Effects of 3-phosphoglycerate and other metabolites on the activation of AMP-activated protein kinase by LKB1-STRAD-MO25

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Ellingson WJ, Chesser DG, Winder WW. Effects of 3-phosphoglycerate and other metabolites on the activation of AMP-activated protein kinase by LKB1-STRAD-MO25. Am J Physiol Endocrinol Metab 292: E400–E407, 2007. First published September 19, 2006; doi:10.1152/ajpendo.00322.2006.—Skeletal muscle contraction results in the phosphorylation and activation of the AMP-activated protein kinase (AMPK) by an upstream kinase (AMPKK). The LKB1-STE-related adaptor (STRAD)-mouse protein 25 (MO25) complex is the major AMPKK in skeletal muscle; however, LKB1-STRAD-MO25 activity is not increased by muscle contraction. This relationship suggests that phosphorylation of AMPK by LKB1-STRAD-MO25 during skeletal muscle contraction may be regulated by allosteric mechanisms. In this study, we tested an array of metabolites including glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, 3-phosphoglycerate (3-PG), glucose 1-phosphate, glucose 1,6-bisphosphate, ADP, carnitine, acetylcarnitine, IMP, inosine, and ammonia for allosteric regulation. ADP inhibited both AMPK and LKB1-STRAD-MO25 activities, but probably is not important physiologically because of the low free ADP inside the muscle fiber. We found that 3-PG stimulated LKB1-STRAD-MO25 activity and allowed for increased AMPK phosphorylation. 3-PG did not stimulate LKB1-STRAD-MO25 activity toward the peptide substrate LKB1-tide. These results have identified 3-PG as an AMPK-specific regulator of AMPK phosphorylation and activation by LKB1-STRAD-MO25.

adenosine 5’-monophosphate-activated protein kinase; adenosine 5’-monophosphate-activated protein kinase kinase; metabolism; glycolysis; STK11

THE AMP-ACTIVATED PROTEIN KINASE (AMPK) is a master metabolic regulator found to regulate fatty acid oxidation (20, 43, 44) and glycogen uptake (2, 11, 17, 20) in skeletal muscle. AMPK senses changes in AMP-to-ATP ratios. An increase in AMP concentration causes a conformational change in AMPK. AMPK senses changes in AMP-to-ATP ratios. An increase in AMP concentration causes a conformational change in AMPK, which allows AMPK to be phosphorylated, and consequently activated, by an upstream kinase, AMPKK, composed of three complexed proteins [LKB1, STE-related adaptor (STRAD), and mouse protein 25 (MO25); see Refs. 9, 32, and 45]. AMPK must be phosphorylated by AMPKK on its activation loop at Thr172 for full activation.

AMPK is activated by the constitutively active kinase complex LKB1-STRAD-MO25 during skeletal muscle contraction (28, 29). AMPK activation increases during a bout of skeletal muscle contraction caused by endurance exercise (41), in situ stimulation (14, 15, 28, 39), or in vitro stimulation (11), whereas the apparent AMPKK activity does not change (14, 28). Furthermore, studies showing skeletal muscle contraction of both trained humans (23) and rats (14) yielded increased in AMPK phosphorylation in the face of no change or decreased measurable AMPKK activity. This relationship seems contradictory, given that one would expect an increased AMPKK activity to accompany the increased AMPK phosphorylation. These findings imply that muscle AMPKK becomes more active during contraction by mechanisms other than covalent modifications that could be detected in extracts of the muscle homogenate. Because LKB1-STRAD-MO25 can phosphorylate at least 14 downstream kinases, substrate level regulation for each of these is conceivable. Allosteric activation of AMPK phosphorylation by undefined modulators must also be considered as a mechanism of specific regulation of downstream targets.

To explore this issue, we considered changes that occur in the skeletal muscle metabolite concentrations during contraction. Previous studies have demonstrated exercise-induced changes in the concentrations of glucose 6-phosphate (G-6-P; see Refs. 12, 13, 16, 26, 30), fructose 6-phosphate (F-6-P; see Refs. 12, 26, 41, 42), fructose 1,6-bisphosphate (F-1,6-P2; see Refs. 13, 30, and 42), 3-phosphoglycerate (3-PG; see Ref. 6), glucose 1-phosphate (G-1-P; see Ref. 12), glucose 1,6-bisphosphate (G-1,6-P2; see Refs. 30 and 41), ADP (12, 26, 33), carnitine (Carn); see Refs. 3, 27, and 38), acetylcarnitine (Acarn; see Refs. 3, 27, and 38), IMP (12, 46, 47), inosine (5, 8, 47), and ammonia (12, 33). Initially, no consideration was given to intracellular compartmentalization or protein binding of the metabolites. In the case of ADP, only a small proportion of the total would be in the free ADP fraction of the sarcoplasm; therefore, additional concentrations were examined. We hypothesized that one or more of these metabolites would demonstrate allosteric control on AMPK activation by interacting either directly on the LKB1-STRAD-MO25 complex, or on AMPK itself, making it a better substrate for phosphorylation by LKB1-STRAD-MO25.

MATERIALS AND METHODS

Buffers. When applicable, dithiothreitol (DTT) and [γ-32P]ATP are added just before use. The following buffers were used: AMPK storage buffer: 50 mM Tris-HCl, 250 mM mannitol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.02% (wt/vol) Brij-35, and 10% vol/vol glycerol, pH 7.4 at 4°C; AMPK phosphorylation buffer: 100 mM HEPES, 200 mM NaCl, 20% glycerol, 2 mM EDTA, 12.5 mM MgCl2, 0.5 mM AMP, 0.5 mM ATP, and 2.0 mM DTT, pH 7.0; AMARA phosphorylation buffer: 40 mM HEPES, 80 mM NaCl, 8% glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl2, 0.2 mM AMP, 0.2 mM ATP, 0.33 mM AMARA peptide, and 0.05 μCi/μl [γ-32P]ATP, pH 7.0; Laemmli’s buffer (18); PBS: 140 mM NaCl, 2.7

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mM KCl, 2.1 mM KH2PO4, and 9.9 mM Na2HPO4, pH 7.3; PBST: PBS with 1% Tween 20.

Materials. General reagents were obtained from Sigma-Aldrich Chemical (St. Louis, MO) unless otherwise stated. G-6-P, F-6-P, F-1,6-P2, 3-PG, G-1-P, G-1,6-P2, ADP, Carn, Acarn, IMP, and inosine were obtained from Sigma-Aldrich Chemical. Ammonium acetate was obtained from Merck (Darmstadt, Germany). Recombinant LKB1-STRAD-MO25 lot no. 28640AU (0.1 mg/ml) and LKB1tide were obtained from Upstate (Charleston, VA). His-bind nickel-binding resin was obtained from Novagen (Madison, WI). Western blotting primary antibodies were obtained from Cell Signal-ling Technologies (Danvers, MA). Resins for chromatographic purification, secondary antibody for Western blotting, and enhancing chemiluminescence (ECL) detection solution were obtained from Amer-sham Biosciences (Piscataway, NJ).

Generation and purification of recombinant AMPK. Bacteria expressing recombinant αβ2γ2- and αβ1γ1-AMPK were prepared as previously described (21, 22, 34, 35). Recombinant αβ2γ2- and αβ1γ1-AMPK was extracted and purified by nickel affinity chroma-tography (36). After buffer exchange into AMPK storage buffer, recombinant AMPK was concentrated to 1 μg/μl before use for experimentation. Recombinant α-312 AMPK protein for Western blot analysis was likewise obtained as previously described (7, 14, 35, 36).

AMPKK and phosphorylated-AMPK activity assays. In all cases, unless otherwise stated, the AMPK used for experimentation was αβ2γ2-AMPK. AMPKK activity was measured in a two-step assay. In the first step, AMPK was phosphorylated and activated by LKB1-STRAD-MO25 (p-AMPK). In the second step, p-AMPK phosphorylated AMARA peptide (*) recombinant αβ2γ2-AMPK was diluted 1:19 in water and αβ1γ1 was diluted 1:9 in water. Recombinant LKB1-STRAD-MO25 (0.1 μg/μl) was diluted 1:39 in AMPK storage buffer. The metabolites G-6-P, F-6-P, F-1,6-P2, 3-PG, G-1-P, G-1,6-P2, ADP, Carn, Acarn, inosine, IMP, and ammonia were individually dissolved in water at 5 mM KCl, 2.1 mM KH2PO4, and 9.9 mM Na2HPO4, pH 7.3; PBST: PBS with 1% Tween 20.

Western blot. The first step of the AMPKK assay was performed, but with a 30-min incubation. After the incubation period, the reaction was stopped by addition of diluted 4× Laemmli’s buffer and water (1:1:2). Proteins in the reaction mixture were then separated by SDS-PAGE at 200 V for 50 min in 7.5% Tris-HCl, 30 μl/well (Criterion Precast Gels; Bio-Rad, Hercules, CA). Proteins were electro-photrolytically transferred from the gels to polyvinylidene difluoride membranes at 100 volts for 60 min. Membranes were blocked in PBST and 5% Blotting Grade Blocker Non-Fat Dry Milk for 1 h. Membranes were incubated overnight at 4°C in PBST, 5% blocking milk, and primary antibody. After incubation with a rabbit anti-P-AMPK antibody, membranes were washed two times with PBST for 10 min and two times with PBS for 5 min. Membranes were then incubated for 1 h at room temperature in PBST, 3% blocking milk, and a secondary antibody. After incubation with a horseradish peroxy-dase-linked anti-rabbit antibody, membranes were washed two times with PBST for 10 min and two times with PBS for 5 min. Membranes were covered with ECL Western Blotting Detection Reagent, enclosed in transparent plastic sheets, and visualized on Classic Blue Autoradiography film. Relative amounts of protein were quantified by measuring spot size and intensity with AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

Statistical analysis of the effects of each metabolite on AMPKK activity, assays testing effects of ADP and 3-PG on AMPKK and p-AMPK activity, assays of AMPKK activity against αβ2γ2-AMPK in the presence of 3-PG, Western blot analysis of 3-PG-mediated AMPKK and α-312 AMPK activation, and AMPKK activity assays against LKB1tide were all compared by one-way ANOVA. Post hoc comparisons were performed using Fisher’s least-significant difference multiple-comparison test. For Western blotting of the inhibitory effect of ADP on AMPKK phosphorylation, and comparisons of maximal velocity (vmax) and substrate concentration at 50% vmax (Ks) of the substrate-activation curve, Student’s t-tests were used to test for statistical significance. For all statistical tests, significance was set at P < 0.05. All statistical procedures were performed using the NCSS statistical program (Kaysville, UT). All data are reported as means ± SE.

RESULTS

To test the hypothesis that an intracellular metabolite demon-strates allosteric control of the phosphorylation of AMPKK, we assayed the AMPKK activity of LKB1-STRAD-MO25 in the presence of each of the following compounds: G-6-P, F-6-P, F-1,6-P2, 3-PG, G-1-P, G-1,6-P2, ADP, Carn, Acarn, inosine, IMP, and ammonia. Each metabolite was assayed at concentrations typical of resting conditions and exercising conditions (Fig. 1, A–D, n = 5–6). Resting concentrations for all metabolites except Carn were determined by taking the lowest values cited in the primary literature. Likewise, exer-cising concentrations were determined by taking the highest values cited. Given that Carn concentrations decreased during exercise, the resting concentration was found by taking the highest value, and the exercising concentration was found by taking the lowest value found in the primary literature. Addition of ADP at both resting and exercising concentrations resulted in a marked inhibition of LKB1-STRAD-MO25 activity, whereas addition of Carn at the resting concentration resulted in slight inhibition of LKB1-STRAD-MO25 activity (Fig. 1B). Addition of 3-PG at the exercising concentration resulted in an increase in LKB1-STRAD-MO25 activity (Fig. 1D). Addition of all other metabolites had no effect on LKB1-STRAD-MO25 activity.

Given that the AMPKK assay is a two-step assay, with the metabolite being added at the beginning of the first step, it is possible that ADP and 3-PG exhibit their effects on p-AMPK rather than LKB1-STRAD-MO25. To test this, two assays were performed. The first was done the same as the previous
assays, but with ADP at concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mM. In the second assay, we fully phosphorylated AMPK and then added ADP at the same concentrations at the start of the second reaction. ADP had an inhibitory effect under both conditions, but the effect was greater in the first assay (Fig. 2A, n = 6). From these curves, it is apparent that, if only the free ADP (i.e., not bound to protein) is available for inhibition of phosphorylation of AMPK by the LKB1 complex, minimal inhibition would be seen between the concentrations of 0.02 and 0.11 mM (free ADP concentration calculated from reference 12).

Because the p-AMPK assay showed ADP to inhibit P-AMPK activity, but demonstrated an inhibition of ~22% less than what was seen in the AMPKK assay, it was possible that ADP was inhibiting both p-AMPK and LKB1-STRAD-MO25 activities. To test this, a Western blot was performed. We incubated LKB1-STRAD-MO25 with AMPK at ADP concentrations of 0 or 0.8 mM, stopped the reaction with Laemmli’s buffer, and Western blotted for p-AMPK (Fig. 2B, n = 8). Addition of 0.8 mM ADP reduced p-AMPK quantities by ~22%.

AMPKK and p-AMPK assays were also performed in the presence of 0, 0.5, 2.5, 5.0, 7.5, and 10.0 mM 3-PG concentrations to test if it stimulates either LKB1-STRAD-MO25 or p-AMPK activities. 3-PG had a stimulatory effect on LKB1-STRAD-MO25 activity but no effect on p-AMPK activity (Fig. 3A, n = 5–6). To validate the AMPKK assay by directly measuring the LKB1-STRAD-MO25 activity toward AMPK, Western blotting was performed. We incubated LKB1-STRAD-MO25 with AMPK at 3-PG concentrations of 0, 0.5, 2.5, 5.0, 7.5, and 10.0 mM, stopped the reaction with Laemmli’s buffer, and Western blotted for p-AMPK. 3-PG stimulated LKB1-STRAD-MO25 activity in the same pattern as was seen in the AMPKK activity assays (Fig. 3B, n = 7–8). To test whether the stimulatory effect that 3-PG has on LKB1-STRAD-MO25 is specific to the AMPK isoform used as a substrate, AMPKK assays were performed using α1β1γ1-AMPK at 3-PG concentrations 0, 0.5, 2.5, 5.0, 7.5, and 10.0 mM. 3-PG was found to have a similar effect on α1β1γ1-AMPK activation as it did on α2β2γ2-AMPK activation. Analysis was also performed on these blots to obtain a Hill coefficient, Vmax, and activation constant (K; Fig. 4). The Vmax was
determined to be 157.0 ± 12.4 units, the $K_a$ was found to be 1.50 ± 0.28 mM 3-PG, and the Hill coefficient was found to be 1.49 ± 0.26. The best-fit line was calculated using the average value at each data point.

To test whether 3-PG is able to cause an LKB1-STRAD-MO25-mediated activation of a non-AMPK substrate, or whether 3-PG has a substrate-specific stimulatory effect, we tested LKB1-STRAD-MO25 activity against the peptide substrate LKB1tide at 3-PG concentrations of 0 and 7.5 mM. Preliminary tests showed that 3-PG had an inhibitory effect on LKB1-STRAD-MO25 activity toward LKB1tide. To ensure that the inhibitory effect was not caused by the sodium found in the 3-PG, additional assays were performed with sodium acetate at 15.0 mM. We found again that 3-PG has an inhibitory effect and that the inhibition is not because of the presence of sodium (Fig. 5, $n = 6$).

We investigated the necessity of the $\beta$- and $\gamma$-AMPK subunits in LKB1-STRAD-MO25 activation of AMPK. Western blotting was done with LKB1-STRAD-MO25 incubated with truncated $\alpha$-312 AMPK in the presence of 0.0, 0.5, 2.5, 5.0,

Fig. 2. Inhibition of AMPKK activity of LKB1-STRAD-MO25 and phosphorylated (p)-AMPK activity by ADP. A: assays of both AMPKK activity and p-AMPK activity in the presence of 0.0, 0.2, 0.4, 0.6, 0.8, or 1.0 mM ADP. B: measurement of ADP inhibition of LKB1-STRAD-MO25 by Western blot analysis of p-AMPK. LKB1-STRAD-MO25 and AMPK were incubated in the presence of 0.0 or 0.8 mM ADP. All control values were normalized to 1. $P < 0.05$, significantly different from AMPKK assay (†) and significant difference from control (*; $n = 5$–8 experiments).

Fig. 3. Stimulatory effect of 3-PG on AMPKK activity of LKB1-STRAD-MO25-mediated AMPK activation. Concentrations of 3-PG used in all experiments were 0.0, 0.5, 2.5, 5.0, 7.5 and 10.0 mM. A: assays of both AMPKK activity and p-AMPK activity in the presence of 3-PG. B: Western blot validation of the stimulatory effect of 3-PG on AMPKK activity. C: stimulation of AMPKK activity by 3-PG is not confined to muscle-specific AMPK. AMPKK assay was performed using $\alpha_2\beta_2\gamma_2$-AMPK as a substrate. Control values were normalized to 1. $P < 0.05$, significantly different from p-AMPK assay and significant difference from control observed at these concentrations (*; $n = 5$–8).
7.5, and 10.0 mM 3-PG. Analysis with anti-p-AMPK antibody detected a progressive increase in p-AMPK as a function of 3-PG concentration, with a maximal increase of approximately threefold seen at 5 mM 3-PG (Fig. 6, n = 8).

Western blots were also done to investigate a possible allosteric interaction of 3-PG. p-AMPK blots were performed at ATP concentrations of 0.0, 0.01, 0.025, 0.05, 0.1, 0.2, and 1.0 mM, and with or without 7.5 mM 3-PG. Both 0.0 and 7.5 mM 3-PG curves demonstrated a sigmoidal curve. The \( V_{\text{max}} \) for LKB1-STRAD-MO25 in the absence of 3-PG was 356 ± 14 relative units, and the \( K_{0.5} \) was 86 ± 14 µM ATP. The \( V_{\text{max}} \) for LKB1-STRAD-MO25 in the presence of 7.5 mM 3-PG was 468 ± 23 relative units, and the \( K_{0.5} \) was 56 ± 5 µM ATP (Fig. 7, n = 8).

**DISCUSSION**

Previous studies dealing with AMPKK activity and AMPK activation during exercise have produced data that appear to be contradictory. Previous studies have demonstrated a contraction-induced increase in phosphorylation of AMPK (24, 15, 39, 43) without concurrent detectable increases in AMPKK activity (14, 28). This discrepancy could be explained by the presence of intramuscular allosteric activators that increase in response to contraction. These activators would not be retained in the polyethylene glycol precipitates utilized for determination of AMPKK activity. Very little is currently known about allosteric regulation of the LKB1-STRAD-MO25/AMPK system. A previous study showed that creatine phosphate acted as an allosteric inhibitor of AMPK (25, 40); however, a recent study from our laboratory produced evidence against that
concept (34). Another report from our laboratory demonstrated that long-chain acyl-CoA esters, such as palmitoyl-CoA, inhibit AMPKK activity and that palmitoylcarbamide may act as an allosteric activator (35). In our current study we tested, in vitro, an array of metabolites at concentrations typical of both resting and exercising conditions in an attempt to discover an allosteric regulator.

From our first set of experiments, our data suggest that Carn, ADP, and 3-PG all have some type of effect on LKB1-STRAD-MO25 activity (Fig. 1, B and D). We first investigated the drastic inhibition seen when LKB1-STRAD-MO25 and AMPK were incubated with ADP. Controlling for the presence of sodium in the ADP, both our AMPKK assay and our p-AMPK assay showed an ADP dose-dependent inhibition (Fig. 2A). Because in the AMPKK assay the ADP was added at the beginning of the first step of the two-step assay and was still present during the second step, ADP could possibly be inhibiting p-AMPK, and not AMPKK. The p-AMPK assay showed a similar decrease in activity, which indicates that ADP is inhibiting the activity of p-AMPK against its substrate, the AMARA peptide. However, there is a discrepancy in the extent that ADP caused inhibition between the AMPKK assay and the p-AMPK assay. At an ADP concentration of 0.8 mM, an ~22% difference is seen between the two assay curves. This difference could be explained by an inhibition of the LKB1-STRAD-MO25 complex as well. Western blot analysis confirmed that ADP indeed does inhibit the phosphorylation of AMPK by LKB1-STRAD-MO25 (Fig. 2B). We conclude that ADP is able to inhibit both p-AMPK and, to a lesser extent, LKB1-STRAD-MO25 activity toward AMPK. We emphasize that free ADP concentrations in the muscle at rest and during exercise are in the range of 0.02–0.11 mM (calculated from values given in Ref. 12). By far, the greatest proportion of ADP would be expected to be bound to proteins. As can be seen from Fig. 2, a relatively small inhibitory effect would be seen in the range of 0.02 to 0.11 mM ADP. Although it is unclear what proportion of the total ADP in the muscle fiber would be available for inhibition of LKB1-STRAD-MO25 or AMPK, we consider it unlikely that ADP is an important inhibitor under physiological conditions. Of course any rise in free ADP would be expected to markedly increase the free AMP concentration via the adenylate kinase reaction. AMP, being a very potent activator, would certainly overwhelm any small inhibitory effect of the rise in free ADP.

Both our AMPKK assays and our Western blots clearly showed that 3-PG acts as an activator of LKB1-STRAD-MO25 activity. Our p-AMPK assay also showed that 3-PG has no significant effect on the activity of activated AMPK. These results suggest that, when an individual undergoes a bout of exercise, the change in the concentration of 3-PG allows for the LKB1-STRAD-MO25 complex to more readily phosphorylate skeletal muscle AMPK. This effect, however, may not be isolated to working skeletal muscle. Our AMPKK assay using α1β1γ2-AMP as a substrate demonstrated a similar stimulatory response to increased 3-PG levels (Fig. 3C). Given that very little information is available on the concentrations of 3-PG during resting and exercising conditions in non-skeletal muscle sources, we cannot declare with certainty that this newfound effect will occur outside skeletal muscle, such as the liver; however, it does remain a possibility.

With the use of Western blot data from Fig. 3B, a Hill coefficient was calculated. While recognizing Western blotting to represent a nonprecise method of quantitation, a crude estimation of the $K_{H}$ and Hill coefficient can be obtained. Although the method was crude, we were able to gain a general understanding from our analysis. Our calculated Hill coefficient of 1.49 ± 0.26 leads us to believe that 3-PG exhibits mild cooperativity. Our calculated $K_{H}$ for 3-PG of 1.50 ± 0.28 lies well within the range of fluctuations in concentration expected to be observed between resting and contracting muscle.

We performed a number of experiments in an attempt to discern where 3-PG was inducing its stimulatory effect. 3-PG has the possibility of acting on either the LKB1-STRAD-MO25 complex, allowing it to phosphorylate AMPK better, or it could act on AMPK itself, causing AMPK to undergo a change, making it a better substrate for phosphorylation by the LKB1-STRAD-MO25 complex. Our evidence suggests that 3-PG enhancement of LKB1-STRAD-MO25 activity is specific to AMPK as a substrate. AMPK is one of 14 kinases that have been found to be directly phosphorylated by LKB1 (19). The peptide LKB1tide corresponds to an amino acid sequence of another of the 14 kinases, NUAK2. LKB1-STRAD-MO25 activity against LKB1tide was inhibited by 7.5 mM 3-PG, suggesting that the effect is specific to AMPK (Fig. 5). Likewise, AMPK phosphorylation was further enhanced when we used the truncated AMPK α-312 subunit as shown by Western blot analysis. When we used the full αβγ-AMPK as a substrate, maximal AMPK phosphorylation yielded an increase of ~160% (Fig. 3B), whereas the maximal increase in phosphorylation of the α-312 AMPK reached nearly 300% (Fig. 6). These data suggest that 1) the β- and γ-AMPK subunits are not necessary for 3-PG-mediated enhancement of LKB1-STRAD-MO25 activity and 2) 3-PG will either bind to the α-AMPK subunit or to the LKB1-STRAD-MO25 complex. In summary, although our data suggest that 3-PG causes a substrate-specific enhancement of LKB1-STRAD-MO25 activity, we cannot definitively conclude that the effect was specific to AMPK. Further investigation of the effect of 3-PG on multiple LKB1 substrates, as well as binding affinity experiments, would need to be performed to make such a conclusion. Furthermore, we were unable to conclude from our data where 3-PG binding was occurring.

Regardless of the location of the 3-PG binding, we present clear evidence as to the type of interaction that 3-PG has with whichever enzyme it binds to (Fig. 7). First, we show activity of LKB1-STRAD-MO25 as a function of ATP concentration. The curve shown in Fig. 7 clearly has a sigmoidal shape, which is consistent with allosteric enzymes. As well, we show that the $V_{max}$ of LKB1-STRAD-MO25 is reached at no more than 1.0 mM ATP. Preliminary tests showed that incubation with concentrations of 2.0 and 4.0 mM ATP yielded velocities that were not significantly different from 1.0 mM ATP (data not shown). LKB1-STRAD-MO25 without any additional modulators was able to reach a $V_{max}$ of 356 ± 14 relative units, with a $K_{0.5}$ of 86 ± 14 μM ATP. At a 3-PG concentration of 7.5 mM, and with the same increasing concentrations of ATP, a sigmoidal curve was also observed. The $V_{max}$ of this curve was 468 ± 23 relative units, with a $K_{0.5}$ of 56 ± 5 μM ATP. Comparing the two curves, the $V_{max}$ of each curve is significantly different from the other, whereas the $K_{0.5}$ of each curve is not significantly different from the other. These results are
consistent with allosteric activators; we believe that the data presented are sufficient to conclude that 3-PG is an allosteric modulator of LKB1-STRAD-MO25 activity. Another point that should be emphasized is that the prevailing ATP concentrations inside the muscle fiber are always far above the \( K_{d} \) and would never be limiting for LKB1-STRAD-MO25 phosphorylation of AMPK.

We now propose a physiological rationale for having 3-PG as an activator of the AMPK pathway. 3-PG could be acting as an energy utilization switch. At the onset of exercise, the predominant energy substrate used in skeletal muscle is glycogen. This glycogen is broken down to G-6-P, which then enters glycolysis. Further down the glycolytic pathway, 3-PG will be formed. As more glycogen is broken down in the first moments of exercise, 3-PG levels will continue to increase and will stimulate LKB1-STRAD-MO25 activity. This increase in LKB1-STRAD-MO25 activity will then activate AMPK and allow for blood glucose to be transported into the muscle. As exercise continues, the 3-PG-induced elevation in AMPK activity will cause phosphorylation and inactivation of acetyl-CoA carboxylase, causing malonyl-CoA to decrease and allowing an increase in fatty acid oxidation as fatty acids become available. In short, this model suggests that 3-PG contributes to AMPK activation, acting as a switch to turn on blood glucose utilization near the beginning of exercise and allowing for more fatty acids to be oxidized as exercise continues over time.

In summary, we found that a majority of the metabolites that we investigated had no effect on LKB1-STRAD-MO25 activity. ADP had a partial inhibitory effect on LKB1-STRAD-MO25 activity but a more pronounced effect on p-AMPK activity. This effect of ADP was determined to be minimal at estimated free ADP concentrations. Carn had an effect on the LKB1-STRAD-MO25 complex or whether the LKB1-STRAD-MO25, whereas 3-PG had a larger effect. Al-

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REFERENCES


23. Nielsen JN, Mustard KJ, Graham DA, Yu H, MacDonald CS, Pilegaard H. Phosphorylation-activity relationships of AMPK and its activators; we believe that the data presented are sufficient to conclude that 3-PG is an allosteric modulator of LKB1-STRAD-MO25 activity. Another point that should be emphasized is that the prevailing ATP concentrations inside the muscle fiber are always far above the \( K_{d} \) and would never be limiting for LKB1-STRAD-MO25 phosphorylation of AMPK.

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In summary, we found that a majority of the metabolites that we investigated had no effect on LKB1-STRAD-MO25 activity. ADP had a partial inhibitory effect on LKB1-STRAD-MO25 activity but a more pronounced effect on p-AMPK activity. This effect of ADP was determined to be minimal at estimated free ADP concentrations. Carn had an effect on the LKB1-STRAD-MO25 complex or whether the LKB1-STRAD-MO25, whereas 3-PG had a larger effect. Although it seems clear that 3-PG is an allosteric modulator of LKB1-STRAD-MO25 activity, we are unsure whether its effect is on the LKB1-STRAD-MO25 complex or whether the effect occurs within the first 312 residues of the \( \alpha \)-AMPK subunit. Physiologically, 3-PG may act as an energy substrate utilization sensor, or in some other capacity. Regardless, the discovery of an LKB1-STRAD-MO25 allosteric modulator appears to clarify why previous studies have shown contradictory evidence of exercise-induced decreases in AMPK activity along with increased AMPK phosphorylation and is generally important as we continue to learn and understand the AMPK signaling cascade. (4, 6, 37)