Integrated effects of multiple modulators on human liver glycogen phosphorylase a

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1Metabolic Research Laboratory and Section of Endocrinology, Metabolism and Nutrition, Minneapolis Veterans Affairs Medical Center, and 2Departments of Medicine and 3Food Science and Nutrition, University of Minnesota, Minneapolis, Minnesota 55417; and 4Pfizer Global Research and Development, Groton Laboratories, Groton, Connecticut 06340

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Ercan-Fang, Nacide, Mary C. Gannon, Virginia L. Rath, Judith L. Treadway, Miriam R. Taylor, and Frank Q. Nuttall. Integrated effects of multiple modulators on human liver glycogen phosphorylase a (HLGPa) activity. Am J Physiol Endocrinol Metab 283: E29–E37, 2002. First published March 5, 2002; 10.1152/ajpendo.00425.2001.—Hepatic glucose production is increased in people with type 2 diabetes. Glucose released from storage in liver glycogen by phosphorylase accounts for ∼50% of the glucose produced after an overnight fast. Therefore, understanding how glycogenolysis in the liver is regulated is of great importance. Toward this goal, we have determined the kinetic characteristics of recombinant human liver glycogen phosphorylase a (HLGPa) (active form) and compared them with those of the purified rat enzyme (RLGPa). The Michaelis-Menten constant (Km) of HLGPa for P1, 5 mM, was about fivefold greater than the Km of RLGPa. Two P1 (substrate) concentrations were used (1 and 5 mM) to cover the physiological range for P1. Other effectors were added at estimated intracellular concentrations. When added individually, AMP stimulated, whereas ADP, ATP and glucose inhibited, activity. These results were similar to those of the RLGPa. However, glucose inhibition was about twofold more potent with the human enzyme. UDP-glucose, glucose 6-phosphate, and fructose 1-phosphate were only minor inhibitors of both enzymes. We reported previously that when all known effectors were present in combination at physiological concentrations, the net effect was no change in RLGP activity. However, the same combination reduced HLGP activity, and the inhibition was glucose dependent. We conclude that a combination of the known effectors of phosphorylase a activity, when present at estimated intracellular concentrations, is inhibitory. Of these effectors, only glucose changes greatly in vivo. Thus it may be the major regulator of HLGP activity.

IN HUMANS, GLUCOSE RELEASED from liver glycogen by the enzyme phosphorylase accounts for ∼50% of the total glucose produced after an overnight fast, significantly contributing to the maintenance of a normal blood glucose concentration (11, 27, 38, 53, 63). Despite increased serum glucose level in people with type 2 diabetes, phosphorylase-mediated glycogenolysis continues to contribute ∼40–50% of overnight glucose production, inappropriate maintaining hyperglycemia (3, 4, 21, 63, 68). Once glycogenolysis decreases, as with extended fasting, the blood glucose concentration decreases dramatically (3, 20, 24, 32, 65). Therefore, understanding how phosphorylase-mediated glycogenolysis is regulated in people with or without diabetes is of considerable importance.

Phosphorylase removes glycosyl units from the terminal branches of glycogen through phosphorylisis, forming glucose 1-phosphate. It is present in two interconvertible forms, phosphorylase a (phosphorylated) and b (unphosphorylated). The a form is the active form. Regulation of phosphorylase is incompletely understood. Phosphorylase has been postulated to be primarily regulated by the interconversion of the active, phosphorylated (phosphorylase a) and inactive, nonphosphorylated (phosphorylase b) forms (6, 17, 28, 33, 43, 64). This important mechanism clearly plays a major role during stress, exercise, hypoxia, and hyperglycemia. However, during the normal feeding and fasting cycle, we and others have shown that phosphorylation/dephosphorylation does not explain the regulation of glycogenolysis (9, 14, 25, 37). Consequently, allosteric, i.e., small-molecular-weight effectors must play a major role in regulating phosphorylase a activity in vivo (14, 45, 48, 50, 51, 67). This postulate is based on two observations. 1) During the normal feeding-fasting cycle in which glycogen is first synthesized and then degraded, measurable phosphorylase a activity does not change (14). Thus another mechanism must exist to explain glycogenolysis in vivo. 2) In vitro phosphorylase a activity is 10- to 100-fold greater than both glycogen synthase activity and the rate of glycogenolysis measured in animal models (13, 14, 19, 56, 57, 67) and in humans (58, 61, 69). If phosphorylase a activity...

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as determined in vitro were the same in vivo, it would be impossible for glycogen to be synthesized postprandially unless there were strong inhibition by other means.

Even though small-molecular-weight effectors are likely to play a regulatory role, we were surprised to find that, when all of the known endogenous small-molecular-weight effectors were added to a rat liver phosphorylase α preparation at estimated physiological concentrations and under simulated in vivo conditions, there was no net change in activity compared with that in the absence of effectors (13). Thus known endogenous effectors of phosphorylase α did not explain the lower-than-expected in vivo enzymic activity in rats. In the present study, we have investigated the possible role of the known effectors of phosphorylase α activity, alone or in various combinations, by use of recombinant human liver phosphorylase α. The results are compared with those obtained previously for the purified rat liver enzyme (13).

Our hypothesis was that known endogenous effectors of phosphorylase α cannot explain phosphorylase α regulation in humans or rats. Thus other as-yet-unidentified endogenous inhibitor or inhibitors are likely to be present and are important in regulation of phosphorylase α.

**EXPERIMENTAL PROCEDURES**

32P-labeled inorganic phosphate (P_i) was purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). [14C]glucose-1-phosphate was purchased from New England Nuclear. All chemicals were obtained from Sigma Chemical (St. Louis, MO). The rabbit liver glycogen used was purified by passage through a mixed-bed ion exchange resin (Amberlite MB-3; Mallinkrodt Laboratory Chemicals, Phillipsburg, NJ).

Recombinant human liver glycogen phosphorylase (HLGP) was expressed in baculovirus (55). Protein was 95% pure, as determined by Coomassie-stained SDS-PAGE.

Specific activity of the active form of HLGP (HLGP_a) was determined using two separate assays, one in the direction of glycogen synthesis and the other in the direction of glycogenolysis. The Tan and Nuttall (62) modification of the method of Gilboe et al. (23) was used to determine phosphorylase α and total phosphorylase activities in the glycogen synthesis direction. A unit represents 1 µmol of [14C]glucose incorporated into glycogen per minute at 30°C under the conditions of the assay.

For the assay in the glycogenolytic direction, a modification of the method of Gevers and Stalmans (22) was used, as described previously (13). We determined the rate of catalysis by phosphorylase a in the glycogenolytic direction for comparison with the observed in vivo glycogenolytic rates. The specific activity of phosphorylase α, determined using the assay in the glycogenolytic direction, was compared with that determined by the widely used assay in the glycogen synthesis direction. For the studies of all allosteric effectors, the assay in the glycogenolytic direction was used. In this method, phosphorylase α activity is determined by incubating purified phosphorylase in the presence of glycogen and 32P-labeled P_i. The radioactivity incorporated into glucose 1-phosphate is then measured. A unit represents 1 µmol of product produced/min at 37°C under the conditions of the assay. The glycogen concentration was saturating.

The Michaelis Menten constant (K_m) for P_i was determined using the assay in the glycogenolytic direction and over a range of P_i concentrations (0.1–10 mM) and in the presence or absence of fixed concentrations of various modulators.

Approximately 55% of the total liver weight is represented by intracellular water. Therefore, intracellular concentrations of the effectors were estimated by dividing their measured concentrations in a liver extract by 0.55 (49). P_i concentrations of 1 and 5 mM were used because they are likely to represent the range of free P_i concentrations in the liver (5, 47).

Results are expressed as percent control. Control (100%) is the activity in the absence of any effectors.

Phosphorylase activity was stable at 37°C; the velocity of the reaction was linear with time and the amount of phosphorylase added. Only 0.03% of the substrate was converted into product over the 3-min incubation time period used in the assay. Thus the conditions approximated an initial velocity (data not shown).

Protein was determined spectrophotometrically by measuring absorption at A_{280}.

Data are presented as means ± SE. Statistics were performed by two-way ANOVA. When the number of observations taken within each treatment was different, ANOVA was done for an “unbalanced” design. Comparison of treatment means with the control was made by Dunnett’s procedure. P < 0.05 was the criterion for statistical significance. The Student’s t-test was used where appropriate.

**RESULTS**

The recombinant phosphorylase was homogenous. On SDS electrophoresis gel, a single band with a molecular mass of ~97 kDa was present. Phosphorylase α represented 84% of the total activity present (Table 1).

The specific activity of HLGP_a in the direction of glycogen synthesis was 53 U/mg protein at saturating concentrations of glycogen and glucose 1-phosphate based on absorption at A_{280}. In the glycogenolytic direction, under a near-saturating concentration of P_i (25 mM) and a saturating concentration of glycogen, specific activity for HLGP_a was 34 U/mg protein (Table 1).

The K_m for P_i, under approximated physiological conditions, i.e., at pH 7.0, 37°C, and in the glycogenolytic direction, was 5 mM (Table 1). In these studies a near-saturating concentration of glycogen (3 mg/ml) was used.

The mean phosphorylase α activities, i.e., control activities representing 100% at concentrations of 1 and 5 mM P_i in the absence of effectors, were 1.3 ± 0.05 and

<table>
<thead>
<tr>
<th>Table 1. Kinetic characteristics of human and rat liver glycogen phosphorylases</th>
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<tbody>
<tr>
<td><strong>Glycogenolysis</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>HLGP_a</td>
</tr>
<tr>
<td>RLGP_a</td>
</tr>
</tbody>
</table>

Values for specific activity are expressed in µmol product produced/min·mg -1·protein -1 and measured under saturating concentration of substrates. HLGP_a and RLGP_a, active human and rat liver glycogen phosphorylases, respectively; K_m, Michaelis-Menten constant.
AMP. AMP is a known stimulator of rat liver phosphorylase a activity. In humans, the physiological concentration of AMP was reported to be 0.3–1 μmol/g wet wt (0.5–1.8 mmol/l intracellular water) (1, 2, 30) (Table 2). AMP at concentrations of 0.05–1 mM increased the HLPa activity ~1.9-fold at a 1 mM P_i concentration and ~1.2-fold at a 5 mM P_i concentration (Fig. 1). AMP was saturating even at a 0.05 mM concentration. At 0.3 mM, AMP reduced the K_m for P_i by ~50% (Table 3). Thus AMP alone is likely to be saturating in vivo under these conditions in humans.

ADP. ADP is an inhibitor of rat phosphorylase a activity (13). In humans, a physiological concentration of ADP as determined chemically was reported to be 0.3–1 μmol/g wet wt (0.5–1.8 mmol/l intracellular water) (1, 2, 30) (Table 2). Values in the range of 0.05–0.2 mM have been reported for free ADP concentration in rat liver by NMR (7, 10, 12, 31, 36). Free ADP concentration in human liver has not been reported. Whether the free ADP is the fraction that binds to phosphorylase a and affects activity in the liver is not known. Therefore, we studied the effects of ADP at concentrations between 0.1 and 10 mM. We included concentrations above the physiological range to gain insight into the binding affinity of ADP to phosphorylase a. ADP inhibited human liver phosphorylase a in a concentration-dependent manner (Fig. 1). The concentration at which the activity is inhibited by 50% (I_0.5) was ~9 mM at 1 mM P_i concentration. This indicates that ADP alone is likely to be a weak inhibitor in vivo. The I_0.5 was not determined at the 5 mM P_i concentration. At an estimated physiological concentration of 3 mM, ADP decreased activity to 85 and 79% of control at 1 and 5 mM P_i, respectively (P < 0.05). Thus the inhibition was modest at a physiological concentration of ADP. At a 0.1 mM concentration, ADP slightly increased activity at both 1 and 5 mM P_i concentration. However, this did not reach statistical significance (P > 0.05). ADP at a 2 mM concentration did not change the K_m for P_i (Table 3).

ATP. ATP is also an inhibitor of rat phosphorylase a activity. A physiological concentration of ATP in humans was reported to be 2.5–3.5 μmol/g wet wt (4.5–6 mmol/l intracellular water), as determined chemically in liver cell extracts, and 2.0–2.9 mmol/l liver volume (~5 mmol/l intracellular water) as measured by NMR (1, 2, 5, 8, 30, 41, 42, 66) (Table 2). In the present study, ATP at concentrations from 3 to 6 mM decreased HLPa activity to ~75–80% of controls at both 1 and 5

Table 2. Estimated physiological concentrations of effectors in rat and human livers

<table>
<thead>
<tr>
<th>Effector</th>
<th>Condition</th>
<th>Rat Liver</th>
<th>Human Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>In extract</td>
<td>0.5</td>
<td>0.5-1.8</td>
</tr>
<tr>
<td></td>
<td>Free AMP</td>
<td>0.05-0.2</td>
<td>ND</td>
</tr>
<tr>
<td>ADP</td>
<td></td>
<td>0.5-1.8</td>
<td>0.5-1.8</td>
</tr>
<tr>
<td>ATP</td>
<td>Fasting</td>
<td>8</td>
<td>ND*</td>
</tr>
<tr>
<td>Glucose</td>
<td>Fasting</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>F-1-P</td>
<td>Fasting</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>0.3-0.5</td>
<td>0.8-1.5</td>
<td></td>
</tr>
<tr>
<td>G-6-P</td>
<td></td>
<td>0.13-0.22</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Concentrations are expressed in mmol/l intracellular water. F-1-P and G-6-P, fructose and glucose 6-phosphate, respectively. *Not determined.

Table 3. Effect on human liver glycogen phosphorylase a K_m for P_i of effectors

<table>
<thead>
<tr>
<th>Effector</th>
<th>Concentration, mM</th>
<th>K_m, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>AMP</td>
<td>0.3</td>
<td>2.3</td>
</tr>
<tr>
<td>ADP</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>ATP</td>
<td>6</td>
<td>6.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>F-1-P</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>
mM P; concentrations (Fig. 1). This decrease was statistically significant (P < 0.05). ATP at a 6 mM concentration increased the K_m for P_i for HLGPa by 1.3-fold (Table 3). ATP was in the form of MgATP, and the Mg^{2+} concentration was always 1 mM higher than the ATP concentration.

Glucose. Glucose is also an inhibitor of rat phosphorylase a activity. The glucose concentration in the liver varies considerably depending on the nutritional state. In rats, it is ~8 mmol/l intracellular water in fasted animals and as high as 20 mmol/l intracellular water after oral glucose administration (49) (Table 2). In humans, liver glucose concentration has not been reported, but it is likely to be similar to that in rats because the blood glucose concentrations are similar. Glucose at 8 mM decreased phosphorylase a activity to 31% of control at a 1 mM P_i concentration and to 56% of control at a 5 mM P_i concentration (P < 0.05). At a 20 mM concentration, the activity was decreased to 10 and 23% of control, respectively (P < 0.05; Fig. 2). Thus glucose at concentrations that are within the physiological range (8–20 mM) strongly inhibited HLGPa activity. Glucose at an 8 mM concentration increased the K_m for P_i for HLGPa by twofold (Table 3).

Fructose 1-phosphate. Fructose 1-phosphate is another known inhibitor of phosphorylase a activity. The fructose 1-phosphate concentration in human liver is usually very low (0.3 mmol/l intracellular water) (2, 30, 65) (Table 2). It may increase to a very high level (1–5 mM or 2–9 mmol/l intracellular water) after intravenous administration of fructose in humans or in people with fructose aldolase deficiency (2, 5). In the present study, we found little inhibition at low fructose 1-phosphate concentrations (0.3–1 mM). At higher concentrations (2–12 mM), there was a concentration-dependent inhibition of phosphorylase a activity. Fructose 1-phosphate at a 12 mM concentration decreased phosphorylase a activity to 27% of control at 1 mM P_i concentration and to 34% of control at 5 mM P_i concentration (Fig. 2). Fructose 1-phosphate at a 2 mM concentration increased the K_m for P_i for human liver phosphorylase a 1.6-fold (Table 3).

UDP-glucose. In humans, in four liver biopsy specimens, the UDP-glucose concentration in the liver was reported to be 0.43–0.82 μmol/g wet wt (~0.78–1.5 mmol/l intracellular water) by HPLC (66) (Table 2). In the present study, UDP-glucose at a concentration of 0.5 mM decreased HLGPa to 77% of control at 1 mM P_i concentration and to 67% of control at 5 mM P_i concentration (P < 0.05). At 2 mM UDP-glucose, the activity was decreased to 38 and 43% of control, respectively (P < 0.05; Fig. 2). Thus UDP-glucose was a modest inhibitor of HLGPa activity.

Glucose 6-phosphate. In the human liver, the physiological concentration of glucose 6-phosphate was reported to be 0.2 μmol/g wet wt (0.4 mmol/l intracellular water) (2) (Table 2). In the present study, there was little inhibition of human phosphorylase a at physiological concentrations (0.1–0.5 mM) (data not shown).

Effect of combinations of effectors on phosphorylase a activity. In subsequent experiments, we determined HLGPa activity at approximate physiological concentrations and in combinations of each of the effectors. The concentrations of each effector were those typically found in rat liver intracellular water (Table 2), because we wished to compare the data with those of rat phosphorylase a and because the concentrations and possible changes under different metabolic conditions are not known for all effectors in humans.

Combination 1 is a mixture of 0.3 mM AMP, 3 mM ADP, 6 mM ATP (in the form of MgATP), 0.5 mM UDP-glucose, 0.3 mM glucose 6-phosphate, 0.3 mM fructose 1-phosphate, and 8 mM glucose. Combination 2 is the same combination of effectors, but the glucose concentration has been increased to 20 mM. Thus the two combinations encompass the glucose concentrations estimated to be present in vivo. As stated previously, the ADP concentration in a rat liver cell extract is ~3 mmol/l intracellular water (47). In humans, in four liver biopsy specimens, it was reported to be 0.77–1.17 μmol/g wet wt (~1.4–2.1 mmol/l intracellular water) by HPLC (66) (Table 2). However, the free ADP concentration in the rat liver as determined by NMR...
may be as low as 0.1 mM (7, 10, 12, 31, 36), and the free ADP concentration in the liver has not been determined in humans. Because the latter may be the ADP concentration that affects phosphorylase \( \alpha \) in vivo, the experiments were repeated using the same combination of effectors at an 0.1 mM instead of a 3 mM ADP concentration.

Both combination 1 and combination 2 inhibited HLGPa activity. With combination 1, the activity was decreased to 34% of control at 1 mM \( P_i \) concentration and to 47% of control at 5 mM \( P_i \) concentration \((P < 0.05)\). With combination 2, the activity was decreased to 17 and 29% of control, respectively \((P < 0.05; \text{Fig. 3A})\). Thus there was a significant inhibition in the presence of all known effectors of phosphorylase \( \alpha \). Furthermore, the inhibition was glucose concentration dependent \((P < 0.05)\).

When the ADP concentration in the combination was lowered to 0.1 mM, inhibition of activity was present but less \((\text{Fig. 3B})\). Compared with the combinations with 3 mM ADP, the difference was statistically significant \((P < 0.05)\). With combination 1, the activity was reduced to 47% of control at 1 mM \( P_i \) concentration and to 64% of control at 5 mM \( P_i \) concentration \((P < 0.05)\). With combination 2, the activity was decreased to 32 and 50% of control, respectively \((P < 0.05)\). A modest but statistically significant glucose concentration-dependent increase in inhibition was again observed when the ADP concentration in the combinations was 0.1 mM \((P < 0.05)\).

DISCUSSION

In the present study, the specific activity of human phosphorylase \( \alpha \) in the direction of glycogen synthesis was 53 U/mg protein based on absorption at \( A_{280} \). In our laboratory, the specific activity of human liver phosphorylase \( \alpha \) in the direction of glycogen synthesis was previously reported to be 40 U/mg of protein in liver biopsy specimens from organ donors \((69)\), i.e., similar to that for the recombinant human enzyme. In an autopsy specimen \((39)\), others have reported it to be only 14.4 U/mg protein \((39)\). This low activity may be attributable to the tissue being obtained 5 h after death \((69)\). We have determined that the rat enzyme specific activity measured in the direction of glycogen synthesis was 22 U/mg protein. Thus it was only \( \sim 50\% \) of that for the human enzyme \((\text{Table 1})\). In contrast, in isolated human hepatocytes, total phosphorylase activity as measured by the method of Stalmans and Hers \((60)\) was reported to be only \( 20\% \) of that from isolated rat hepatocytes \((35)\). Why the activity was less in human hepatocytes is unexplained.

The velocity of the reaction in the opposite direction, i.e., in the direction of glycogenolysis, has been known to be slower than that in the synthetic direction \((40)\). In the present study, the specific activity of human phosphorylase \( \alpha \) in the direction of glycogenolysis also was less than that in the direction of glycogen synthesis \((34 \text{ vs. } 53 \text{ U/mg protein; Table 1})\).

Another difference between the human and rat enzymes was the \( K_m \) for \( P_i \). The HLGPa \( K_m \) for \( P_i \) under approximated physiological conditions, i.e., at pH 7.0, 37°C, and in the glycogenolytic direction, was about fivefold greater than that for the rat liver enzyme \((13)\).

Several effectors of phosphorylase \( \alpha \) have been defined previously. These bind to the enzyme at five separate sites. AMP, ADP, ATP, and glucose 6-phosphate bind at a nucleotide-binding site. The substrates, glycogen, \( P_i \), and the product, glucose 1-phosphate, all bind to the catalytic site. Glucose, fructose 1-phosphate, and UDP-glucose also bind to this site. Glycogen binds to a glycogen storage site as well as the catalytic site \((43)\). A purine site was originally identified by the binding of caffeine. Caffeine is a strong inhibitor of rat liver phosphorylase \( \alpha \), and the inhibition is synergistic with glucose \((15, 34)\). The natural ligand for this site is unknown. Recently, a new allosteric site was identified by the binding of a new type of inhibitor of human liver glycogen phosphorylase \( \alpha \) \((54)\). This site is referred to as the “indole inhibitor” site. The inhibition was synergistic with caffeine. The natural ligand for this site is also unknown.

AMP is the only known stimulator of rat liver phosphorylase \( \alpha \). In the present study, we demonstrated...
that AMP also stimulated human liver phosphorylase \(a\) (Fig. 1). The stimulation by AMP for the human enzyme (≈1.9-fold) was similar to that for the rat isozyme. The results are similar to those reported for human liver phosphorylase \(a\) from an autopsy specimen 5 h after death (39). In the latter study, AMP reduced the \(K_m\) for glucose 1-phosphate by 50%.

ADP, ATP, and UDP-glucose all inhibited human liver phosphorylase \(a\) modestly and in a concentration-dependent manner (Figs. 1 and 2). A concentration-dependent inhibition by ADP was also demonstrated using highly purified rat enzyme (13). However, the \(I_{0.5}\) for the rat enzyme at a 1 mM \(P_i\) concentration was approximately one-half of that for the human enzyme (4 vs. 9 mM). Thus ADP was a stronger inhibitor for the rat isozyme than for the human isozyme (\(P < 0.05\)). The inhibition by ATP was similar for both the human and the rat enzymes.

In contrast, UDP-glucose was a markedly stronger inhibitor of human liver phosphorylase \(a\) than the rat enzyme (Fig. 2). The usual concentration of UDP-glucose in rat liver is 0.3–0.5 mmol/l intracellular water (46–48) (Table 2). The rat enzyme was not inhibited at all at a 0.5 mM UDP-glucose concentration. However, at a 0.5 mM concentration, the human enzyme was inhibited by 1–1.5 (Fig. 2). Fructose 1-phosphate inhibition was similar for both the human and rat enzymes. In both cases, a significant inhibition was demonstrated only at fructose 1-phosphate concentrations of 1–1.5 mM and greater, i.e., at concentrations likely to be present only after a large oral or intravenous dose of fructose (Fig. 2). Glucose 6-phosphate was a minor inhibitor of both enzyme species.

In the present study, we demonstrated that glucose was a strong inhibitor of human liver phosphorylase \(a\). The magnitude of inhibition was about twofold greater for the human enzyme than for the rat enzyme (13). Intracellular AMP, ADP, and ATP concentrations are quite stable in vivo except under pathological conditions. However, the physiological intracellular glucose concentration varies considerably (between 8 and 20 mM) (49). Thus glucose may be an important regulator of phosphorylase activity in vivo, particularly in humans.

When all of the known effectors were present at their presumed intracellular concentrations, a strong net inhibition of human liver phosphorylase \(a\) was observed. The inhibition was dependent on ADP concentration. When the ADP concentration in the combination of effectors was 3 mM, the magnitude of inhibition was similar to that seen with glucose alone (Figs. 2 and 3). Thus, in the presence of all known effectors, glucose canceled the stimulatory effect of AMP and was regulating. How these two compounds interact is unknown. When the ADP concentration in the combination of effectors was lowered to 0.1 mM, the inhibition was less than that with 3 mM ADP (\(P < 0.05\)) but was still significant compared with the control (\(P < 0.05\)) (Fig. 3). Thus glucose was regulatory regardless of the concentration of ADP in the combination. Nevertheless, inhibition by glucose was greater at higher concentrations of ADP.

Liver phosphorylase exists as a homodimer. However, the crystallographic structure has only been determined for the human enzyme (55). The dimer can be thought of as having two sides (faces). The side containing the catalytic site is bound to the glycogen particle (55). The enzyme is known to undergo complex tertiary and quaternary changes upon ligand binding (18). By binding to the catalytic site, glucose may modulate the transmission to the catalytic site of conformational changes from the nucleotide site as well as the purine and the indole sites. The nucleotide site binds a stimulator and several inhibitors. We refer to this very complex regulatory mechanism as a multiplex, site-site regulation of enzymic activity.

The results of these effectors, when added together, were significantly different from those with the rat enzyme (13). With the rat enzyme, when the known effectors were added together at their presumed intracellular concentrations, the net effect was no change in phosphorylase activity compared with that in the absence of effectors. Thus inhibitors canceled the stimulatory effect of AMP, and glucose was not regulating.

Overall, the human and rat liver phosphorylase proteins are 94% identical (29, 44, 59). Specifically, with one exception, all residues that bind AMP (nucleotide-binding site) are conserved. The catalytic and the purine sites are identical. Glycogen-binding sites are 67 (minor site) to 94% (major site) conserved. A new allosteric (indole inhibitor) site was identified in the human liver and rabbit skeletal muscle isozymes (52, 54). The amino acid sequence of this site is also identical in rat and human enzymes (29).

Despite a high degree of similarity between the rat and human liver phosphorylase proteins, the regulation by glucose in the presence of all known effectors of phosphorylase is different. Differential regulation by allosteric effectors of different isozymes of phosphorylase is not uncommon. For example, with one exception, all residues that bind AMP are conserved within the rat liver and rabbit skeletal muscle phosphorylase isozymes (29). However, it has been well documented that their response to AMP stimulation is considerably different. In the absence of AMP, skeletal muscle phosphorylase \(b\) is inactive. AMP lowers the \(K_m\) significantly and activates skeletal muscle phosphorylase \(b\) (26). In contrast, the \(b\) form of the hepatic isozyme has an affinity for all substrates that is exceedingly low. AMP lowers the \(K_m\) modestly but not to levels that are close to the physiological concentrations. In the present study, we determined the \(K_m\) for phosphorylase \(b\) to be >600 mM. AMP at a 0.3 mM concentration did not lower the \(K_m\). Therefore, liver phosphorylase \(b\) can be considered to be inactive in vivo at all times both in humans and in rats (62).

Similarly, AMP has strikingly different effects on the saturation kinetics of human and rat liver phosphorylase \(b\) enzymes, even though the AMP-binding site is
highly conserved (39, 62). AMP increases the maximum velocity ($V_{\text{max}}$) of the human enzyme 10-fold without affecting the affinity for the substrate ($K_m$). With the rat enzyme, AMP lowers the $K_m$ but does not affect the $V_{\text{max}}$. Presumably, long-range transitions are being affected differently in the two enzymes and are thus affecting the catalytic activity. A structural basis for these differences has been proposed (55).

In the present study, recombinant human phosphorylase $a$ activity has been compared with that purified from rat liver. We cannot exclude the possibility that the purification process itself may account for some of the differences observed in this study. There were no posttranslational modifications in the recombinant human phosphorylase except for phosphorylation.

The inhibition of human liver phosphorylase $a$ by 8–20 mM glucose in the presence of other known effectors at their physiological concentrations cannot entirely explain the smaller-than-expected in vivo phosphorylase $a$ activity. Therefore, the mechanisms by which phosphorylase $a$ activity is inhibited in vivo remain to be determined. One possible mechanism may be the presence of another, as-yet-unidentified inhibitor that would increase the inhibition by glucose. In this regard, ligands that bind to the purine site (15, 34) and/or to the recently identified indole inhibitor site (54) may synergize with glucose in inhibiting phosphorylase $a$. The natural ligands for these sites are unknown. Preliminary data indicate that endogenous purines, which presumably bind to the purine site, may interact with glucose in regulating phosphorylase $a$ activity (16).

It is possible that combinations of all of these metabolites may also modulate the stimulatory effect of glucose on phosphorylase phosphatase activity and thus the amount of phosphorylase $a$ present. This also remains to be determined.

In summary, human liver phosphorylase $a$ is regulated differently from the rat enzyme by the same allosteric effectors. The inhibition by glucose in the presence of all known effectors of phosphorylase $a$ is stronger and may explain in part the mechanism by which phosphorylase $a$ activity is inhibited in vivo, at least in the fed state. Additional mechanisms must also have a role, because the activity measured, even at its most inhibited state, still likely exceeds the activity of the active form of liver glycogen synthase and the rate of observed glycogenolysis in rats ($\sim 0.2 \mu\text{mol}\cdot\text{ml}^{-1}\cdot\text{min}^{-1}$) (14) and in humans ($\sim 0.160 \mu\text{mol}\cdot\text{ml}^{-1}\cdot\text{min}^{-1}$) (58, 61, 69).

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