Synergistic Regulation of Phosphorylase $\alpha$ by Glucose and Caffeine*

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Kinetic studies of both liver and muscle phosphorylase $\alpha$ demonstrate that caffeine and glucose inhibit the binding of glucose-1-phosphate to the enzyme in a synergistic competitive and nonexclusive manner. Inhibition studies for numerous other caffeine analogs show that the muscle enzyme has a relaxed specificity for this negative effector. The liver enzyme is more discriminating by preferential binding of methylated oxypurines. Physiological concentrations of AMP and ATP, which affect the enzyme activity at a separate site, prevent glucose from effectively inhibiting the enzyme. The addition of the second synergistic ligand improves the binding of glucose. These data suggest that glucose homeostasis as regulated by phosphorylase may be dependent on a second ligand and that the role of glucose in this physiological process may have been overestimated. A structural rationalization of this synergistic response is discussed with reference to the crystal structure of the muscle enzyme.

The concentration of circulating glucose has long been considered to be responsible for the regulation of glucose homeostasis by the liver (1). The work of Hers and co-workers (2, 3) indicates that liver phosphorylase $\alpha$ (EC 2.4.1.1), as the glucose receptor, is largely responsible for the overall control of hepatic glycogen metabolism via activation of glycogen synthetase-phosphatase and, thus, glycogen homeostasis.

The recent report of Kasvinsky et al. (4) identified a previously undescribed negative effector (nucleoside) site in the muscle phosphorylase $\alpha$ monomer. Preliminary experiments indicated that the site is synergistic with respect to glucose inhibition of phosphorylase $\alpha$ activity (4). It was suggested that the nucleoside site may function physiologically to regulate phosphorylase phosphatase as does glucose.

In the present communication we describe kinetic studies which demonstrate a functional nucleoside site in liver phosphorylase $\alpha$. By the use of caffeine, which binds preferentially at this inhibitory site, we have examined the synergistic relationship between this negative effector site and the glucose binding site. The effect of physiological concentrations of AMP and ATP on glucose inhibition was examined. The data are consistent with the interpretation that glucose may not be the only major physiological regulator of phosphorylase activity and, therefore, of glycogen metabolism in the liver. X-ray crystallographic structural changes in the muscle enzyme are documented which could account for the synergism noted.1 It is proposed that the liver enzyme is analogous in the structural transitions required for caffeine-glucose inhibition.

MATERIALS AND METHODS

Rabbit muscle phosphorylase $\alpha$ was prepared by the method of Krebs et al. (5), as described previously (4). Bovine and rabbit liver phosphorylase $\alpha$ were prepared similarly, but from liver phosphorylase $\beta$, which had been isolated by hydrophobic chromatography (6, 7).

Prior to use, the phosphorylase was dissolved and purified on a column of Sephadex G-25 (8). Protein concentration of the purified enzymes was determined from absorbance measurements at 280 nm using the absorbance index $E_{280}$ of 13.2 (9). Rabbit liver glycosyl (type III), purchased from Sigma, was purified on a Dowex 1-Cl column and assayed by the method of Dishe as described by Ashwell (10).

Caffeine, theophylline, allopurinol, riboflavin, FAD, FMN, and biotin were obtained from Sigma. Folic acid and xanthopterin were from Chemical Dynamics.

Initial reaction rates were determined by the Fiske-Subbarow protocol biochemistry in the direction of saccharide synthesis as described by Engers et al. (11). Reaction mixtures were 0.5 ml and contained 2 mM sodium $\beta$-glycerophosphate (pH 6.8), 0.15 mM EDTA, 1 mM dithiothreitol, 28 mM glycogen, and 2 to 4 μg of phosphorylase. For the liver enzymes 2 mM 2-[bis(2-hydroxyethyl)aminio]ethanesulfonic acid (Bis) (pH 6.8) was substituted for $\beta$-glycerophosphate. Rabbit liver phosphorylase was assayed in the presence of 170 mM glycogen. Enzyme and glycogen were preincubated for 15 min at 30°C before initiating the enzymatic reaction with glucose-1-P.2

RESULTS

Allosteric inhibition of glucose-1-P binding to muscle phosphorylase $\alpha$ has been demonstrated for many purine nucleosides or derivatives. These include AMP, IMP, inosine, hypoxanthine, xanthine, adenosine, adenine, and the methylated oxypurines, caffeine and theophylline (4). All appear to function by binding at the nucleoside site since AMP is not required for inhibition.

Apparent inhibition constants for various other heterocyclic fused ring compounds are presented in Table I. This table shows an unusual feature of the negative effector locus of the muscle enzyme, namely, that it has relaxed specificity and allows binding and functioning for diverse chemical structures. Various purine analogues function as does the isooxazoline ring of riboflavin and the pteridine ring of folic acid. This is surprising in view of the disparate size and shape of these fused ring systems. Liver phosphorylase, on the other hand, appears to be considerably more selective in terms of the nature of the ligand bound. Although the methylated oxypurines, caffeine and theophylline, and allopurinol remain strong inhibitors of the liver enzyme, inosine, FMN, and folic acid have no effect.

Kinetic results for inhibition of both muscle and liver phosphorylase $\alpha$ by caffeine or glucose, or both, are presented in Fig. 1. Both caffeine and glucose show the expected apparent competitive inhibition with the substrate, glucose-1-P, and

1 The abbreviation used is: glucose-1-P, α-D-glucopyranose-1-phosphate.
the positive effector, AMP (4). The nucleotides AMP and ATP were included in these studies at their presumed physiological concentrations (12-14) since we have demonstrated that the allosteric activator site for nucleotide is spatially well separated from, but allosterically linked to, the nucleoside binding site (4). ATP, which is a powerful competitive inhibitor of the activator AMP in phosphorylase from both muscle and liver (15-17), has little effect on the activity of phosphorylase a in the absence of AMP. The apparent inhibitor of the activator AMP in phosphorylase from both muscle separated from, but allosterically linked to, the nucleoside binding site (4). ATP, which is a powerful competitive inhibition of glucose-1-P binding to rabbit muscle and liver phosphorylase a under such conditions is 61 and 51 mM, respectively, at saturating glycogen (data not shown). Thus, when ATP is included in the reaction mixture, it is probably not acting at the nucleoside site; but rather, is competitive with the activator AMP (16).

Fig. 1 indicates that glucose is not a very effective inhibitor of muscle or liver phosphorylase a activity in the presence of either AMP alone or AMP plus ATP, at their presumed physiological concentration. The synergistic combination of glucose and caffeine, however, gives more effective inhibition, even in the presence of AMP alone.

In the nomenclature of Segel (18), caffeine and glucose affect phosphorylase a by cooperative (synergistic) competitive inhibition (in a nonexclusive manner). A Dixon plot of reciprocal velocity versus glucose at increasing concentrations of caffeine and fixed glucose-1-P (and saturating glycogen) for the liver enzyme is presented in Fig. 2. This plot clearly demonstrates the nonexclusivity of caffeine and glucose binding to rabbit liver phosphorylase a and is consistent with the crystallographic picture already seen with the muscle enzyme.

Data obtained with the muscle enzyme gives a primary plot which is qualitatively identical to that of the liver enzyme. The primary plot is presented in Fig. 3 from which the dissociation constant for glucose (\(K_g = 2.9\) mM) and the interaction constant (\(\alpha = 0.3\)) between glucose and caffeine were obtained. However, a replot of the slopes versus the corresponding caffeine concentration yields a curve (Fig. 3, inset). A Hill plot (data not shown) of \(\log (v/v_0 - v)\) versus the log of caffeine concentration, where \(v_0\) is the velocity at constant substrate plus caffeine and \(v_0\) is the velocity minus the inhibitor, yields a slope of -1.4, suggesting that the nucleoside sites in the dimer of active phosphorylase may be showing cooperative binding. Since we cannot accurately extrapolate \(aK_c\) (the dissociation constant for caffeine in the presence of saturating glucose) from the replot of slopes in Fig. 3 (inset), the dissociation constant for caffeine in the absence of glucose was determined and, using this independently derived value, \(aK_c\) was calculated.

According to the concerted symmetry model for allostery (19), the effect of caffeine on phosphorylase a may be regarded as inhibition in an exclusive binding \(K\) system where we assume that glucose-1-P binds exclusively to the \(R_0\) state (\(C = 0\)) and caffeine binds exclusively to the \(T_0\) state. Thus, caffeine as the allosteric inhibitor displaces the \(T_0 = R_0\) equilibrium towards \(T_0\). The constants \(K_c\) and \(L\) may be determined from a plot of \(\sqrt{L} = K_c (0.2\) mM caffeine). A replot (Fig. 4, inset) of the y-intercepts versus glucose-1-P concentration yields \(K_c\) (2.0 mM glucose-1-P), the intercept on the x axis, and \(1/\sqrt{L} (L = 1.0)\), the intercept on the y axis. The data are clearly linear when \(n = 2\) and appear to be consistent with the allosteric model for caffeine inhibition. The interaction constant between caffeine and glucose (\(\alpha = 0.3\)) may now be used to calculate \(aK_c\), 0.06, the dissociation constant for caffeine in the presence of saturating glucose. This value is identical to that extrapolated from Fig. 3 (inset).

The variation in the apparent \(K_c\) for caffeine inhibition of phosphorylase a as a function of glucose concentration is presented in Fig. 5. These data were obtained from Lineweaver-Burk plots as a function of glucose-1-P in the presence and absence of the inhibitor. The limit values obtained are similar to those obtained from the Dixon plot (Fig. 2). The variation in apparent \(K_c\) may be viewed simply as a measurement of the interaction between the glucose and caffeine binding sites. It is striking that the largest effect of glucose on the binding of caffeine occurs in the region of physiological interest (0 to 5 mM). Data for the muscle enzyme were collected in the presence of AMP and ATP. As previously determined, value, \(aK_c\) was calculated.

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reported for IMP and AMP (4), the limiting $V_{\text{max}}$ of these inhibition studies (at saturating glucose-1-P) was characteristically that of the AMP activated form of phosphorylase $a$. Caffeine did not appear to displace AMP under the conditions used. In the case of the liver enzyme, no nucleotides were included in the incubations. Thus, the caffeine effect on the liver enzyme could not be due to inhibition of AMP binding. When AMP or ATP, or both, were included in the liver enzyme incubations, the resulting kinetics were complex. Lineweaver-Burk plots no longer showed classical competitive inhibition but indicated that caffeine was acting as a mixed inhibitor. We believe that this is partially the result of direct competition between caffeine and AMP at the allosteric activator site. Such a result would be consistent with the observed $V_{\text{max}}$ and $K_m$ effects and the fact that the liver enzyme binds AMP at least 50-fold more weakly than that from muscle (17), where simple competitive inhibition is always found.

**Fig. 2 (left).** A Dixon plot of reciprocal velocity versus glucose concentration at different fixed caffeine concentrations and 2.0 mM glucose-1-P for rabbit liver phosphorylase. Caffeine concentrations are as follows: ○, none; ■, 0.25 mM; △, 0.6 mM; ○, 1.0 mM; ●, 2.0 mM. The dissociation constant for glucose in the presence of caffeine, $aK_G$, equals 1.8 mM glucose. The interaction factor = 0.2 and $K_c$, the dissociation constant for glucose = 9.1 mM. The replot of slopes versus caffeine concentration is shown in the inset. The dissociation constant for glucose in the presence of caffeine, $K_{G-C}$, equals 1.8 mM glucose and allows the calculation the dissociation constant for caffeine, $K_{C-G}$ = 0.08 mM caffeine.

**Fig. 3 (center).** A Dixon plot of reciprocal velocity versus glucose concentrations for muscle phosphorylase $a$ at different fixed caffeine concentrations and 7.0 mM glucose-1-P. Caffeine concentrations are as follows: ○, none; △, 0.25 mM; ■, 0.6 mM; ●, 1.0 mM; ○, 2.0 mM. The replot of slopes versus caffeine concentration is shown in the inset. The calculation of dissociation constants is discussed in the text.

**Fig. 4 (right).** The determination of $K_C$ and $L$, the dissociation constant for caffeine binding and the allosteric constant, for muscle phosphorylase $a$. Caffeine inhibition was assumed to be inhibition in an exclusive $K$ system and $n$, the number of caffeine or nucleoside binding sites was two. A replot of the y-intercepts (inset) yields $K_C$ and $L$ as discussed in the text.

**DISCUSSION**

Liver phosphorylase $a$ has been shown to have a dominant role in the control of glucose homeostasis. It responds directly to serum glucose levels. The pathway from inhibition of phosphorylase to activation of glycogen synthetase is complex and incompletely understood; however, it has been demonstrated in vivo and in vitro that glucose inhibition of phosphorylase $a$ makes the enzyme a better substrate for the phosphatase. The latter enzyme is then able to cleave the phosphate from serine-14 of phosphorylase $a$ to form inactive phosphorylase $b$ (1, 3). Recent evidence suggests that this phosphatase is also the activator of glycogen synthetase (20) but functions maximally only when its inhibitor, phosphorylase $a$, is nearly depleted. Thus, when serum glucose levels rise, phosphorylase $a$ levels drop prior to activation of glycogen synthetase, which finally channels serum glucose into storage glycogen in the liver (1). We believe that the crucial control of the phosphatase is regulated by the concentration of active phosphorylase $a$; which in turn regulated by the concentrations of several metabolites, including glucose, AMP, ATP, Pi, and glycogen.

The effects of glucose on the conformation and function of phosphorylase $a$ are mimicked by caffeine. For example, at 1 mM caffeine in liver filtrates, phosphorylase $a$ is rapidly converted to phosphorylase $b$ and this conversion is accelerated by glucose (21-23). The details of the mechanism of this caffeine effect on both muscle and liver phosphorylase are not understood and it has been assumed to be the result of direct competition with the allosteric activator AMP (24, 25). Recent studies from our laboratory using x-ray crystallographic and kinetic studies of muscle phosphorylase $a$ document the fact that caffeine is a potent negative effector which binds at a site distinct from the two sites that bind glucose or AMP (4, 26). Unfortunately, the only crystallographic data available have been collected on the muscle enzyme and muscle does not contribute directly to blood glucose homeostasis as does the liver. Considerable evidence exists, however, which indicates a structural and functional homology between phosphorylase $a$ isolated from muscle and liver. Such evidence includes kinetic (15, 16, 27), primary structure (28), and quaternary structural (29) studies.
The present communication describes kinetic studies which demonstrate the existence of a functional nucleoside site (caffeine) in liver phosphorylase α. The kinetic analyses, consistent with the crystallographic results obtained by the use of the muscle enzyme, show that caffeine and glucose inhibit phosphorylase α from both liver and muscle synergistically, in a competitive nonexclusive manner (Figs. 2 to 4). Although there is a strong homology between the muscle and liver enzymes, the finding of cooperativity in caffeine binding to the muscle enzyme suggests that the enzymes are not structurally identical in the subunit interactions between the caffeine sites. Similar conclusions were made by Feldmann et al. (29) in their study of muscle-liver hybrids where the hybrids were active but not allosterically competent.

AMP, at its presumed physiological concentration, suppresses the effect of glucose on phosphorylase α from both muscle and liver sources (Fig. 1). Even in the presence of 7.0 to 9.0 mM ATP, a powerful inhibitor of AMP activation, glucose alone is not a very effective inhibitor of phosphorylase α. Only in the presence of caffeine does the enzyme show the inhibitory response we might associate with the proposed regulatory function in glycogen metabolism via phosphorylase or synthetase phosphatase. In this regard it is worth noting that caffeine is routinely added to assays of phosphorylase phosphatase by the indirect method (e.g. phosphorylase α activity remaining) (23). Thus, some of the responses of phosphorylase phosphatase to glucose, which have been well documented, although they are qualitatively correct, have probably been exaggerated by the synergistic relationship between the two ligands (27, 30). The demonstration of a site (binding caffeine), whose function appears to be similar to and synergistic with that binding glucose, suggests that glucose may not be the sole physiological regulator of glucose homeostasis in the liver. The finding that glucose in the presence of physiological concentrations of nucleotides has little effect on phosphorylase supports this view.

There is a simple structural rationalization for the mechanism of synergistic inhibition of caffeine and glucose which is based on the 3-Å resolution x-ray structure of muscle phosphorylase α. This will be presented in detail elsewhere. Briefly, however, the binding pocket for caffeine is about 10 Å away from the glucose molecule and is in an extended hydrophobic slot on the side of the protomer. This pocket (in the muscle enzyme) shows a relaxed specificity and binds more than a dozen related fused-ring compounds (Table I; cf. Ref. 11). The synergism with glucose may be due to the fact that part of the hydrophobic binding pocket (Phe 285) is formed from a loop of 56 amino acids (residues 242 to 297) which we have shown must move about 5 Å in order for glucose to be replaced by the substrate, glucose-1-P, in our crystals. This structural change, which occurs on activation of this allosteric enzyme, is the subject of another paper.1 Fig. 6 is an α-carbon drawing of the protomer showing the disposition of the two ligands and the loop which is involved in the conformational change. The atomic coordinates for glucose and caffeine were determined from 2.5-Å resolution difference Fouriers using standard procedures.

The kinetic data presented here indicate that there are considerable specificity differences between enzyme which originates from muscle and that from liver. Of the ligands tested in the liver system, the larger ring systems, which are the most powerful inhibitors of the muscle enzyme, are without effect (Table I). Even the inhibitory effects of the methylated oxypurines and allopurinol are reduced in the liver system. We believe that the liver enzyme must show greater specificity because liver tissue is generally believed to be freely permeable to glucose. Muscle tissue on the other hand usually does not contain high concentrations of free glucose (31). Thus, in the latter case, partial inhibition of phosphorylase caused by tight ligand binding at the nucleoside site might “sensitize” muscle phosphorylase to low concentrations of free glucose entering the cells. These are, of course, simplistic
suggestions and are probably further complicated by the concentration of AMP, ATP, and ADP.

There is no doubt about the synergistic inhibition (and consequent dephosphorylation to form phosphorylase b) of phosphorylase a by caffeine and glucose in vitro and in liver homogenates (22). That they also function in this fashion to produce a hypoglycemic response in vivo following the conversion of phosphorylase a to b is difficult to prove. Feinberg et al. (32) have reported a hypoglycemic response in normal human subjects to orally ingested coffee in the presence of glucose. This response, in the light of the well known hypoglycemic effects of caffeine via elevated 3':5'-cAMP (33) is paradoxical and implies that a different receptor for caffeine is operating and that this caffeine receptor is synergistic with respect to glucose. Since no change in serum insulin levels with caffeine could be demonstrated, the mechanism of this hypoglycemic response is not established. Drugs other than caffeine also induce hypoglycemia and may act via inhibition of phosphorylase. For example, allopurinol (Table I), a drug with similar effects to caffeine, shows a dangerous hypoglycemic side effect when co-administered with hypoglycemic drugs (34).

The binding site for caffeine on phosphorylase a likely has some physiological role other than a response to after-dinner coffee. Its function suggests that it interacts with some physiological ligand(s) to indirectly promote glycogen synthesis, as does glucose, by inactivating phosphorylase a. What this ligand might be is not yet known. An exciting possibility would be a role for indirect response to hormonal signals at the cell surface. In fact, Witters and Avruch (35) have suggested that insulin activation of liver glycogen synthetase in isolated hepatocytes is solely the result of its ability to inactivate phosphorylase a. In addition, insulin was shown to be synergistic with glucose. The demonstration of a functional nucleoside (caffeine) site in liver phosphorylase in this paper, which is synergistic with glucose (the only such site known), encourages us to suggest that, physiologically, insulin may act to inhibit phosphorylase a activity by inducing an effect which may act by binding at the locus of caffeine binding.

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