Use of Arginine Compounds To Examine the Role of an Essential Arginine in the Mechanism of Glycogen Phosphorylase†

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ABSTRACT: The possible role of arginine in the mechanism of muscle glycogen phosphorylase was studied by examining the effect of arginine compounds. Guanidino compounds with an aromatic group inhibit native phosphorylase *b*, reduced phosphorylase *b*, phosphorylase *b'*, and phosphorylase *a*. The inhibition was found to be uncompetitive with respect to glucose 1-phosphate and noncompetitive toward glycogen for phosphorylase *b*. This is consistent with a kinetic mechanism where the inhibitor binds after the substrate, glucose 1-phosphate. In the presence of citrate and L-cysteine, *N*-α-tosylarginine methyl ester, a good inhibitor, promotes the removal of tightly bound pyridoxal phosphate. Potato phosphorylase has many similarities to the muscle enzyme, but it lacks the regulatory sites and does not have a polysaccharide storage site [Shimomura, S., & Fukui, T. (1980) *Biochemistry 19*, 2287]. *N*-α-Tosylarginine methyl ester inhibited the potato enzyme, was uncompetitive with glucose 1-phosphate, and was competitive with starch; therefore, it seems likely that TAME is binding near the active site of both the potato and muscle enzyme. The different inhibitory patterns with respect to polysaccharide for potato and muscle phosphorylase can be explained by the absence of the polysaccharide storage site on the potato enzyme. Inhibition by arginine compounds is related to the pK_a of the guanidino function, i.e., the lower the pK_a value, the greater the inhibition. On the basis of these studies and those of Dreyfus et al. [Dreyfus, M., Vandenbunder, B., & Buc, H. (1980) *Biochemistry 19*, 3834], who found that Arg-568 was essential for activity, we suggest that arginyl compounds inhibit when an unprotonated guanidino group competes for the "binding site" of Arg-568.

The mechanism for α-glucan phosphorylases (EC 2.4.1.1) remains unknown despite the vast amount of information known about its regulation. It is known that the coenzyme pyridoxal 5'-phosphate is required for activity (Graves & Wang, 1972). The function of pyridoxal 5'-phosphate in rabbit muscle phosphorylase is unlike that of other pyridoxal 5'-phosphate requiring enzymes in that the coenzyme is tightly bound (Shaltiel et al., 1966) and 60–70% activity remains after reduction by $NaBH_4$ (Fischer et al., 1958; Snell & di Mari, 1970). The amino acid sequence has been determined (Titani et al., 1977), and the X-ray structure is known (Sygusch et al., 1977; Weber et al., 1978; Johnson et al., 1979). However, even the X-ray structure and sequence have not proven a mechanism and, indeed, even raise additional questions regarding the proximity of the phosphate group of the substrate glucose 1-phosphate and the phosphate of the coenzyme (Johnson et al., 1980; Kasvinsky et al., 1978b; Sygusch et al., 1977). Since both of these phosphate groups seem to be in the dianion state in the active form of the enzyme (Parrish et al., 1977; Feldmann & Hull, 1977), it seems strange that these negative charges should be located so close together.

On the basis of arginine modification studies it has been suggested that arginine is important in the active site (Vandenbunder et al., 1979; Dreyfus et al., 1980; Li et al., 1977). Dreyfus et al. (1980) and Vandenbunder et al. (1979) have suggested that there is an essential arginine near the AMP[1] site at the N-terminal region as well as in the active site.

The X-ray structure shows that the sequence of residues 567–574 includes two Lys-Arg sequences and this octapeptide is near the phosphate groups of the coenzyme and the substrate (Johnson et al., 1980).

Early work in our laboratory with the denaturant guanidine hydrochloride showed enzyme inactivation at concentrations much lower than that required for unfolding of the enzyme. This suggested some specific binding sites for the guanidinium ion. Because of the presence of the guanidinium side chain of arginine, we examined the effect of arginine and related compounds as well as other guanidinium compounds on different forms of rabbit muscle phosphorylase.

Materials and Methods

Preparation of Enzymes. Rabbit skeletal muscle glycogen phosphorylase *b* was isolated by the method of Fischer & Krebs (1962). Muscle phosphorylase *a* was prepared from phosphorylase *b* by phosphorylation with rabbit muscle phosphorylase kinase (Krebs, 1966; Brostrom et al., 1971). Phosphorylase *b'* was obtained from rabbit muscle phosphorylase *a* by limited proteolysis with trypsin (Graves et al., 1968). The protein concentration of rabbit muscle phosphorylase was measured spectrophotometrically by using the extinction coefficient $E^{1\%}_{10mm}$ at 280 nm of 13.2 (Kastenschmidt et al., 1968). Potato phosphorylase was prepared by the method of Lee (1960) as modified by Staerke & Schlenk (1967). An extinction coefficient $E^{1\%}_{10mm}$ at 280 nm of 11.2 was used for determining enzyme concentrations (Franken et al., 1972).

Assay of Enzymes. Rabbit muscle phosphorylase was assayed in the direction of glycogen synthesis according to the method of Illingworth & Cori (1953). The assay contained 1% glycogen, 16 mM G-1-P, 1 mM AMP, 20 mM β-glycerophosphate, and 15 mM 2-mercaptoethanol at pH 6.8 and 30 °C. AMP was omitted for assays of phosphorylase *a*. Potato

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[1] Abbreviations used: PLP, pyridoxal 5'-phosphate; G-1-P, glucose 1-phosphate; AMP, adenosine 5'-monophosphate; EDTA, ethylenediaminetetraacetic acid; LAME, L-arginine methyl ester; BAEE, *N*-α-benzoyl-L-arginine ethyl ester; BAA, *N*-α-benzoyl-L-arginine amide; TAME, *N*-α-tosyl-L-arginine methyl ester; MGBG, methyl glyoxal bis(guanylhydrazone) dihydrochloride; Gdn-HCl, guanidine hydrochloride.

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Table I: Activity of Different Forms of Phosphorylase in the Presence of Guanidine Compounds[a]

		activity (%)				
					a	
	concn (mM)	native *b*	reduced *b*	*b'*	−AMP	+AMP
L-arginine methyl ester	10	130	124	111	90	nd[c]
N-α-acetylarginine methyl ester	10	97	100	98	92	nd
N-α-tosyllysine methyl ester	10	98	95	80	nd	85
L-arginine	10	104	107	93	101	97
canavanine	10	67	62	64	70	66
N-α-benzoylarginine ethyl ester	10	44	62	45	26	nd
N-α-tosylarginine methyl ester	10	54	55	50	26	39
N-α-benzoylarginine amide	10	77	82	65	50	55
N-α-benzoylarginine	5	68	74	70	70	70
4-guanidinobenzoic acid	5	20	22	26	19	21
2-guanidinobenzimidazole[b]	3.1	50	nd	nd	nd	nd
β-guanidinopropionic acid	12.5	100	95	96	100	95
guanidine hydrochloride	50	55	68	nd	nd	nd

[a] One hundred percent activity is the activity observed in the absence of these compounds. The assays were done at 30 °C in 1% glycogen, 16 mM glucose 1-phosphate, and 1 mM AMP at pH 6.8. [b] Assay was done in 1.5% Me_2SO because of low solubility. For this reason, no experiments were done with other forms of phosphorylase. [c] Not determined.

phosphorylase was assayed in the direction of glycogen synthesis in 10 mM G-1-P, 1% starch, 0.15 mM EDTA, 30 mM maleate, and 0.25 M NaCl (ionic strength 0.32), pH 6.3 at 30 °C. The amount of phosphate released from G-1-P was determined by the Fiske–SubbaRow method (1925). Assays were stopped by the addition of the ammonium molybdate–sulfuric acid stopping reagent. There was a tendency for some cloudiness to appear after the addition of the stopping reagent at high concentrations of the arginine compounds. This was eliminated by the use of 12% Me_2SO in the stopping reagent. The presence of Me_2SO had no effect on the standard curve for phosphate concentration.

Potentiometric titrations were done with a Beckman Phasar I pH meter fitted with a Beckman Future Model 39504 electrode. The titrations were done at 30 °C. CO_2-free NaOH was prepared, protected with soda lime, and standardized at 0.1104 M with potassium acid phthalate. All the compounds titrated were dried in a vacuum desiccator for 3–4 h at 20 °C and dissolved in boiled CO_2-free H_2O at 10 mM. Solutions were stirred with a stream of nitrogen.

G-1-P, AMP, L-arginine methyl ester (LAME), *N*-benzoyl-L-arginine ethyl ester (BAEE), β-guanidinopropionic acid, 4-guanidinobenzoic acid, and L-cysteine were obtained from Sigma Chemical Co. The following compounds were purchased from Vega Fox: *N*-α-benzoyl-L-arginine, *N*-α-benzoyl-L-arginine amide hydrochloride (BAA), *N*-α-tosyl-L-lysine methyl ester, and *N*-α-tosyl-L-arginine methyl ester (TAME). Canavanine was isolated in our laboratory by Michael Hurst by the procedure of Rosenthal (1977). *N*-α-Acetyl-L-arginine methyl ester was from by Chemalog. L-Arginine was from California Foundations. Glycogen was obtained from Sigma and purified by the method of Anderson & Graves (1973). 2-Guanidinobenzimidazole was obtained from Aldrich.

Results

The effect of some arginine compounds on the activity of various forms of phosphorylase is shown in Table I and is seen to vary from inhibition to stimulation. The effect of these compounds is similar for all forms of the enzyme. For significant inhibition, it was necessary to have both an aromatic and a guanidino group present.

The inhibition with TAME was found to be completely reversible up to 12.5 mM, and there was no time dependence on the inhibition. Early in these studies, we felt that the

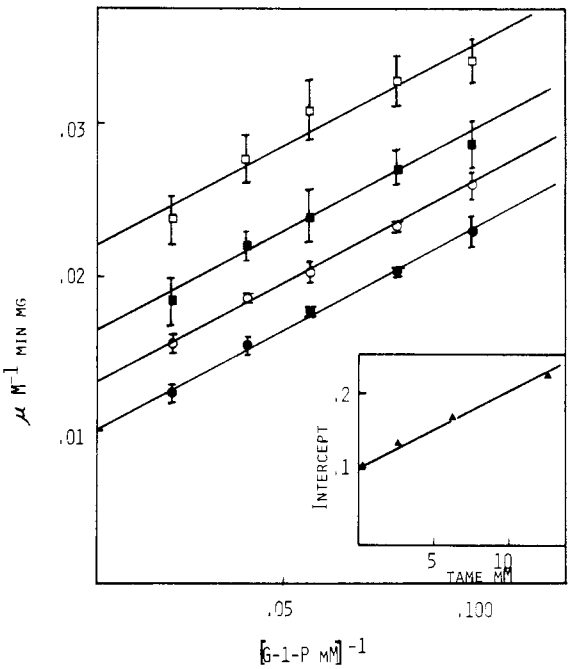

FIGURE 1: Effect of TAME on the kinetics of phosphorylase *b* with respect to G-1-P. Assay was done in 1% glycogen and 1 mM AMP. G-1-P concentrations ranged from 10 to 50 mM. TAME concentrations were 0 (●), 2.5 (○), 6.25 (■), and 12.5 mM (□). Each point is the mean ± 1 SD of values obtained at phosphorylase *b* concentrations of 4.2, 3.1, and 6.3 μg.

requirement for an aromatic ring suggested inhibition at the nucleoside site found by Kasvinsky et al. (1978b). Inhibitors that bind at the nucleoside site show competitive kinetics with G-1-P, and the X-ray structure shows the site to be at the edge of the active-site pocket (Kasvinsky et al., 1978b; Johnson et al., 1979).

The kinetics of the reaction in the presence of TAME are shown in Figure 1. The presence of parallel lines on the Lineweaver–Burk plot suggests uncompetitive kinetics. This is consistent with a model in which the inhibitor binds when G-1-P is present. It is difficult to prove whether the lines are truly parallel. However, even if the reaction is not strictly uncompetitive, the pattern suggests that TAME actually tightens the binding of G-1-P. The inhibition is definitely not competitive; therefore, TAME is not binding at the caffeine site. Similar uncompetitive kinetics were also found with respect to G-1-P with guanidine hydrochloride with phos-

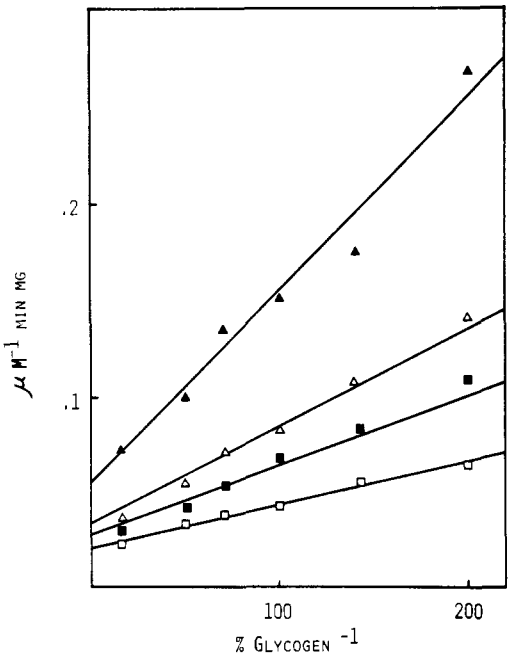

FIGURE 2: Effect of BAEE on the kinetics of phosphorylase *b* with respect to glycogen. Assay was done in 16 mM G-1-P, 1 mM AMP, and 4.2 μg of phosphorylase *b*. Glycogen concentrations ranged from 0.005% to 0.06%. BAEE concentrations were 0 (□), 2.5 (■) 5.0 (△), and 10 mM (▲).

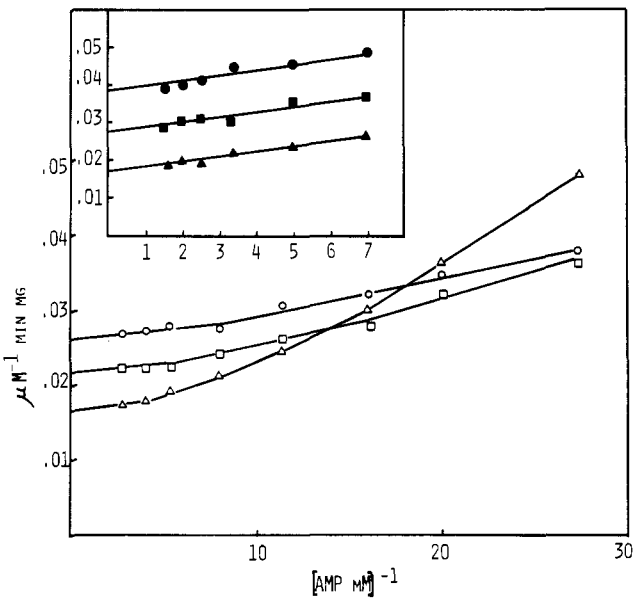

FIGURE 3: Effect of TAME on phosphorylase *b* with respect to AMP. Assay was in 16 mM G-1-P, 1% glycogen, and 5 μg of phosphorylase *b*. AMP concentrations ranged from 27.5 to 375 μM. TAME concentrations were 0 (△), 6.25 (□), and 12.5 mM (○). (Inset) Effect of BAEE at AMP concentrations ranging from 0.14 to 6 mM. BAEE concentrations were 0 (▲), 5 (■), and 10 mM (●). Enzyme concentration was 4.8 μg.

phorylase *b*, BAEE and phosphorylase *b*, benzoylarginine and phosphorylase *b*, and BAEE and reduced phosphorylase *b*.

The kinetics with glycogen showed noncompetitive kinetics with BAEE, as shown in Figure 2. This type of inhibition with respect to glycogen was obtained by using Gdn-HCl and phosphorylase *b*, Gdn-HCl and reduced phosphorylase *b*, BAEE and reduced phosphorylase *b*, and BAEE and phosphorylase *a*.

The effect of the inhibitors on AMP kinetics (Figure 3) again seems to indicate an uncompetitive inhibition at higher AMP concentrations. This result was found with BAEE and

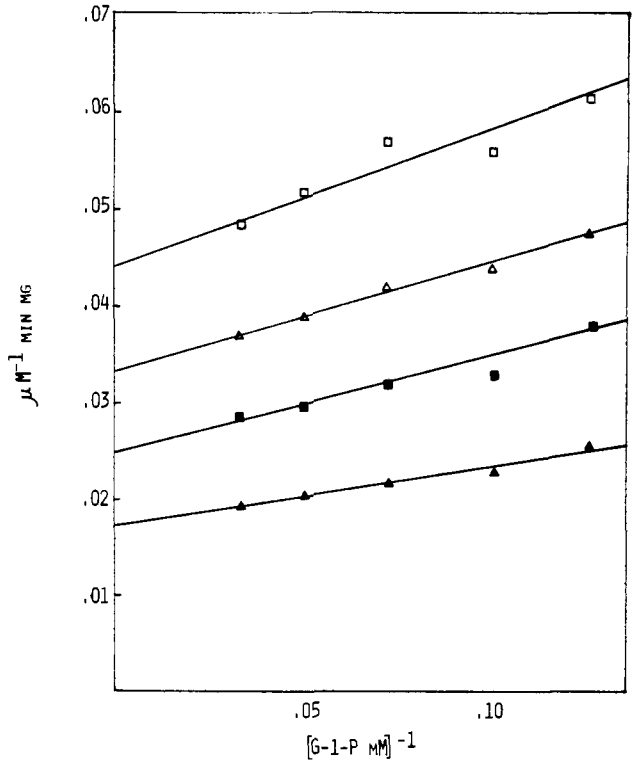

FIGURE 4: Effect of BAEE on kinetics of phosphorylase *a* with respect to G-1-P. Enzyme concentration was 5.5 μg. G-1-P concentrations ranged from 0.8 to 30 mM. BAEE concentrations were 0 (▲), 2.5 (■), 5 (△), and 7.5 mM (□).

with TAME. This is shown in the inset of Figure 3. Although detailed kinetic studies have not been done, these results are consistent with a mechanism where AMP binds to the enzyme before the substrate G-1-P.

Although the inhibition of these different compounds is about the same for all forms of the enzyme (Table I), there is a significant difference in that phosphorylase *a* (in contrast to phosphorylase *b*) showed noncompetitive kinetics with G-1-P in the presence of BAEE and TAME (Figure 4). The kinetic pattern with G-1-P in the presence of TAME was obtained with and without 1 mM AMP, and a noncompetitive pattern was obtained, indicating little or no change in K_m for G-1-P.

Since it seemed possible that these inhibitors were binding at the active site, we examined the effects of TAME on the characteristics of the active site. Spectral data obtained in the 300–500-nm range showed little effect at pH 6.0 or pH 6.8. We then examined the effect of TAME on the resolution of phosphorylase *b*. The loss of coenzyme from phosphorylase *b* occurs only in the presence of a deforming buffer (imidazole citrate) and a trapping reagent, L-cysteine.

When high (0.4 M) imidazole concentrations were used, neither TAME nor G-1-P showed significant effects on the rate of resolution. When low (50 mM) imidazole concentrations were used, G-1-P and TAME did not seem to affect the rate of resolution, but a different equilibrium value was reached in each case. This is shown in Figure 5A. G-1-P (10 mM) seems to stabilize the holoenzyme at about 60%, while TAME (5 mM) significantly increased the proportion of the apoenzyme to 90%. The equilibrium value was not a function of the presence or absence of AMP but was a function of protein concentrations (Figure 5B).

We then tried to replace imidazole with TAME as the deforming agent. Figure 5C shows the loss of activity of phosphorylase *b* in 100 mM citrate buffer and 40 mM cysteine at pH 6 and indicates that TAME at low concentrations (10 mM) could effectively promote resolution. Activity could be

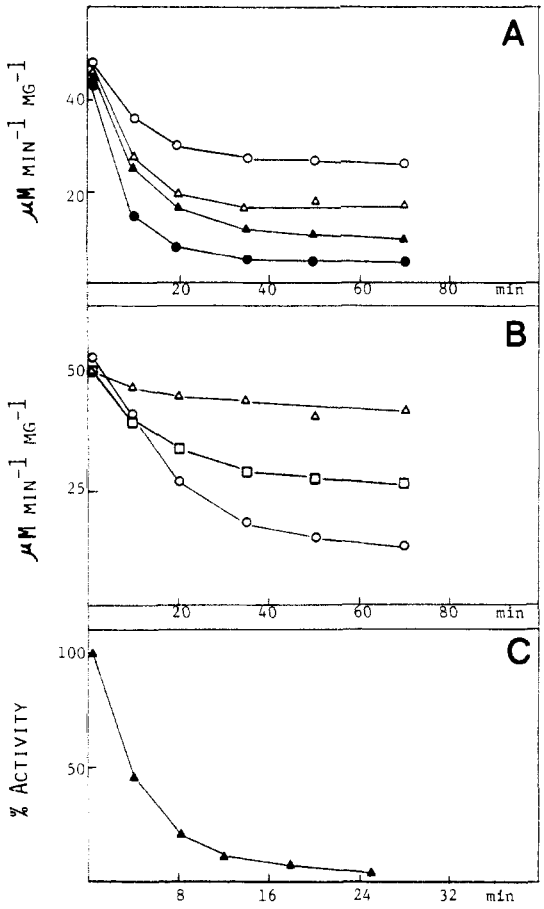

FIGURE 5: (A) Resolution of phosphorylase *b* in 50 mM imidazole citrate buffer, 40 mM L-cysteine, 1 mM AMP, and 2.3 mg/mL phosphorylase *b* at pH 6, 30 °C. Activity is measured by removing 20-μL aliquots and diluting into 0.25 mL of 0.08 M β glycerophosphate–0.06 M β mercaptoethanol, pH 6.8, buffer. Twenty seconds after dilution 100 μL is used in a 0.4-mL total volume activity assay. Additions to the incubation mixture are 0 additions (▲), 10 mM G-1-P (○), 5 mM TAME (●), 10 mM G-1-P, and 5 mM TAME (△). (B) Effect of protein concentration on the resolution of phosphorylase *b*. Incubation and assay conditions were the same as in (A). Phosphorylase concentrations in the incubation mixture were 4.6 (△), 2.3 (□), and 1.15 mg/mL (○). (C) Ability of 10 mM TAME to aid in resolution of phosphorylase *b*. Incubation was in 100 mM citrate buffer, and 40 mM L-cysteine, pH 6 at 30 °C. Percent activity is percent of control, which had no TAME in the incubation mixture.

recovered by incubation with PLP before assay, indicating that resolution had occurred rather than a nonspecific loss of activity. No loss of activity was seen in the absence of L-cysteine. L-Arginine methyl ester had no effect on the resolution in the absence of TAME, nor did it modify the effect of TAME.

The effect of TAME on the activity of phosphorylase plus the type of inhibition and the effect on resolution suggested that it was acting near the active site. However, strong evidence exists to show definite communication between the N-terminal region and the PLP site (Janski & Graves, 1979; Yan et al., 1979). Therefore additional evidence is needed to prove that the inhibitor is binding at the active site and not at the regulatory sites.

Potato phosphorylase has been shown to have many similarities to the muscle enzyme, particularly in regard to the coenzyme (Shimomura et al., 1980). However, it does not have an active and inactive form; it is not affected by AMP, ATP, and DTT (Gold et al., 1971) and is not inhibited at the nucleoside site (Graves et al., 1980). It uses starch rather than glycogen and does not have a polysaccharide storage site (Shimomura & Fukui, 1980). Thus, the potato enzyme seems to be a good model for rabbit muscle phosphorylase stripped

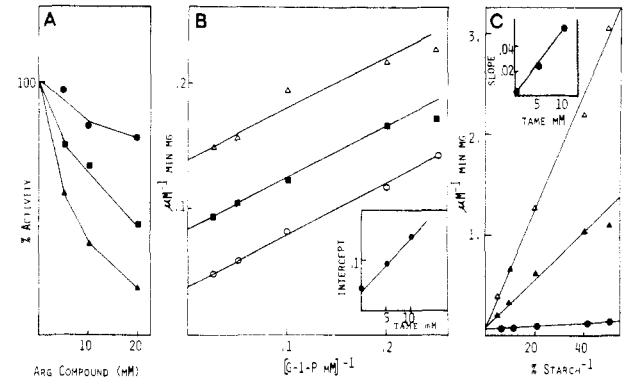

FIGURE 6: Effect of arginine compounds on potato phosphorylase. (A) Effect of various concentrations of arginine compounds on the potato phosphorylase activity: TAME (▲), BAA (■), and LAME (●). (B) Effect of TAME on the kinetic pattern of potato phosphorylase with respect to G-1-P. Assays were done in 1% starch. Enzyme concentration was 23.5 μg. G-1-P concentrations ranged from 4 to 40 mM. TAME concentration was 0 (○), 5 (■), and 10 mM (△). (C) Effect of TAME on the kinetic pattern of potato phosphorylase with respect to starch. G-1-P concentration was 10 mM. Enzyme concentration was 23.5 μg. Starch concentrations ranged from 0.02% to 0.2%. TAME concentration was 0 (●), 5 (▲), and 10 mM (△).

of its allosteric effector sites. If guanidino compounds are binding at the active site, we would expect that they would inhibit the potato enzyme. If they bound the allosteric sites of the muscle enzyme, we would expect to see no effect with the potato enzyme.

Figure 6A shows that TAME is an inhibitor of the potato enzyme with 35% activity at 10 mM. The kinetics again seem to be uncompetitive with respect to G-1-P (Figure 6B), which suggests binding near the active site.

One difference between the potato phosphorylase and muscle phosphorylase is the presence of the glycogen storage site in the muscle enzyme. Kasvinsky et al. (1978a) have suggested that after binding occurs at the storage site, nonreducing ends of glycogen bind at a second binding site, the active site. Shimomura & Fukui (1980) found, by affinity acrylamide gel electrophoresis of PLP analogue reconstituted potato phosphorylase, that the primary binding site for starch is near the PLP site. When we examined the type of inhibition of TAME with starch and potato phosphorylase, we found that TAME was competitive with starch (Figure 6C). This indicates that TAME is binding near the active-site region.

Since the differences in the observed effects with similar guanidino compounds (Table I) seemed larger than expected, we examined the possibility of an effect of the pK_a of the guanidino group. Titrations of TAME and BAA resulted in pK_a values of 9.39 ± 0.03 and 11.75 ± 0.04, respectively. This suggested that an unprotonated arginine group may be more inhibitory. Attempts to titrate BAEE were unsuccessful owing to the instability of the pH, which was probably due to hydrolysis of the ester linkage. It was possible to titrate TAME since it was completely titrated at pH 10.3. The ethyl ester of BAEE was more easily hydrolyzed than the methyl group, while the amide showed no evidence of hydrolysis.

Discussion

The fact that guanidino-containing compounds inhibit various forms of muscle phosphorylase and potato phosphorylase suggests that the inhibitors operate through a common mechanism. The uncompetitive kinetics observed with respect to glucose-1-P for phosphorylase *b* and potato phosphorylase suggests that the binding site for inhibitors is not formed or is poorly formed until the substrate, glucose-1-P, binds. *N*-

α-Tosylarginine methyl ester, a good inhibitor, is far more effective than imidazole in promoting the resolution of tightly bound pyridoxal phosphate. Because of these results and the fact that TAME is a competitive inhibitor with respect to polysaccharide for potato phosphorylase, we suggest that guanidino compounds influence phosphorylase activity by binding in the active-site region.

The difference in the kinetics of inhibition with respect to polysaccharide for muscle and potato phosphorylase does not mean that different mechanisms are involved. The kinetic studies of Kasvinsky et al. (1978a) suggest that polysaccharide binds in a compulsory order to muscle phosphorylase. The first interaction is believed to occur at the storage site followed by interaction of the nonreducing ends with glycogen phosphorylase. Although Shimomura & Fukui (1980) have suggested that the polysaccharide site may consist of two subsites in potato phosphorylase, no polysaccharide storage site like that of the muscle enzyme has been identified. When the random rapid equilibrium model for potato phosphorylase is used, the following equilibrium expressions are important: E + P $\rightleftarrows$ EP, K_{ip}; E + G $\rightleftarrows$ EG, K_{iG}; EP + G $\rightleftarrows$ EPG, K_G; EG + P $\rightleftarrows$ EPG, K_p; and EP + I $\rightleftarrows$ EPI, K_I; E is the enzyme, G is the polysaccharide, P is G-1-P, and I is the inhibitor. The rate equation is

$$\frac{V_m}{V} = \frac{K_{iG}K_P}{(G)(P)} + \frac{K_P}{(P)} + \frac{K_G}{(G)}\left(1 + \frac{I}{K_I}\right) + 1$$

This predicts uncompetitive kinetics with G-1-P and competitive kinetics with starch (Figure 6B,C). With muscle phosphorylase, which has a glycogen storage site, two additional dissociation constants must be considered, EPG + I $\rightleftarrows$ EPGI, K_I', and EPI + G $\rightleftarrows$ EPGI, K_G'. In this case, the complexes indicated by EG, EPG, or EPGI represent binding of the polysacchride G at the storage site. The rate equation is similar except the term I/K_I' is added and predicts uncompetitive kinetics for G-1-P (Figure 1) and noncompetitive kinetics for glycogen (Figure 2).

Our data indicate that for an arginine compound to be an inhibitor it must have a hydrophobic group. This could be a steric effect or an effect on the pK_a of the guanidino group. Our titration of the inhibitor TAME resulted in a pK_a of 9.39, considerably lower than the value of 12.5 commonly assigned to arginine. Titration of BAA (a less effective inhibitor) gave a pK_a of 11.7, which is still lower than that of arginine, which had no effect on the activity. Canavanine, an amino acid in which the methylene group adjacent to the guanidino group has been replaced with an oxygen, has a pK_a of 7.4 (Tomiyama, 1935) and was a good inhibitor. Guanidinobenzimidazole was a good inhibitor and has a pK_a of 8.5 (Witt & Roskoski, 1980). Thus, in general, arginine compounds with a lower pK_a are better inhibitors.

The involvement of arginine in the active site of phosphorylase has been shown by chemical modification studies of Dreyfus et al. (1980). In the presence of AMP, these authors found Arg-568, to be modified but in the absence of AMP and glucose-1-P, the reaction was blocked. In a recent study, Patthy & Thèsz (1980) have suggested that at neutral to alkaline pH, α-dicarbonyl compounds react preferably with the unprotonated form of the guanidino group. The selectivity of modification of arginyl groups in proteins by α-dicarbonyl compounds was explained by effects of the environment on the pK_a of a guanidino group, and they proposed that the strong positive electric potential of an anion binding site lowers the pK_a of an essential arginine. The hypothesis of Patthy & Thèsz (1980) would suggest that Arg-568 is unprotonated (or at least a significant portion). Because we find that arginine compounds with lower pK_a values inhibit uncompetitively with respect to G-1-P, we suggest that these compounds inhibit by replacing the unprotonated Arg-568, which has been found essential to the mechanism.

Various factors could contribute to a lower pK_a value for an arginyl group in the active site of phosphorylase. First, the sequence Lys-Arg$_{568}$-Ile-His-Glu-Tyr-Lys-Arg contains a cluster of basic groups. Proximity of positive charge could lower the pK_a value, and Walsh (1979) has suggested that the low pK_a value of 5.9 for an ε-amino group of a lysyl residue in the active site of acetoacetate decarboxylase (Schmidt & Westheimer, 1971) is due to an adjacent lysyl group. Second, α-helical structure could contribute to the character of the anion binding site. The X-ray structure shows several α helices near the active site (Johnson et al., 1979), and Hol et al. (1978) proposed that the N-terminal end of an α helix has considerable positive charge and often aids in the alignment of anions at the active site. Ploegman et al. (1979) calculated a decrease in the pK_a of cysteine of approximately 3.5 pH units in rhodanese, which has two α helices. Thus, it is not impossible that the pK_a for arginine could be lowered significantly.

Johnson et al. (1980) have proposed a model for glycogen phosphorylase that is attractive because of its suggestion of a mode of nonproductive binding of G-1-P and the ability to accommodate the nonreducing end of glycogen into the active site. However, the model does not account for the essential character of Arg-568. Our kinetic data would be consistent with trapping G-1-P in a nonproductive manner.

Cotton et al. (1973) have found that methylguanidine will form complexes with phosphate. Earlier we suggested that upon binding of G-1-P, arginine forms hydrogen bonds bridging the phosphate groups of the substrate and coenzyme (Miller et al., 1980). Although these proposals had a charge localized at the guanidino group, the concept of a guanidino group bridging two phosphates may occur with a shared proton and provide a means of proton transfer in the active form of the enzyme. The function of the essential arginine has been examined for many enzymes, and data for several enzymes seem to indicate that arginine may have a catalytic function rather than a binding function since the substrate or coenzyme will also bind the modified protein (Fujioka & Takata, 1981; Kremer et al., 1980; Bleile et al., 1975; Yang & Schwert, 1972).

Although we cannot prove any mechanism by these data, our data strongly suggest a direct role for an unprotonated arginine in the mechanism. It has long been hoped that an intermediate could be observed in the phosphorylase reaction, and some means must exist for stopping the reaction at some point on the way to products. It seems that these compounds bind at the active site after the substrate is bound and allow observation of more of the reaction profile.

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Purification and Characterization of Hypoxanthine–Guanine Phosphoribosyltransferase from *Saccharomyces cerevisiae*†

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ABSTRACT: Hypoxanthine–guanine phosphoribosyltransferase (HPRT) was purified 12 000-fold to homogeneity from yeast by a three-step procedure including acid precipitation, anion-exchange chromatography, and guanosine 5′-monophosphate affinity chromatography. The enzyme is a dimer consisting of two, probably identical, subunits of M_r 29 500. The enzyme recognizes hypoxanthine and guanine, but not adenine or xanthine, as substrates. An antiserum against both native and denatured enzyme has been raised and shown to be specific for the enzyme. The antiserum has no affinity for Chinese hamster or human HPRT but does recognize subunits of yeast HPRT as well as some cyanogen bromide fragments of the enzyme.

Hypoxanthine–guanine phosphoribosyltransferase[1] (HPRT; EC 2.4.2.8) catalyzes the conversion of hypoxanthine and guanine to their respective nucleotides. This enzyme from mammalian sources has proved to be of great interest for the study of mutation in cultured mammalian cells (Caskey & Kruh, 1979). Deficiency of the enzyme in humans causes

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[1] Abbreviations used: HPRT, hypoxanthine–guanine phosphoribosyltransferase; GMP, guanosine 5′-monophosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; DTT, dithiothreitol; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride.

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