The Glucoamylase Inhibitor Acarbose Is A Direct Activator of Phosphorylase Kinase[†]

Owen W. Nadeau, Weiya Liu, Igor G. Boulatnikov, Jessica M. Sage, Jennifer L. Peters and Gerald M. Carlson[*]

Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160

Supporting Information

Contents

1. Proteins and reagents

PhK was purified from the psoas muscle of New Zealand White rabbits (*1*), dialyzed against 50 mM Hepes (pH 6.8), 0.2 mM EDTA and 10% sucrose, and stored at -80 ºC. The mAbs against the α, β and γ subunits of PhK were previously described (*2, 3*), and the anti-calmodulin mAb was from Zymed. All other secondary conjugates were from Southern Biotechnology. Acarbose was purchased from Tocris. The S-peptide was synthesized and purified at the Alberta Institute.

2. Phosphorylation assays

PhK autophosphorylation ± acarbose. Phosphate incorporation into theregulatory α and β subunits of PhK was carried out essentially as previously reported [Kumar *et al.*, 2004], with the following modifications. Rabbit muscle PhK was diluted to 40 μg/ml (30.8 nM) in the reaction buffer containing 67 mM β-glycerophosphate, 50 mM Tris (pH 6.8 or pH 8.2), 10 mM $Mg(CH_3CO_2)_2$, 1.5 – 1.8 mM [γ-^{32}P]ATP (400 – 480 Ci/mol), 0.09 mM EGTA, 0.2 mM $CaCl_2$ and 7 mM β-mercaptoethanol ± acarbose (250 μM). Reactions were initiated by addition of the enzyme in 40 mM β-glycerophosphate buffer (pH 6.8, 0.1 mM EGTA, 28 mM β-mercaptoethanol) and carried out at 30 ^{o}C. At appropriate time intervals, 20-μl aliquots were either spotted onto P81 filters, washed with 0.5% H_3PO_4, and counted in a scintillation counter or mixed with an equal volume of 2× electrophoresis sample buffer and the subunits resolved by 1D SDS-PAGE. Gels were stained with Coomassie, destained, dried, and exposed to a phosphor screen.

Phosphorylation of S-peptide by PhK. S-peptide conversion was carried out in 50 mM Tris/50 mM β-glycerophosphate buffer (pH 6.8), 12 mM $Mg(CH_3CO_2)_2$, 1.0 mM [γ-^{32}P]ATP, 0.1 mM EGTA, 0.2 mM $CaCl_2$, 100 μM S-peptide ± 250 μM acarbose. Reactions were initiated by addition of PhK (10 μg/ml)

and carried out at 30 ^{o}C for 2 min. Aliquots (20 μl) were spotted onto P81 filters, washed with 0.5% H_3PO_4 and counted on a scintillation counter.

3. Analysis of acarbose binding by PhK.

Fluorescence emission spectra of PhK ± acarbose were collected in 1-cm, quartz cuvettes from 295 to 405 nm at 1-nm intervals with a scan rate of 5 nm/sec on a PTI 814 fluorescence spectrometer (PTI Photon Technology International) equipped with an internal Peltier-type temperature controller. The instrument was set to emit at a 280-nm excitation wavelength, with 5-nm excitation and 5-nm emission slit widths, and data collection at 25 °C with a 300-sec equilibration time between data collection intervals. Final concentrations in the assays were: PhK (100 μg/mL), β-glycerophosphate buffer (50 mM, pH 6.8 or 8.2), EDTA (0.1 mM) and acarbose (1, 2.5, 5, 20, 60, 100, and 250uM). One minute prior to fluorescence measurements, PhK was added to solutions containing the indicated concentrations of acarbose and mixed. After each acarbose measurement, a negative acarbose control containing the same amount of PhK was measured to correct for minor photo-bleaching of the kinase. The fractional bound acarbose f_{ac} was determined from the following equation:

$$f_{ac} = [\text{acarbose}] / K_D + [\text{Acarbose}]$$

where $f_{ac} = |F_c - F_{ac}|$. F_c and F_{ac} were the relative fluorescence peak intensities measured for PhK in the absence and presence of the indicated concentrations of acarbose, respectively. Plots of the differences were hyperbolic and fitted by non-linear least square methods using KaleidaGraph graphical package (Synergy software).

4. Chemical Crosslinking

PhK was crosslinked with GMBS essentially as described (5), with crosslinking initiated by addition of GMBS and carried out at 30 ºC for 2.5 min at pH 8.2 in 50 mM β-glycerophosphate, 0.2 mM EDTA. Final concentrations of the αβγδ protomer of PhK and GMBS in the reaction were 0.47 and 4.7 μM, respectively. The reaction was terminated by addition of an equal volume of SDS buffer [0.125 M Tris (pH 6.8), 20% glycerol, 5% β-mercaptoethanol, 4% SDS], followed by brief vortexing. The PhK subunits were separated on 6-18% linear gradient polyacrylamide gels and stained with Coomassie Blue. Western blotting of the proteins was performed on PVDF membranes with subunit-specific mAbs as previously described (5). All crosslinking reactions were performed at least twice using different preparations of PhK.

References

1. King, M. M., and Carlson, G. M. (1981) Synergistic activation by Ca^{2+} and Mg^{2+} as the primary cause for hysteresis in the phosphorylase kinase reactions. *J. Biol. Chem.* 256, 11058-11064.

2. Wilkinson, D. A., Marion, T. N., Tillman, D. M., Norcum, M. T., Hainfeld, J. F., Seyer, J. M., and Carlson, G. M. (1994) An epitope proximal to the carboxyl terminus of the alpha-subunit is located near the lobe tips of the phosphorylase kinase hexadecamer. *J. Mol. Biol.* 235, 974-982.

3. Wilkinson, D. A., Norcum, M. T., Fizgerald, T. J., Marion, T. N., Tillman, D. M., and Carlson, G. M. (1997) Proximal regions of the catalytic gamma and regulatory beta subunits on the interior lobe face of phosphorylase kinase are structurally coupled to each other and with enzyme activation. *J. Mol. Biol.* 265, 319-329.

4. Kumar, P., Brushia, R. J., Hoye, E., and Walsh, D. A. (2004) Baculovirus-mediated overexpression of the phosphorylase b kinase holoenzyme and αγδ and γδ subcomplexes, *Biochemistry* 43, 10247-10254.

5. Nadeau, O. W., Anderson, D. W., Yang, Q., Artigues, A., Paschall, J. E., Wyckoff, G. J., McClintock, J. L., and Carlson, G. M. (2007) Evidence for the location of the allosteric activation switch in the multisubunit phosphorylase kinase complex from mass spectrometric identification of chemically crosslinked peptides. *J. Mol. Biol.* 365, 1429-1445.

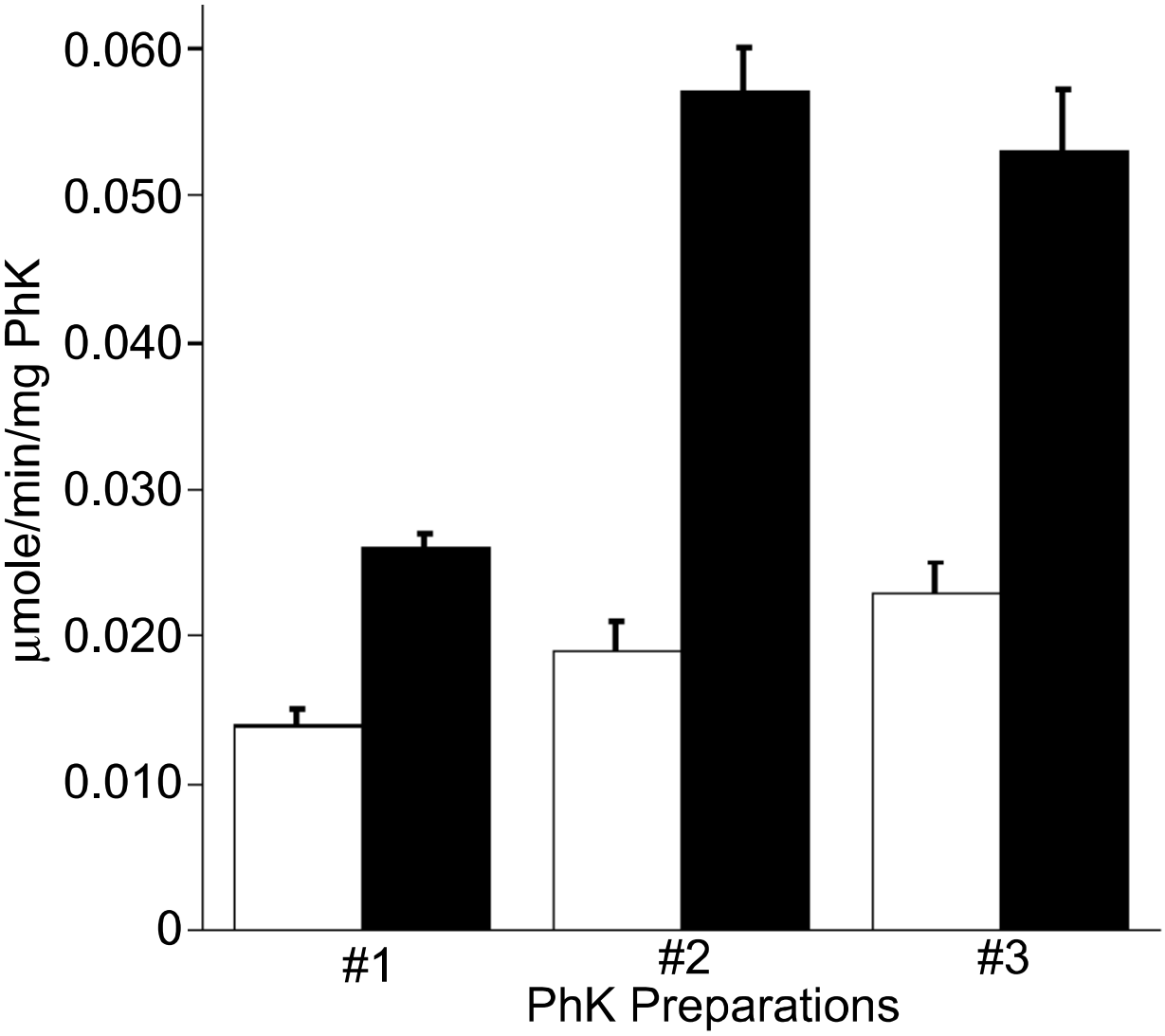

Figure S1. Phosphorylation of S-peptide ± acarbose. The activity of PhK was determined in the absence (empty bar) and presence (filled bar) of 250 µM acarbose. All measurements were carried out in triplicate for each of the three different PhK preparations.