Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia

A-S. Marsin*†, L. Bertrand*†, M.H. Rider*, J. Deprez*, C. Beauloye*, M.F. Vincent‡, G. Van den Berghe‡, D. Carling§ and L. Hue*

Background: The role of protein phosphorylation in the Pasteur effect – the phenomenon whereby anaerobic conditions stimulate glycolysis – has not been addressed. The AMP-activated protein kinase (AMPK) is activated when the oxygen supply is restricted. AMPK acts as an energy-state sensor and inhibits key biosynthetic pathways, thus conserving ATP. Here, we studied whether AMPK is involved in the Pasteur effect in the heart by phosphorylating and activating 6-phosphofructo-2-kinase (PFK-2), the enzyme responsible for the synthesis of fructose 2,6-bisphosphate, a potent stimulator of glycolysis.

Results: Heart PFK-2 was phosphorylated on Ser466 and activated by AMPK *in vitro*. In perfused rat hearts, anaerobic conditions or inhibitors of oxidative phosphorylation (oligomycin and antimycin) induced AMPK activation, which correlated with PFK-2 activation and with an increase in fructose 2,6-bisphosphate concentration. Moreover, in cultured cells transfected with heart PFK-2, oligomycin treatment resulted in a parallel activation of endogenous AMPK and PFK-2. In these cells, the activation of PFK-2 was due to the phosphorylation of Ser466. A dominant-negative construct of AMPK abolished the activation of endogenous and cotransfected AMPK, and prevented both the activation and phosphorylation of transfected PFK-2 by oligomycin.

Conclusions: AMPK phosphorylates and activates heart PFK-2 *in vitro* and in intact cells. AMPK-mediated PFK-2 activation is likely to be involved in the stimulation of heart glycolysis during ischaemia.

Addresses: *Hormone and Metabolic Research Unit and ‡Laboratory of Physiological Chemistry, Université Catholique de Louvain and Christian de Duve Institute of Cellular Pathology, 1200 Brussels, Belgium. §Cellular Stress Group, MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, DuCane Road, London W12 ONN, UK.

Correspondence: Louis Hue
E-mail: hue@horm.ucl.ac.be

†A-S.M. and L.B. contributed equally to this work.

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Background

To maintain its function, the heart requires a constant supply of fuels and oxygen. When the oxygen supply is not limited (normoxic conditions), these fuels are completely oxidised in the mitochondria (reviewed in [1]). Myocardial ischaemia induces a shift to anaerobic metabolism, with a rapid stimulation of glycolysis [2,3]. This anaerobic stimulation of glycolysis, called the Pasteur effect, can occur in different mammalian tissues. The role of protein phosphorylation in this phenomenon has not been addressed. In the heart, a protein kinase that is activated by ischaemia could be responsible for the stimulation of glycolysis in this condition.

The AMP-activated protein kinase (AMPK) is a protein kinase that is activated in anaerobic conditions. AMPK is a heterotrimeric protein composed of a catalytic subunit (α) and two regulatory subunits (β and γ) [4,5]. Isoforms of all three subunits have been identified [6,7], but the physiological significance of the isoforms is unclear. In the heart, the catalytic α2 isoform is more abundant than α1 [6]. AMPK is activated by an increase in the intracellular AMP:ATP ratio and so acts as a sensor of the energy state ('fuel gauge') of the cell (for a review, see [8]). The AMP:ATP ratio is known to increase when the oxygen supply is restricted or in the presence of inhibitors of mitochondrial oxidative phosphorylation. In various tissues (liver, adipose tissue and skeletal muscle), AMPK phosphorylates and inactivates several key enzymes in energy-consuming biosynthetic pathways, thereby conserving ATP. This is the case for acetyl-CoA carboxylase (ACC), 3-hydroxy-3-methylglutaryl-CoA reductase [9], glycogen synthase [10] and creatine kinase [11], which control the synthesis of fatty acids, cholesterol, glycogen and phosphocreatine, respectively. In skeletal and cardiac muscle, AMPK is thought to mediate the recruitment of glucose transporters (GLUT4) by an unknown mechanism that is distinct from that of insulin [12,13]. Our hypothesis is that myocardial hypoxia stimulates glycolysis in a concerted manner, which involves GLUT4 recruitment and 6-phosphofructo-2-kinase (PFK-2) activation, and is mediated by AMPK.

PFK-2 is a bifuntional enzyme, termed PFK-2/fructose-2,6-bisphosphatase (FBPase-2), which controls the synthesis and degradation of fructose 2,6-bisphosphate, respectively. Fructose 2,6-bisphosphate is a potent stimulator of 6-phosphofructo-1-kinase (PFK-1), a key enzyme of glycolysis and, as such, it is considered as a ubiquitous glycolytic signal (for a review, see [14]). Distinct isozymes of PFK-2

exist in mammalian tissues. They are encoded by different genes and differ in molecular mass, kinetic and immunological properties, and phosphorylation by protein kinases (for reviews, see [14,15]). The heart (H) isozyme contains a carboxy-terminal regulatory domain that is absent in the liver (L) isozyme and can be phosphorylated on both Ser466 and Ser483 *in vitro* by protein kinase B (PKB), cyclic AMP-dependent protein kinase (PKA), p70 ribosomal S6 kinase ($p70^{S6k}$) and mitogen-activated protein kinase-activated protein kinase–1 (MAPKAPK-1) [16]. Phosphorylation activates PFK-2 with no effect on FBPase-2 activity. Phosphorylation of Ser466 is sufficient to increase the V_{max}, whereas phosphorylation of both Ser466 and Ser483 is required to decrease the K_m for fructose 6-phosphate of PFK-2 [17]. In accordance with its capacity to be phosphorylated by several protein kinases, PFK-2 is activated in hearts stimulated by adrenaline or insulin, or submitted to high workload, and correlates with the stimulation of glycolysis observed under these conditions [1].

Here, we studied whether heart PFK-2 could be a substrate of AMPK and we investigated the effect of anaerobic conditions on AMPK and PFK-2 activity in perfused rat hearts. We also transfected human embryonic kidney (HEK)-293 cells with cDNA constructs encoding recombinant wild-type bovine heart PFK-2 and both wild-type and dominant-negative forms of AMPK, in order to assess the role of AMPK in the activation of heart PFK-2 in anaerobic conditions.

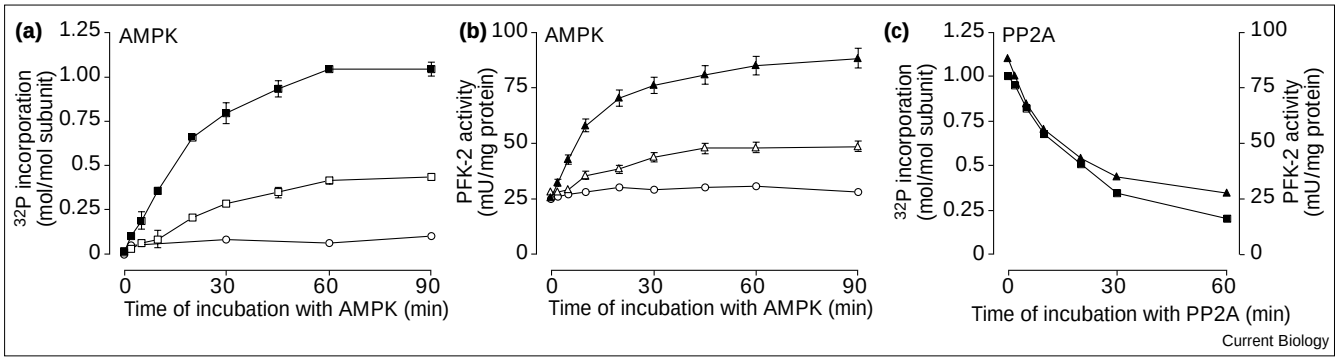
Results

Phosphorylation and activation of heart PFK-2 *in vitro* by AMPK

Recombinant bovine heart PFK-2 carrying a carboxy-terminal hexa-histidine tag (His_6–PFK-2) was phosphorylated by rabbit liver AMPK with a stoichiometry close to 1 mole phosphate incorporated per mole enzyme subunit. The rate of phosphorylation was stimulated by 0.2 mM AMP (Figure 1a) and correlated with PFK-2 activation (Figure 1b). Similarly, protein phosphatase 2A (PP2A)-mediated dephosphorylation of PFK-2 that had been previously phosphorylated by AMPK correlated with PFK-2 inactivation (Figure 1c). Such a correlation between activation and phosphorylation has already been described for heart PFK-2 phosphorylated by PKB, $p70^{S6k}$ and MAPKAPK-1 [16]. PFK-2 activation by AMPK resulted from a 2.5-fold increase in V_{max} (49 ± 5 to 123 ± 10 mU/mg protein; $n = 3$), with little (less than 20%) or no change in K_m for fructose 6-phosphate and MgATP or in IC_{50} for magnesium citrate (see Supplementary material). In contrast, phosphorylation of His_6–PFK-2 by PKB increased the V_{max} (twofold) but also decreased the K_m for fructose 6-phosphate (twofold) and decreased the sensitivity towards magnesium citrate inhibition (twofold). Phosphorylation of His_6–PFK-2 did not affect FBPase-2 activity (see Supplementary material).

To identify the site(s) phosphorylated by AMPK, His_6–PFK-2 was maximally phosphorylated with Mg[γ-^{32}P]ATP and AMPK. After purification by SDS–PAGE and digestion with trypsin, peptides were separated by reversed-phase high-pressure liquid chromatography (HPLC). A single radioactive peak was detected (Figure 2a) and its radioactivity was decreased (twofold) when phosphorylation was performed without AMP (data not shown). The elution time of this peak was the same (Figure 2a,b) as that of the first peak obtained after phosphorylation of His_6–PFK-2 by PKB and which contained phosphorylated Ser466 [17]. The residue phosphorylated by AMPK was

Figure 1



Time-dependent changes in phosphorylation state and activity of His_6–PFK-2 incubated with (a,b) AMPK and (c) PP2A. (a) Purified wild-type His_6–PFK-2 (0.16 mg/ml) was incubated with 0.1 mM Mg[γ-^{32}P]ATP and purified AMPK (0.6 U/ml) with (shaded squares) or without (unshaded squares) 0.2 mM AMP in a final volume of 50 µl. Unshaded circles correspond to a control incubated without AMPK. At the indicated times, aliquots (5 µl) were removed for SDS–PAGE and phosphorimaging for measurement of ^{32}P incorporation. (b) Same protocol as in (a) with 1 mM MgATP (non-radioactive) in a final volume of 0.1 ml with (shaded triangles) or without (unshaded triangles) 0.2 mM AMP. At the indicated times, aliquots (10 µl) were removed for PFK-2 assay. The results are the means ± SEM for three separate determinations. (c) His_6–PFK-2 was first phosphorylated as described above and then dephosphorylated with PP2A (50 U/ml). Aliquots were removed for the measurement of ^{32}P incorporation (squares) and PFK-2 activity (triangles); results are the means for two separate experiments.

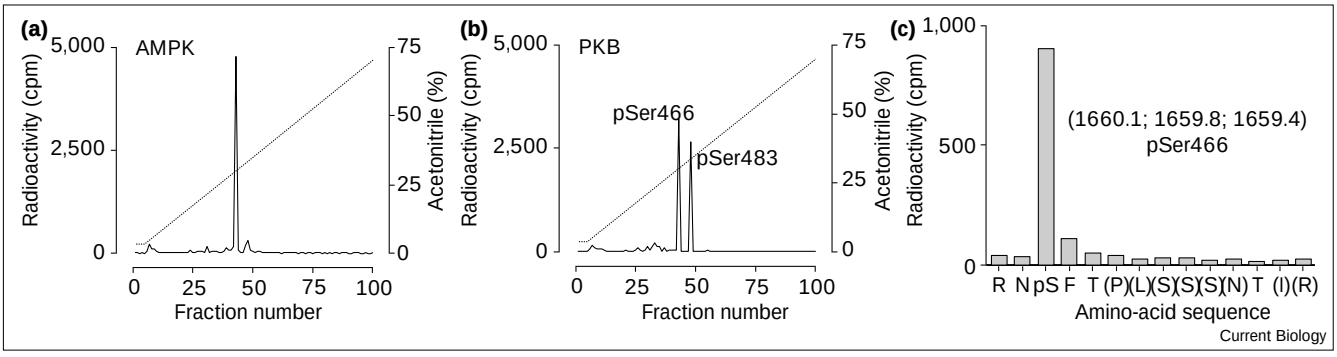
identified by analysing the labelled peak by matrix-assisted laser desorption-ionisation mass spectrometry (MALDI-MS), nanoelectro-spray ionisation tandem mass spectrometry (nanoESI-MS/MS) and by solid-phase sequencing (Figure 2c). The labelled peptide contained the sequence ^{464}RNSFTPLSSSNTIR^{477} in which the burst of radioactivity occurred at Ser466 (Figure 2c). In agreement with the identification of Ser466 as the phosphorylated residue, a constitutively active S466E mutant of His_6–PFK-2 was not phosphorylated and could not be further activated by AMPK (data not shown). This mutant could, however, be phosphorylated by PKB on Ser483 [17]. The recognition motif for phosphorylation by AMPK, as deduced from sequences surrounding its known phosphorylated sites, contains hydrophobic residues at P–5 and P+4 (where P is the phosphorylated site) and a single basic residue at P–3 or P–4 [8]. This motif differs slightly from that in endothelial nitric oxide synthase (eNOS), which contains hydrophobic residues at P–4 and P+3 [18]. The phosphorylation site in heart PFK-2 (VRMRRN***S***FTPLS; the phosphorylated Ser is bold and italicized) shares similarities with both motifs. It contains hydrophobic residues at P–4 and P+4 and a basic residue at P–3.

In the experiments described above, we used AMPK purified from rabbit liver, which contains both α1βγ and α2βγ complexes, similar to the enzyme purified from rat liver [19]. To study the relative specificity of the individual isoforms, we isolated α1βγ- and α2βγ-specific complexes by immunoprecipitation from rat liver and compared their activity using either His_6–PFK-2 or ACC as a substrate. Both isoforms phosphorylated His_6–PFK-2 at the same rate, which was about half the rate of phosphorylation of ACC (data not shown). We also compared the kinetic properties towards SAMS, a known synthetic substrate derived from ACC [20], and the peptide MR6 (containing Ser466 of cardiac PFK-2). The V_{max} and K_m values for SAMS were the same for both isoforms. For the MR6 peptide, the V_{max} and K_m of α2βγ were twice that of α1βγ. The latter differences compensated for each other, such that the value of k_{cat}/K_m (sometimes called 'catalytic efficiency') was the same. Nevertheless, this value was 3–4 times lower than for SAMS, confirming that ACC is a better substrate of AMPK than His_6–PFK-2 (see Supplementary material). We also studied the kinetic properties of the AMPK isoforms towards a peptide containing a serine residue in a sequence homologous to that surrounding Ser466 in heart PFK-2. This sequence is found in a PFK-2 isozyme present in some tumour cells [21] and placenta [22]. This peptide (MR47) was equally phosphorylated by both AMPK isoforms, although the efficiency was about 10 times less than that observed with the MR6 peptide (see Supplementary material).

Activation of AMPK and PFK-2 in perfused rat hearts

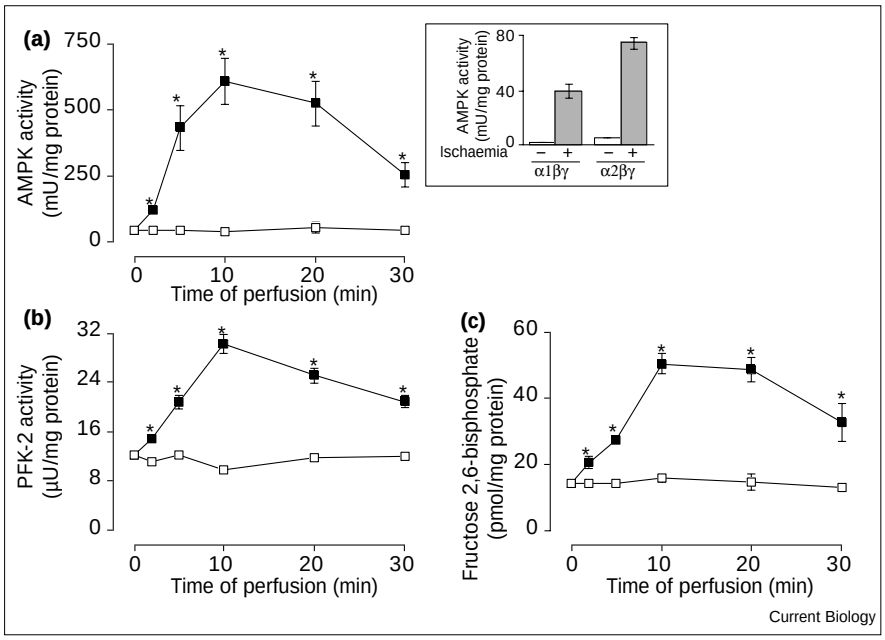
Perfused rat hearts were submitted to different periods of no-flow ischaemia to study whether AMPK activation would correlate with PFK-2 activation and with an increase in fructose 2,6-bisphosphate concentration. AMPK, which was almost completely inactive under aerobic conditions, was activated 10-fold after 10 minutes of ischaemia (both AMPK isoforms were activated) and its activity returned towards basal levels after 30 minutes of ischaemia (Figure 3a). Ischaemia activated PFK-2 and increased fructose 2,6-bisphosphate concentration, both processes following AMPK activation with a slight delay (Figure 3). The activation of AMPK and PFK-2 by ischaemia was transient. Remarkably, this correlated with

Figure 2



HPLC profile and solid-phase sequencing of ^{32}P-labelled tryptic peptides of His_6–PFK-2 phosphorylated *in vitro* by AMPK and PKB. His_6–PFK-2 was phosphorylated by (a) AMPK or (b) PKB and 0.1 mM Mg[γ-^{32}P]ATP. After digestion with trypsin, peptides were separated by reverse-phase HPLC using a linear gradient of acetonitrile (dashed line). The means of two separate experiments are shown. The peaks corresponding to the peptides containing phosphorylated Ser466 and Ser483 (pSer466, pSer483) are indicated in (b). (c) The radioactive peptide from His_6–PFK-2 phosphorylated by AMPK was analysed by solid-phase sequencing (1000 cpm), MALDI-MS and nanoESI-MS/MS to identify the phosphorylated residue(s). The three numbers in parentheses correspond to the peptide mass calculated from the sequence, measured by MALDI-MS and by nanoESI-MS/MS, respectively. The cycle numbers were replaced by the amino-acid sequence established by nanoESI-MS/MS (amino acids not identified are in parentheses).

Figure 3



Activation of (a) AMPK and (b) PFK-2, and increase in (c) fructose 2,6-bisphosphate concentration in ischaemic rat hearts. After a 20 min equilibration, rat hearts were perfused for the indicated periods of time under normoxic conditions (white squares) or submitted to a no-flow ischaemia (black squares) by interrupting the perfusion flow. (a) Total AMPK activity, (b) PFK-2 activity and (c) fructose 2,6-bisphosphate concentration were measured in freeze-clamped hearts. Inset, isoform-specific AMPK activity was measured in samples taken after 10 min of perfusion and immunoprecipitated with anti-α1 or anti-α2 antibodies. Values are the means ± SEM for at least four different hearts. Asterisks indicate values significantly different ($p < 0.01$, unpaired *t*-test) from the corresponding control value.

the stimulation of heart glycolysis by ischaemia, which was also transient and disappeared during prolonged and severe ischaemia [23]. The transient nature of the phenomenon probably explains our previous failure to observe an increase in fructose 2,6-bisphosphate concentration in hearts submitted to ischaemia/anoxia for 30 minutes [24].

Perfusion for 10 minutes with 5 μM oligomycin or 3 μM antimycin, or under anoxic or ischaemic conditions activated both AMPK (at least 10-fold) and PFK-2 (2–3-fold) and doubled the concentration of fructose 2,6-bisphosphate (Table 1). Maximal effects were already obtained with 0.5–1 μM of both inhibitors (data not shown). The extent of activation of AMPK correlated with the increase in the AMP:ATP ratio (see Supplementary material). In contrast, perfusion with 5-aminoimidazole-4-carboxamide (AICA) riboside had no effect on AMPK and PFK-2 activity, and on fructose 2,6-bisphosphate concentration. AICA riboside is known to activate AMPK in liver after its phosphorylation into AICA ribotide (also called ZMP), an analogue of AMP responsible for AMPK activation [25,26]. However, in hearts perfused with AICA riboside, the concentration of ZMP, ZDP and ZTP were barely detectable (data not shown), in agreement with previous work performed on rabbit cardiomyocytes [27]. The lack of effect of AICA riboside on AMPK activity in heart differs from the results reported for isolated papillary heart muscle cells [13]. Whatever the reason for this discrepancy, we did not detect any significant accumulation of ZMP, consistent with the lack of AMPK activation.

The kinetic properties of PFK-2 from normoxic or ischaemic hearts were compared. The V_{max} of PFK-2 from ischaemic hearts was twice that of normoxic hearts. No significant changes in K_m for MgATP and fructose 6-phosphate, or in IC_{50} for magnesium citrate could be detected. Therefore, ischaemia induces changes in kinetic properties of PFK-2 *in vivo* that are similar to those observed after phosphorylation of His_6–PFK-2 by AMPK *in vitro* (see Supplementary material).

Activation of AMPK and PFK-2 in cell lines

We first compared the effect of 1 mM AICA riboside or 0.5 μM oligomycin on endogenous AMPK activity in four different cell lines (CHO, FTO-2B, HEK-293 and COS-7). AMPK activation by oligomycin was observed in all cell lines, whereas AICA riboside activated AMPK (more than twofold) only in FTO-2B and CHO cells (see Supplementary material and Figure 4a). The failure to detect an activation of AMPK following incubation with AICA riboside appears to be due to a lack of accumulation of ZMP within the cells (D.C., unpublished results), as in the perfused hearts.

The effects of AICA riboside or oligomycin on transfected His_6–PFK-2 activity were then studied in FTO-2B and HEK-293 cells. In such experiments, the treatment lasted for only 10 minutes and was therefore too short to affect the enzyme content, as verified by immunoblots. Endogenous PFK-2 activity was unaffected by AICA riboside or oligomycin treatment (data not shown). Transfection of His_6–PFK-2 in FTO-2B cells resulted in a sevenfold

Table 1

Activation of AMPK and PFK-2 and increase in fructose 2,6-bisphosphate concentration and AMP:ATP ratio in hearts submitted to various conditions.

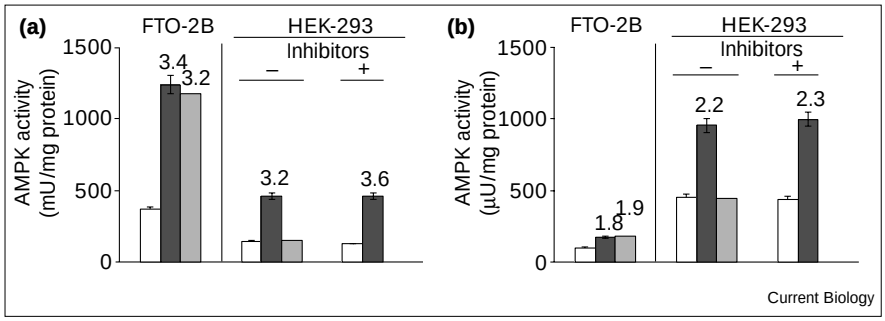
Condition of perfusion	AMP:ATP ratio	AMPK activity (mU/mg protein)	PFK-2 activity (μU/mg protein)	Fructose 2,6-bisphosphate concentration (pmol/mg protein)
Normoxic control	0.03 ± 0.01	119 ± 10	12.2 ± 0.5	14.3 ± 0.4
Ischaemia	0.25 ± 0.05*	911 ± 87*	25.4 ± 0.6*	46.3 ± 4.4*
Anoxia	0.48 ± 0.04*	1468 ± 162*	27.8 ± 2.7*	44.3 ± 1.9*
AICA riboside	0.04 ± 0.01	114 ± 8	11.8 ± 0.9	14.7 ± 1.5
DMSO control	0.06 ± 0.01	276 ± 63	12.5 ± 0.5	14.3 ± 0.7
Oligomycin	1.32 ± 0.14*	2099 ± 288*	26.5 ± 1.2*	34.8 ± 3.1*
Antimycin	0.55 ± 0.09*	1279 ± 34*	23.3 ± 2.7*	35.5 ± 2.3*

After a 20 min equilibration period, rat hearts were perfused for 10 min under the following conditions: anoxia (obtained by equilibrating the perfusion buffer with a 95% N_2/5% CO_2 gas phase), ischaemia, 5 μM oligomycin, 3 μM antimycin, 1 mM AICA riboside or control conditions (normoxia for anoxia, ischaemia and AICA riboside; DMSO for inhibitors of oxidative phosphorylation). Values are the means ± SEM for at least four different hearts; *$p < 0.01$ (unpaired *t*-test) compared with the control value.

overexpression (endogenous, 13 ± 3 μU/mg protein; transfected with 10 μg DNA, 97 ± 5 μU/mg protein; $n = 3$). Incubation for 10 minutes with 1 mM AICA riboside or 0.5 μM oligomycin increased transfected PFK-2 activity (Figure 4b). In HEK-293 cells, the activity of transfected His_6–PFK-2 was about 60 times that of endogenous PFK-2 (endogenous, 12 ± 1 μU/mg protein; transfected with 10 μg DNA, 706 ± 13 μU/mg protein; $n = 3$). In these cells, 0.5 μM oligomycin activated transfected PFK-2 after 10 minutes of incubation (Figure 4b). This activation was not due to other protein kinases such as PKB, MAPKAPK-1 and $p70^{S6k}$ because it was not antagonised by PD 98059 (50 μM), rapamycin (100 nM) or wortmannin (100 nM), which prevent the activation of these kinases (Figure 4).

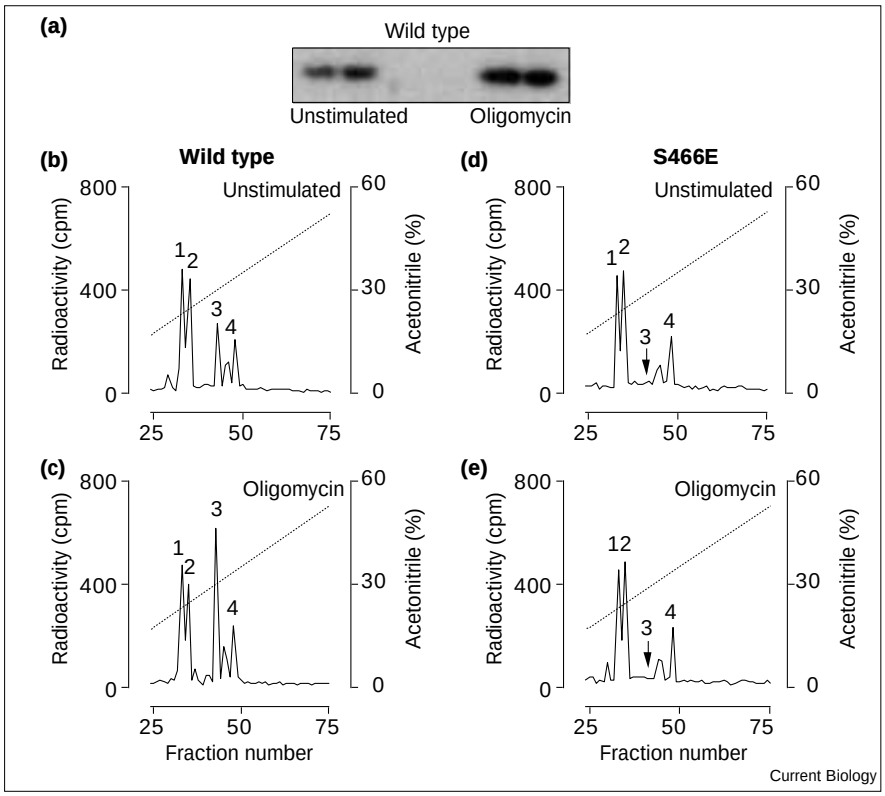
To determine the *in vivo* phosphorylation site(s) involved in the oligomycin-induced activation of PFK-2, HEK-293 cells were transfected with His_6–PFK-2. After labelling with ^{32}P, the transfected cells were incubated for 10 minutes with or without 0.5 μM oligomycin. Immunoprecipitation and SDS–PAGE of transfected His_6–PFK-2 followed by autoradiography revealed a single ^{32}P-labelled band with the expected M_r of 61,000 (Figure 5a). Its labelling was increased about twofold in oligomycin-treated cells. The labelled band was digested with trypsin and peptides were separated by reverse-phase HPLC (Figure 5b). The elution profile from unstimulated cells displayed two major peaks (peaks 1 and 2) followed by two minor peaks (peaks 3 and 4). This profile was similar to that previously described [17]. The first two peaks correspond to peptides containing phosphorylated Ser522 and Ser528, which may represent MAP kinase(s) sites and which do not change PFK-2 activity [17]. Oligomycin increased the labelling of peak 3 (Figure 5c). The elution time of this peak corresponds to that of the peptide containing phosphorylated Ser466 *in vitro* (compare Figures 2b and 5c). The S466E His_6–PFK-2 mutant (10 μg DNA) was also transfected in HEK-293 cells. Oligomycin did not activate the S466E His_6–PFK-2 (control, 665 ± 15 μU/mg protein; oligomycin, 656 ± 23 μU/mg protein; $n = 3$). The elution profile of radioactive peaks obtained with this mutant indicated that

Figure 4



Activation of (a) endogenous AMPK and (b) transfected His_6–PFK-2 by AICA riboside and oligomycin in FTO-2B and HEK-293 cells. FTO-2B and HEK-293 cells were transfected with a vector expressing wild-type His_6–PFK-2 (10 μg DNA). The cells were incubated for 10 min under control conditions (white columns), with 0.5 μM oligomycin (dark grey columns) or with 1 mM AICA riboside (light grey columns) and with inhibitors (50 μM PD 98059, 100 nM rapamycin and 100 nM wortmannin) as indicated. These inhibitors were added 20 min before oligomycin treatment. The content of His_6–PFK-2 was not affected by oligomycin or AICA riboside, as verified by western blots (data not shown). The extent (-fold) of oligomycin-induced activation of AMPK and PFK-2 is given on top of the columns when statistically significant ($p < 0.01$, unpaired *t*-test). Values are the means ± SEM for three different preparations.

Figure 5



(a) Phosphorylation of wild-type His_6–PFK-2 and (b–e) HPLC profiles of ^{32}P-labelled tryptic peptides of wild-type His_6–PFK-2 or the S466E mutant from transfected HEK-293 cells incubated with or without oligomycin. (a) Transfected His_6–PFK-2 from ^{32}P-labelled HEK-293 cells incubated with or without oligomycin (10 min) was immunoprecipitated and subjected to SDS–PAGE and autoradiography. After tryptic digestion of immunoprecipitated ^{32}P-labelled (b,c) wild-type His_6–PFK-2 or (d,e) S466E mutant, peptides were separated by reverse-phase HPLC using a linear gradient of acetonitrile (dashed line). The numbered peptides are defined in the text.

peak 3 was absent and that no other oligomycin-induced radioactive peak was detected (Figure 5d,e).

To assess the role of AMPK in the activation of PFK-2 by oligomycin, the effect of co-expression of PFK-2 with a catalytically inactive mutant of α1 AMPK (α1DN AMPK) was studied in HEK-293 cells. Expression of α1DN AMPK in primary rat hepatocytes acted as a dominant-negative inhibitor of endogenous AMPK by competing for the binding of the β and γ subunits [28]. We verified that transfection with α1DN AMPK abolished the oligomycin-induced activation of both endogenous and cotransfected wild-type (α1βγ and α2βγ) AMPK in HEK-293 cells (Figure 6a).

Having confirmed the dominant-negative character of α1DN AMPK, we tested whether α1DN AMPK could interfere with the effect of oligomycin on His_6–PFK-2 activation. In the absence of α1DN AMPK, there was a time-dependent and concomitant activation of both AMPK and transfected His_6–PFK-2 by oligomycin (Figure 6b,c). Furthermore, immunoblotting with antibody raised against phosphorylated Ser466 revealed a similar time-dependent phosphorylation of His_6–PFK-2 on Ser466 (Figure 6d). In contrast, when α1DN AMPK was coexpressed, phosphorylation and activation of transfected His_6–PFK-2 by oligomycin was almost completely abolished (Figure 6b–d).

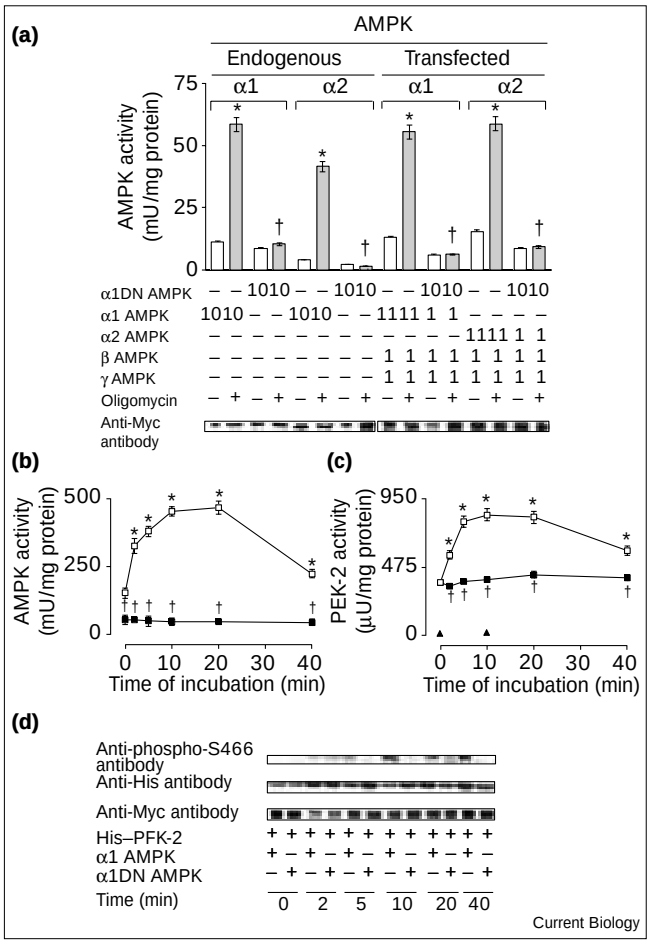
Taken together, these results provide strong evidence that the oligomycin-induced activation of heart PFK-2 is due to phosphorylation by AMPK. The data support our hypothesis that AMPK mediates PFK-2 activation under conditions leading to ATP depletion in the heart.

Discussion

AMPK activates heart PFK-2 by phosphorylating Ser466. Our *in vitro* and *in vivo* studies fulfil the criteria put forward by E.G. Krebs [29] to demonstrate that phosphorylation of a target protein has physiological relevance. The experimental evidence can be summarised as follows: first, PFK-2 is phosphorylated *in vitro* by AMPK to a significant extent; second, AMPK-phosphorylated PFK-2 can be dephosphorylated by PP2A; third, phosphorylation correlates with PFK-2 activation; fourth, AMPK activation in heart or cells in culture correlates with PFK-2 activation; fifth, the same site, Ser466, is phosphorylated by AMPK *in vitro* and in intact cells; and, finally, PFK-2 activation by oligomycin in transfected cells is abolished by a dominant-negative mutant of AMPK. Heart PFK-2 therefore represents a new substrate of AMPK and is activated by AMPK in anaerobic conditions. It is noteworthy that PFK-2 is only

Figure 6

Inhibition of the oligomycin-induced activation of (a,b) AMPK and (c) His_6–PFK-2 in HEK-293 cells cotransfected with the α1DN AMPK mutant. HEK-293 cells were cotransfected with the different constructs as indicated. All α AMPK constructs incorporated sequences encoding the Myc epitope tag. (a) Effect of α1DN AMPK on the oligomycin-induced activation of endogenous and cotransfected α1βγ and α2βγ AMPK. These wild-type AMPK isoforms were obtained by cotransfection of three constructs (encoding α1 AMPK or α2 AMPK, β AMPK and γ AMPK). To obtain the same amount of Myc-tagged α AMPK, α1 or α2 AMPK subunit was overexpressed in control cells. The amount (μg) of transfected DNA is indicated. Cells were incubated with or without 0.5 μM oligomycin for 10 min. AMPK activity was measured after immunoprecipitation with anti-α1 or anti-α2 antibodies for endogenous AMPK and with anti-Myc antibody for transfected AMPK. Results are the means for three different preparations. Immunoblots of transfected AMPK (anti-Myc antibody) are shown. (b,c) Effect of α1DN AMPK (shaded squares) on oligomycin-induced activation of (b) total AMPK activity and (c) cotransfected His_6–PFK-2 activity. The α1 AMPK subunit was taken as control (unshaded squares). Cells were incubated with 0.5 μM oligomycin for the indicated periods of time. The triangles indicate endogenous PFK-2 activity in non-transfected cells. Values are the means for three different cell preparations. Amount of transfected DNA: His_6–PFK-2, 5 μg; α1 AMPK, 5 μg; α1DN AMPK, 5 μg. (d) Immunoblots of phosphorylated heart PFK-2 (anti-phospho-S466 antibody), transfected PFK-2 (anti-His antibody, Sigma), and transfected AMPK (anti-Myc antibody, Boehringer) were performed on samples taken from the cells shown in (b,c). The times are indicated below the blots. Asterisks indicate values significantly different ($p < 0.01$, unpaired *t*-test) from the corresponding zero time; crosses indicate values significantly different ($p < 0.01$, unpaired *t*-test) from cells without the α1DN construct.



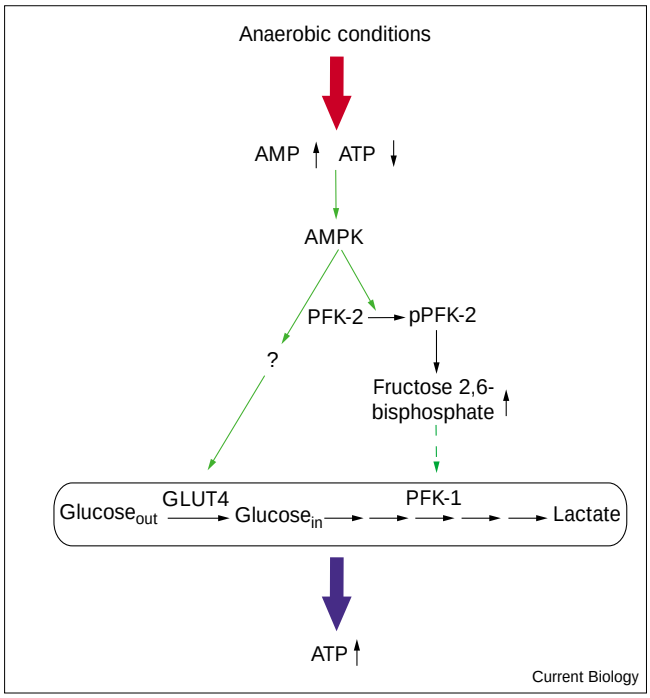
the second substrate for AMPK known to be activated following phosphorylation, the first being eNOS [18].

AMPK is known to inactivate key enzymes in biosynthetic pathways, thereby conserving energy. Until recently, however, AMPK was not known to stimulate ATP production. Our results indicate that this could occur under anaerobic conditions in the heart. The AMPK-mediated activation of PFK-2 stimulates the flux through PFK-1 by increasing fructose 2,6-bisphosphate concentration. This would be expected to lead to a stimulation of ATP production through glycolysis. To our knowledge, this is the first demonstration of protein phosphorylation being involved in the Pasteur effect. This phenomenon is superimposed on the well-known and direct stimulation of PFK-1 by changes in adenine nucleotide concentrations (particularly a fall in ATP and a rise in AMP). Therefore, the increase in AMP:ATP ratio stimulates PFK-1 through two mechanisms: a direct allosteric stimulation of PFK-1 and an indirect mechanism involving the phosphorylation of PFK-2 by AMPK.

Activation of PFK-1 and PFK-2 is unable to stimulate glycolysis by itself [1]. Overall flux can only increase if the supply of glucose 6-phosphate is also increased. Therefore, besides PFK-1/PFK-2 activation, the stimulation of glucose transport by AMPK is an intrinsic part of the concerted mechanism by which AMPK stimulates heart glycolysis (Figure 7).

PFK-2 activation by AMPK is restricted to tissues containing the heart isozyme of PFK-2. Heart and renal medulla express the heart isozyme, but this is not the case for liver or skeletal muscle. The liver and muscle PFK-2 isozymes do not contain the carboxy-terminal AMPK phosphorylation site. Therefore, in anoxic livers, no increase in fructose 2,6-bisphosphate and PFK-2 would be expected, and in fact a fall in fructose 2,6-bisphosphate content has been observed [30]. A similar observation was reported in hepatocytes incubated with AICA riboside [31] and in skeletal muscle submitted to anaerobic contraction [32]. The difference in regulation between skeletal and cardiac muscle is intriguing and the reasons for this discrepancy are not known. However, one would expect PFK-2 activation by anoxia to hold true for cancer cells that contain the inducible PFK-2 [21] or for the placental PFK-2 isozyme [22]. Our results indicate that these isozymes contain a carboxy-terminal sequence that can be phosphorylated by AMPK, possibly to a lesser extent than heart PFK-2, on the basis of peptide phosphorylation. Activation of inducible PFK-2 by AMPK together with induction of this PFK-2 isozyme could contribute to maintain the high glycolytic rate that is a

Figure 7



Mechanism of stimulation of heart glycolysis by AMPK under anaerobic conditions. Green arrow, activation by covalent modification; dashed green arrow, allosteric stimulation; pPFK-2, phosphorylated form of heart PFK-2. The question mark indicates an indirect and still unknown mechanism.

characteristic feature of many tumours (Warburg effect) [21]. In conclusion, the activation of heart PFK-2 by AMPK and possibly of other PFK-2 homologues offers a new interpretation for the hypoxia-induced stimulation of glycolysis.

Materials and methods

Materials

Mutant (S466E) and wild-type recombinant heart PFK-2/FBPase-2 (His_6–PFK-2, previously called BH1$(His)_6$ [17]), total AMPK [33] and $\alpha1\beta\gamma$ and $\alpha2\beta\gamma$ AMPK isoforms [19] were purified as described. The constructs encoding wild-type and mutant His_6–PFK-2 [17], and $\alpha1$, $\alpha2$, $\beta1$ and $\gamma1$ AMPK subunits [34] have been described. A rabbit polyclonal antibody (anti-phospho-S466 antibody) was raised against the phosphorylated peptide RRN(S_p)FTP (corresponding to residues 463–469 of heart PFK-2 in which Ser466 was phosphorylated). Peptides called MR6 (^{459}PVRMRRNSFT^{468} of heart PFK-2), MR47 (^{455}PLMRRNSVT^{463} of inducible PFK-2 [21]) and SAMS, a synthetic peptide derived from ACC [20], were synthesised by V. Stroobant (Ludwig Institute for Cancer Research, Brussels, Belgium).

In vitro *studies*

Methods for measurement of phosphorylation and dephosphorylation of His_6–PFK-2 by purified AMPK and PP2A, respectively, the changes in kinetic properties induced by phosphorylation, and the identification of phosphorylation sites have been described [16,17].

In vivo *studies*

Hearts from fed male Wistar rats were perfused (Langendorff method) as described [24]. Perfusion conditions are detailed in the legends to the figures and tables. At the indicated times, hearts were freeze-clamped and stored at –80°C. The frozen hearts were homogenised (Ultra-Turrax) at 0–4°C in 5 volume (v/w) of buffers used for the assay. For AMPK measurements, the buffer contained 50 mM Hepes at pH 7.6, 50 mM KCl, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 mM β-glycerophosphate, 100 nM microcystin and protease inhibitors (4 μg/ml leupeptin, 1 mM benzamidine hydrochloride, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin, 0.2 mM phenylmethanesulfonyl fluoride and 4 μg/ml trypsin inhibitor). For PFK-2 assays, the buffer contained 20 mM Hepes at pH 7.5, 30 mM KCl, 20 mM NaF, 5 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol, 100 nM microcystin, 0.1 mM fructose 6-phosphate, 0.3 mM glucose 6-phosphate and the protease inhibitors indicated above. High-speed supernatants (20,000 × *g*, 30 min) were taken for the assays. For measurements of the AMP:ATP ratio and fructose 2,6-bisphosphate, the hearts were homogenised in perchloric acid [31] or alkali [35], respectively.

Cell culture studies

The transfection protocols were a modified calcium phosphate procedure [17] for HEK-293 cells and the lipofectamine method (following instructions provided by the manufacturer GIBCOBRL) for FTO-2B cells. When confluent, the cells were deprived of serum for 16 h and then incubated as described in the legends to the figures and tables. At the end of the incubation period, the medium was rapidly removed and the cells were immediately lysed in 0.8 ml ice-cold lysis buffer (50 mM Hepes at pH 7.5, 0.2% Triton X-100, 50 mM KF, 1 mM potassium phosphate, 5 mM EDTA, 5 mM EGTA, 1 μM microcystin, 1 mM Na_3VO_4, 15 mM β-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine hydrochloride and 1 μg/ml leupeptin), scraped, frozen in liquid nitrogen and stored at –80°C. Phosphorylation and identification of the phosphorylated residue of transfected PFK-2 was realised as described [17]. In experiments using the dominant-negative AMPK (α1DN AMPK), transfection of wild-type α1, which is not active if expressed in the absence of the β and γ subunits [19], was used as a control. This control gave results similar to those obtained following transfection with PFK-2 without cotransfected wild-type α1 AMPK (data not shown).

Enzyme and metabolite measurements

Total AMPK activity was assayed after precipitation with 10% (w/v) polyethylene glycol 6000, whereas the activity of the $\alpha1\beta\gamma$ or $\alpha2\beta\gamma$ isoforms of endogenous AMPK was assayed after immunoprecipitation with anti-α1 or anti-α2 antibodies [19]. All αAMPK constructs incorporated sequences encoding the Myc tag. The activity of these transfected AMPKs was assayed after immunoprecipitation with anti-Myc antibodies (Boerhinger). In all cases, conditions were set up to obtain maximal immunoprecipitation, and the activity was linear as a function of time and of protein concentration. AMPK activity was measured by phosphorylation of the synthetic peptide SAMS in the presence of 0.2 mM AMP [4]. PFK-2 was measured in a 20% (w/v) polyethylene glycol 6000 precipitate (perfused hearts) or in crude extract (cultured cells). Unless otherwise stated, PFK-2 activity [36] was measured at pH 7.1 with 1 mM fructose 6-phosphate, 3 mM glucose 6-phosphate and 5 mM MgATP. Fructose 2,6-bisphosphate was measured as described [35] and purine nucleotides were measured after their separation by HPLC [31].

Other methods

Proteins were measured by Coomassie Blue staining or by the ninhydrin reaction after total alkaline hydrolysis, with bovine serum albumin as a standard. Kinetic constants were calculated by computer fitting of the data to a hyperbola describing the Michaelis–Menten equation by non-linear least-square regression. One unit of enzyme activity corresponds to the formation of 1 μmol (PFK-2) or 1 nmol (protein kinases and PP2A) of product per minute under the assay conditions.

Supplementary material

Supplementary material including a figure showing the correlation between AMPK activity and AMP:ATP ratio in perfused rat hearts submitted to anaerobic conditions, and four tables presenting changes in

kinetic properties of heart PFK-2 *in vitro* and *in vivo*, a comparison of the kinetic properties of the α1βγ and α2βγ AMPK isoforms on synthetic peptides and AMPK activation in different cell lines is available at http://current-biology.com/supmat/supmatin.htm.

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