was 1127.84, measured by electrospray mass spectrometry, indicating that chymotrypsin had cleaved the Leu-Thr bond to give the monophospho form of the peptide TRRPSFSQA. Further, the yield of phenylthiohydantoin-derivatives at cycles 1 and 7 was as expected for Thr and Ser, respectively, but the recovery of phenylthiohydantoin-Ser at cycle 5 was poor. Since phosphoserine undergoes near complete β-elimination during the Edman sequencing cycle, this is consistent with Ser to phospho-Ser as the site of phosphorylation. It is apparent that the presence of phospho-Ser prevented the chymotryptic cleavage at the Phe-Ser bond. The phospho-ADR1 peptide LKKLTRRPS(F)FSQA (mass, 1609.4; theoretical, 1610.8) was also digested to completion with carboxypeptidase Y and was analyzed by time of flight mass spectrometry (Fig. 2B). Following digestion, the residual phosphopeptide corresponding to the peptide LKKLTRRPS(F)FSQA had a mass of 1324.4 (theoretical, 1325.7). The same starting material was digested with trypsin. Following digestion, the residual phosphopeptide corresponding to the peptide RPS(F)FSQA had a mass of 871.6 (theoretical, 871.3) (Fig. 2C). These results demonstrate that neither the Thr nor the Ser232 is phosphorylated, in agreement with the Edman sequencing result.

Since ADR1 is phosphorylated at Ser230 by the cAMP-dependent protein kinase, this raised the possibility that the specificity properties of the two protein kinases may be related. The high affinity binding of the PKI(5–22) peptide to the cAMP-dependent protein kinase depends on Phe11 at the P6 position and an Arg at the P–6 position. We therefore extended the NH$_2$ terminus of the SAMS peptide rat acetyl-CoA carboxylase (63–84)PH$_2$GGR to include both a Phe at the P–11 position and Gly-Arg at the P–6 position, analogous to the PKI(5–22) peptide; but these substitutions did not improve the kinetics of peptide phosphorylation (Table III). These results demonstrate that fusing the high affinity peptide specificity determinants of PKI onto the SAMS peptide does not make it a better substrate for the AMPK. Previously, it was suggested

### Table III

**Comparison of kinetics of peptide phosphorylation**

Peptide phosphorylation was assayed as described under “Experimental Procedures.” AMP (70 μM). The phosphorylation of the peptides was determined using purified enzyme, and the $V_{max}$ values were corrected using the SAMS peptide as an internal standard. Phosphorylation sites in these peptides are shown in boldface, and substituted residues are underlined.

| Peptide | Sequence | AMP | $K_m$ | $V_{max}$ |
|---------|----------|-----|-------|----------|
| rACoAC | (73–87)$^{67}$R$^{68–77}$ | HMR-SAMSGLHLVKRR | + | 33.3 ± 2.7 | 8.1 ± 1.5 |
| rACoAC | (63–84)P$^{75}$G$^{76}$R$^{77}$ | ISALQFLAGMRSMSSGLHLV | + | 27.0 ± 6.0 | 1.6 ± 0.16 |
| HMG-CoAR | (860–875) | HLVKSHMINRSSKINL | + | 46.0 ± 1.3 | 5.0 ± 0.8 |
| ADR1 | (225–234)P$^{229}$ | LKKLTRRPFSQ | + | 3.7 ± 0.5 | 4.8 ± 0.3 |
| cACoAC | (74–98) | HMRP-SMSGLHLVKQRDRKKDVQ | + | 12.3 ± 6.8 | 4.1 ± 0.7 |
| cACoAC | (79–98) | MMGLHLVKQRDRKKDVQ | + | Not a substrate | |
| cACoAC | (74–88)R$^{87–88}$ | HMRP-SMSGLHLVKRR | + | 42.5 ± 2.9 | 22.6 ± 3.8 |
| cACoAC | (74–88)S$^{87–88}$ | HMRP-SMSGLHLVKRR | + | 36.4 ± 12.7 | 11.5 ± 2.8 |
| cACoAC | (74–88)A$^{74–88}$ | HMRP-SMSGLHLVKRR | + | 61.8 ± 15.7 | 11.9 ± 1.4 |
| cACoAC | (74–98)$^{77}$A$^{79}$ | HMRPASMSGLHLVKQRDRKKDVQ | + | 49.0 ± 4.7 | 5.7 ± 1.1 |
| cACoAC | (1194–1208)R$^{1207–1208}$ | PTNLRMFSASNLR | + | 63.0 ± 15.0 | 4.4 ± 1.2 |
| cACoAC | (1209–1233)R$^{1222–1223}$ | GMTHAVSVDVLLRR | + | 55.4 ± 5.0 | 3.8 ± 1.0 |
| yACoAC | (1151–1165)R$^{1164–1165}$ | GMNRKBSVDSLRR | + | 9.7 ± 1.2 | 8.1 ± 1.0 |
| yACoAC | (1136–1150)S$^{1149–1150}$ | PSAAFTPFFTVKSR | + | Not a substrate | |
| Phos kinase a | 5101–1023 | PFRLLISITF | + | Not a substrate | |

$^{a}$ rACoAC and yACoAC, rat and yeast acetyl-CoA carboxylase peptides, respectively; Phos kinase a, phosphorylase kinase a subunit peptide; HMG-CoAR, HMG-CoA reductase peptide.
that the presence of a hydrophobic residue at the P-4 position was important for phosphorylation by the AMPK (14), based on substitutions in the SAMS peptide. However, in the case of the ADR1 peptide, a Gln is present at this position; it is therefore not consistent with the requirement for NH2-terminal residues, even though it is shown in boldface, and substituted residues are underlined.

Effect of methionine oxidation on peptide substrate phosphorylation

Peptides were oxidized as described under "Experimental Procedures." The phosphorylation of the peptides was determined using purified enzyme, and the Vmax values were corrected using the SAMS peptide as an internal standard. The kinetic values for the reduced parent peptides, given in parentheses (Column 1), are taken from Table III. Phosphorylation sites in these peptides are shown in boldface, and substituted residues are underlined.

Comparison of kinetics of peptide phosphorylation with AMPK α1 and AMPK α2

The kinetics of phosphorylation of SAMS, HMG-CoAR (861–876), and ADR1(222–234)(P229) peptides by AMPK α1 and AMPK α2 were compared. AMPK α1 and AMPK α2 were purified from rat liver as described under "Experimental Procedures." Phosphorylation sites in these peptides are shown in boldface, and substituted residues are underlined. Velocities were calculated for the catalytic subunit of both isoforms, expressed as Kcat and the AMPK α1 holoenzyme, expressed as Vmax. Vmax values were not determined for the α2 isoform because the concentration of the α2 holoenzyme was not measured, only that of the catalytic subunit.

Phosphorylation of Oxidized SAMS Peptide—The effect of oxidation on SAMS peptide phosphorylation was investigated because the SAMS peptide contains 2 Met residues. The SAMS peptide was exquisitely sensitive to oxidation (Table IV) with Met at the P-4 position increasing 6-fold and the Vmax reduced to 6%. In general, oxidation of peptides with Met at the P-5 position reduced their rates of phosphorylation; however, the Vmax was not always most affected, because peptide yeast acetyl-CoA

Peptide Sequence AMPK isom. Km Vmax Kcat

rCoAc(73–87)A77R86–87 HMRSAMSGLLVKRR α1 46.0 ± 0.6 14.0 ± 1.8 30.6

ADR1(222–234)(P229) LKKLTRPS(P)FAQ α1 4.5 ± 0.7 9.3 ± 1.2 20.7

HMG-CoAR (861–876) HLVKSHMHRKSKINL α1 39 4.2 8.7

that the presence of a hydrophobic residue at the P-4 position was important for phosphorylation by the AMPK (14), based on substitutions in the SAMS peptide. However, in the case of the ADR1 peptide, a Gln is present at this position; it is therefore not consistent with the requirement for NH2-terminal residues, even though it is shown in boldface, and substituted residues are underlined. The ADR1 peptide does, however, have an Arg at position P-3 and a Leu at position P-5 consistent with the requirement of a basic residue and hydrophobic residue at these positions, respectively, as was assessed with analogs of the SAMS peptide (14). Truncation of the cCoAc peptide to Met78 abolished its capacity to act as a substrate. This is consistent with the requirement for NH2-terminal residues, including a hydrophobic residue at the P-5 position. Substitution of the chicken acetyl-CoA carboxylase peptide (74–88)R87–88 with Ala and Ile at positions P-2 and P-1, respectively, reduced the Vmax for peptide phosphorylation slightly without having a marked effect on the apparent Km. The chicken sequence has a Pro at position P-3, and substitution of this with Ala had no effect on the kinetics of peptide phosphorylation.
phosphorylation of acetyl-CoA carboxylase by AMPK is not observed. The amount of $[^32]P$ phosphate transferred to the acetyl-CoA carboxylase was measured as described under “Experimental Procedures” and quantitated using liquid scintillation counting of excised SDS-PAGE gel bands.

carboxylase (1151–1165)R$^{1164–1165}$GMNRAVSVDLSYRR, had an increased $K_m$ from 9.7 to 181 $\mu$M following oxidation. The cACoAC (1194–1208)$^{2207–8}$PTLNRMSFASNLRRR had only a 2-fold increase in its $K_m$, following oxidation and a negligible increase in the $V_{max}$. These results indicate that Met at the $P^{-7}$ position is not as critical for substrate recognition as the $P^{-5}$ position.

Comparison of AMPK $\alpha_1$ and $\alpha_2$ Specificities—It was of interest to compare the specificities of the AMPK $\alpha_1$ and $\alpha_2$ isoenzymes. The SAMS peptide, rat acetyl-CoA carboxylase (73–87)A$^{77}$R$^{86–87}$ and HMG-CoA reductase (861–876) were all phosphorylated by AMPK $\alpha_1$, but with greatly reduced velocities (Table V). The apparent $K_m$ values for rat acetyl-CoA carboxylase (73–87)A$^{77}$R$^{86–87}$ and HMG-CoA reductase (861–876) were three-fold higher than with AMPK $\alpha_2$; in contrast, the apparent $K_m$ for ADR1 (222–234)$^{P^{229}}$ was the same for both isoenzymes. Despite having essentially the same $K_m$ for the ADR1 (222–234)$^{P^{229}}$, the affinity column is highly selective for the $\alpha_1$ isoform. This may occur with a mechanism where the rate constants for dissociation or association of the $\alpha_2$/peptide complex are higher or lower than for the $\alpha_1$/peptide complex but subsequent steps in the reaction path have a more dominant effect on the $K_m$. The large differences in the velocities observed between the isoenzymes with peptide substrates were also seen with purified acetyl-CoA carboxylase as substrate (Fig. 3). As shown in Fig. 3, both isoforms of rat liver acetyl-CoA carboxylase (AcoAC-280 kDa and AcoAC-265 kDa, respectively) are phosphorylated by AMPK $\alpha_1$ (28). These results suggest that the AMPK $\alpha_2$ isoform may have a specificity similar to that of AMPK $\alpha_1$ but is in an inactive form when isolated from the liver. Analysis of the $\alpha_2$ by immunoblotting (Fig. 1D) reveals that it is not proteolyzed. Further, it seems unlikely that it is inactivated by postextraction dephosphorylation because the $\alpha_1$ isoform is active in the extract. Since there are such large differences in the velocities of the AMPK $\alpha_1$ and $\alpha_2$ isoforms isolated from rat liver, then the earlier specificity studies (14, 15) using partially purified enzyme containing both isoenzymes predominantly reflected the activity of the AMPK $\alpha_1$ isoform.

The AMPK $\alpha_1$ isoform is likely to be important in the control of hepatic lipid metabolism but it seems more likely that the AMPK $\alpha_2$ isoform is under separate control. The low velocity of the $\alpha_2$ isoform is consistent with its being in an inactive form, possibly dephosphorylated on the activation loop. It is not yet known whether both isoforms of the AMPK share the same upstream-activating kinases. While our results demonstrate that the AMPK shares a higher level of peptide substrate specificity overlap with the cAMP-dependent protein kinase than was previously recognized, they are nevertheless clearly different. The amino acid substitutions that ensure very high affinity binding of PKI had no effect on recognition of the SAMS peptide substrate by AMPK.

REFERENCES
1. 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
2. Hardy, D. G. (1992) Biochim. Biophys. Acta 1123, 231–238
3. Moore, F., Weekes, J., and Hardie, G. D. (1991) Eur. J. Biochem. 199, 691–697
4. Gillespie, J. G., and Hardie, D. G. (1992) FEBS Lett. 306, 59–62
5. Weekes, J., Hawley, S. A., Corton, J., Shugar, D., and Hardie, D. G. (1994) Eur. J. Biochem. 219, 751–757
6. Hawley, S. A., Selbert, M. A., Goldstein, E. G., Edelman, A. M., Carling, D., and Hardie, D. G. (1995) J. Biol. Chem. 270, 27186–27191
7. Davies, S. P., Helps, N. R., Cohen, P. T. W., and Hardie, D. G. (1995) FEBS Lett. 377, 421–425
8. Sato, R., Geldstein, J. L., and Brown, M. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9261–9265
9. Colman, R. J., and Carlson, M. (1986) Science 233, 1175–1180
10. Woods, A., Munday, M. R., Scott, J., Yang, X., Carlson, M., and Carling, D. (1994) J. Biol. Chem. 269, 19509–19515
11. 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
12. Gao, G., Fernandez, C. S., Stapleton, D., Auster, A. S., Widmer, J., Dyck, J. R., Kemp, B. E., and Witters, L. A. (1996) J. Biol. Chem. 271, 8675–8684
13. Davies, S. P., Carling, D., and Hardie, D. G. (1989) Eur. J. Biochem. 186, 123–128
14. Weekes, J., Bell, K. L., Caudwell, F. B., and Hardie, D. G. (1995) FEBS Lett. 374, 325–329
15. Dale, S., Wilson, W. A., Edelman, A. M., and Hardie, G. (1995) FEBS Lett. 361, 191–195
16. Carling, D., Clarke, P. R., Zammit, V. A., and Hardie, D. G. (1989) Eur. J. Biochem. 186, 129–136
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
18. Kemp, B. E., Faux, M. C., Means, A. R., House, C., Tiganis, T., Hu, S.-H., and Witters, L. A. (1994) J. Biol. Chem. 269, 191–195
19. Pearson, R. B., Mitchelhill, K. I., and Kemp, B. E., in (1993) Protein Kinases: Frontiers in Molecular Biology (Woodgett, J. R., ed) pp. 30–67, IRL Press/Oxford University Press, Oxford
20. Pearson, R. B., Mitchelhill, K. I., and Kemp, B. E., in (1993) Protein Phosphorylation: A Practical Approach (Hardie, G. D., ed), pp. 265–291, Oxford University Press, Oxford, U. K.
21. Laemmli, U. (1970) Nature 227, 680–685
22. Tupper, J. P., and Witters, L. A. (1982) Biochim. Biophys. Acta 715, 162–169
23. Woodgett, J. R. (1991) Methods Enzymol. 200, 169–178
24. Palmer, C. J., Scott, B. T., and Jones, L. R. (1991) J. Biol. Chem. 266, 11126–11130
25. Kemp, B. E., and Pearce, P. R. (1991) Methods Enzymol. 200, 121–134
26. Denis, C. L., Kemp, B. E., and Zoller, M. J. (1991) J. Biol. Chem. 266, 17092–17095
27. Stapleton, D., Gao, G., Michell, B. J., Widmer, J., Michell, K. I., Teh, T., House, C. M., Witters, L. A., and Kemp, B. E. (1994) J. Biol. Chem. 269, 28243–28246
28. Carling, D., and Hardie, D. G. (1989) Biochim. Biophys. Acta 1012, 81–86
29. Bianchi, A., Evans, J. L., Iverson, A. J., Nordlund, A.-C., Watts, T. D., and Witters, L. A. (1990) J. Biol. Chem. 265, 1502–1509