Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 254, No. 9, Issue of May 10, pp. 3166–3169, 1979
Printed in U.S.A.

The Effect of Solvents on Nucleotide Regulation of Glycogen Phosphorylase*

(Received for publication, January 22, 1979; and in revised form, March 9, 1979)

Ronald J. Uhing, Alvin M. Janski,‡ and Donald J. Graves§

From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011

SUMMARY

The activity of glycogen phosphorylase is controlled by two nucleotide sites. We have found that organic solvents affect the regulatory properties of phosphorylase by altering the binding at these two sites. At the activator site, the K_a for AMP is lowered 10-fold in the presence of 10% 1,2-dimethoxyethane while, at the inhibitor site, the K_i for caffeine is increased 6-fold. The stimulation of activity by organic solvents is highly dependent on the enzyme's activity state. Phosphorylase *b*, which has a requirement for a nucleotide activator, loses this requirement in the presence of organic solvents, while the active form of the enzyme, phosphorylase *a*, is only slightly stimulated by organic solvents. The activation profile obtained with rabbit liver phosphorylase suggests that differences in the properties of this enzyme from rabbit muscle phosphorylase might be explained by a change in the relative affinity for AMP at the two nucleotide sites. The results also suggest that 1,2-dimethoxyethane may be useful to determine accurately the activities of different forms of liver phosphorylase.

INTRODUCTION

Activation of glycogen phosphorylase *b* by AMP is an important regulatory mechanism. X-ray crystallography of phosphorylases *b* and *a* show that AMP binds at a site close to the monomer interface (1, 2). Early studies suggested that a second nucleotide site exists on phosphorylase (3) and x-ray crystallography has shown that this site is found in a crevice of the protein some 30 Å away from the AMP binding site (4). AMP does not bind effectively to this site but IMP, nucleosides, and purine analogs, *e.g.* caffeine and theophylline, do and recent studies show that binding of these compounds competitively inhibits the binding of the substrate glucose-1-P[1] (4). The binding sites are distinct for glucose-1-P and IMP, but they are in close proximity. The exact role that this site has in the regulatory processes of phosphorylase action is not understood.

We sought a mechanism to study the significance of this second nucleotide site and found that, through the use of organic solvents, we could desensitize the enzyme to inhibition by compounds like IMP and caffeine. We have used organic solvents to probe further into control mechanisms of glycogen phosphorylase and found that solvents can activate phosphorylase *b* even in the absence of nucleotides. This report characterizes the action of organic solvents on different forms of glycogen phosphorylase derived from muscle and liver.

MATERIALS AND METHODS

Rabbit skeletal muscle glycogen phosphorylase *b* was isolated by the method of Fischer and Krebs (5) and recrystallized at least three times before use. Rabbit liver glycogen phosphorylase *b* was isolated as described by Appleman *et al.* (6). Muscle phosphorylase *a* and liver phosphorylase *a* were prepared from their respective phosphorylase *b* forms by phosphorylation with rabbit skeletal muscle phosphorylase kinase (7). Phosphorylase concentrations was determined by using an absorbance index $\epsilon^{1\%}_{10\,mm}$ at 280 nm of 13.2 (8). Phosphorylase activity was measured in the direction of glycogen synthesis according to the method of Illingworth and Cori (9). The assay system contained 1% glycogen, 16 mM glucose-1-P, 10 mM β-glycerophosphate, and 7.5 mM 2-mercaptoethanol at pH 6.8 and 30°C. The concentrations of nucleotide used in the assay are described in the text.

AMP, IMP, caffeine, and glucose 1-phosphate were obtained from Sigma Chemical Co. and 1,2-dimethoxyethane from Eastman Kodak Co. Shellfish glycogen was purified by the method of Anderson and Graves (10).

RESULTS

Inhibition of the AMP-induced activation of rabbit muscle phosphorylase *b* by various hydrophobic compounds has been described by Anderson and Graves (10). Soman and Philip (11) have found a direct correlation between the ability of various aromatic compounds to inhibit phosphorylase and their hydrophobicity. Recently, the binding of various hydrophobic compounds such as caffeine and adenosine to a nucleoside inhibitor site on phosphorylase *a* has been described (4). The apparent hydrophobic nature of these binding sites has prompted our use of organic solvents to further examine the nucleotide control of phosphorylase.

A representative list of the organic solvents examined is presented in Table I. It was found that most of the solvents tested, which were miscible with water, resulted in an activation of phosphorylase *b* in the presence of 1 mM AMP, while the cyclic compounds tested, tetrahydrofuran and dioxane, inhibited the enzyme. The most pronounced activation was found to be with methyl ethers of glycols. There appeared to be no direct correlation between the extent of activation of phosphorylase and the dielectric constant of the medium (*e.g.* glycerol, which has a lower dielectric constant than water, inhibits, while dimethyl sulfoxide, which at the concentration used does not significantly alter the dielectric constant, activates). However, most of the solvents that activate have lower dielectric constants than water.

Further studies were initiated with the most potent activator 1,2-dimethoxyethane because preliminary results indicated that it was effective in reducing inhibition by hydrophobic compounds. The effect of 10% 1,2-dimethoxyethane (v/v) on the kinetic parameters is summarized in Table II. Inclusion

* This work was supported by Research Grant GM-09587 from the National Institutes of Health, United States Public Health Service. This is Journal Paper J-9410 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project 2120. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address, Laboratory of Metabolism, National Institute of Alcohol Abuse and Alcoholism, 12501 Washington Ave., Rockville, Md. 20852.

§ To whom requests for reprints should be addressed.

[1] The abbreviation used is: glucose-1-P, α-D-glucopyranose 1-phosphate.

of the solvent increases the maximal velocity of phosphorylase *b* obtained in the presence of 1 mM AMP by 19%. The stimulation of phosphorylase by 1,2-dimethoxyethane is highly dependent upon the enzyme's activity state. Phosphorylase *a* in the presence of 1 mM AMP is only slightly activated by 1,2-dimethoxyethane while, in the absence of AMP, it shows an activation profile similar to that of phosphorylase *b* in the presence of 1 mM AMP (Fig. 1). Phosphorylase *b* no longer shows an absolute requirement for nucleotide when activity is measured in the presence of 1,2-dimethoxyethane (Fig. 1*A*). In the presence of 20% 1,2-dimethoxyethane (v/v) and in the absence of any nucleotide activator, phosphorylase *b* exhibits a maximal velocity of 46 IU/mg or 74% of that obtainable in the presence of saturating AMP (data not presented). The K_m for the substrates glucose-1-P and glycogen were decreased slightly with the inclusion of 1,2-dimethoxyethane (Table II). The presence of the organic solvent also eliminated the homotropic cooperativity for glucose-1-P throughout the concentration range utilized (data not presented).

The most dramatic effect of 1,2-dimethoxyethane is on the nucleotide binding sites of phosphorylase. Besides the aforementioned ability to serve as an activator in the absence of a nucleotide, 1,2-dimethoxyethane also affects the binding parameters directly (Table II). In the presence of 10% 1,2-dimethoxyethane, the K_a for AMP is lowered 10-fold. The solvent also eliminates the cooperativity of AMP binding. 1,2-Dimethoxyethane exerts an opposite effect on the nucleoside inhibitor site recently described by Kasvinsky *et al.* for phosphorylase *a* (4). Nucleoside binding at this site is competitive with glucose-1-P. For phosphorylase *b*, the K_i for caffeine binding at this site is increased 6-fold with the inclusion of 10% 1,2-dimethoxyethane.

The results obtained on the nucleotide binding sites suggested that this organic solvent could serve as a useful probe to examine the different nature of these sites and possible controls of phosphorylase activity by differential binding at either an activator or inhibitor site on the same molecule. The different effects of 1,2-dimethoxyethane on the two nucleotide sites can be seen by using a single nucleotide, IMP (Fig. 1*A*). Whereas, the AMP-induced activity is only slightly affected by 1,2-dimethoxyethane, the IMP-induced activity increases from 1.9 IU/mg in the absence of organic solvent to 46.1 IU/mg at 12.5% 1,2-dimethoxyethane. IMP has about equal affinities for the two nucleotide sites ($K_i \simeq K_a \simeq 2$ mM) so that decreased binding at the inhibitor site and increased binding at the activator site is seen with increasing 1,2-dimethoxyethane concentrations. At the highest concentrations of 1,2-dimethoxyethane, IMP activation is only slightly below that of AMP. These results are consistent with those of Black and Wang (12), which showed that, in the presence of high glucose-1-P, IMP activation of phosphorylase *b* was comparable to that of AMP.

TABLE I

Activation of phosphorylase b by organic solvents

Phosphorylase *b* (13 μ/ml) was assayed as described under "Materials and Methods" in the presence of 1 mM AMP and 10% v/v of the indicated solvents.

Solvent (10% v/v)	Activity ratio
Water	1.00
Methanol	1.22
Ethanol	1.10
Glycerol	0.87
Ethylene glycol	0.93
1,2-Propanediol	1.12
2-Methoxyethanol	1.28
1,2-Dimethoxyethane	1.34
1,1-Dimethoxyethane	1.30
Dimethoxymethane	1.24
Trimethylorthoformate	1.24
2-Butoxyethanol	0.00
Acetone	1.24
Dimethyl sulfoxide	1.24
Tetrahydrofuran	0.16
Dioxane	0.44

TABLE II

The effect of 1,2-dimethoxyethane on the kinetic parameters of phosphorylase b

Kinetic Parameter	In the absence of 1,2-dimethoxyethane	In the presence of 10% 1,2-dimethoxyethane (v/v)
K_m glucose-1-P[a] (mM)	3	2
V_{max}[b] (IU/mg)	62	74
K_m glycogen[c] (%)	0.019	0.013
K_a AMP[d] (μM)	79	8
K_i caffeine[e] (mM)	0.3	1.8

[a] Measured in the presence of 1 mM AMP, 1% glycogen, 20 mM β-glycerophosphate, 10 mM 2-mercaptoethanol, 10 μg/ml of enzyme, and glucose-1-P concentrations between 1 and 15 mM at pH 6.8 and 30°C.

[b] Extrapolated from plots of (velocity)$^{-1}$ *versus* (glucose-1-P)$^{-1}$ from *a*.

[c] Measured in the presence of 1 mM AMP, 16 mM glucose-1-P, 15 mM β-glycerophosphate, 11 mM 2-mercaptoethanol, 13 μg/ml of enzyme, and glycogen concentrations between 0.0025% and 0.0625% at pH 6.8 and 30°C.

[d] Measured in the presence of 1% glycogen, 16 mM glucose-1-P, 15 mM β-glycerophosphate, 11 mM 2-mercaptoethanol, 13 μg/ml of enzyme, and AMP concentrations between 10 and 100 μM in the absence of 1,2-dimethoxyethane or between 0.25 and 12.5 μM in the presence of 1,2-dimethoxyethane at pH 6.8 and 30°C.

[e] Measured in the presence of 1% glycogen, 1 mM AMP, 20 mM β-glycerophosphate, 15 mM 2-mercaptoethanol, 10 μg/ml of enzyme, glucose-1-P concentrations between 2 and 10 mM, and caffeine concentrations between 0.1 and 0.5 mM in the absence of 1,2-dimethoxyethane or between 1 and 7 mM in the presence of 1,2-dimethoxyethane at pH 6.8 and 30°C.

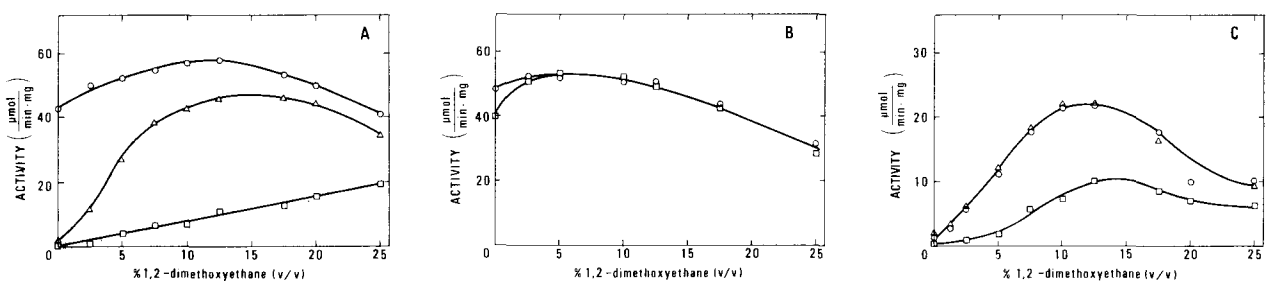

FIG. 1. 1,2-Dimethoxyethane activation of glycogen phosphorylase. Enzymatic activity was measured as described under "Material and Methods" in the presence of the indicated amounts of 1,2-dimethoxyethane. The assay mixture also contained: *A*, 13 μg/ml of rabbit skeletal muscle phosphorylase *b* in the presence of no nucleotide (□), 1 mM AMP (○), or 1 mM IMP (△); *B*, 13 μg/ml of rabbit skeletal muscle phosphorylase *a* in the presence of no nucleotide (□) or 1 mM AMP (○); *C*, 15 μg/ml of rabbit liver phosphorylase *b* in the presence of no nucleotide (□), 5 mM AMP (○), or 5 mM IMP (△).

As mentioned, the activation by 1,2-dimethoxyethane is dependent upon the activity state of the enzyme. The most dramatic effect is the induction of phosphorylase *b* activity in the absence of a nucleotide. Under these same conditions, phosphorylase *a* is only slightly stimulated (Fig. 1*B*). The extent of stimulation is comparable to that of phosphorylase *b* in the presence of saturating AMP. Phosphorylase *a* in the presence of 1 mM AMP exhibits a maximal stimulation of only 9% with 1,2-dimethoxyethane compared with a 35% maximal stimulation of phosphorylase *b* under the same conditions.

Fig. 1*C* depicts the activity of rabbit liver phosphorylase *b* in the presence of no nucleotide, 5 mM IMP, and 5 mM AMP with increasing concentrations of 1,2-dimethoxyethane. The activation patterns for the two nucleotides exhibit identical profiles. The profiles exhibit similar patterns at 1 mM nucleotide. The maximum activation of liver phosphorylase *b* by 1,2-dimethoxyethane in the presence of AMP or IMP is similar to the activity of liver phosphorylase *a* in the absence of nucleotide. Liver phosphorylase *a* activity is not stimulated by 10% 1,2-dimethoxyethane.

DISCUSSION

Rabbit skeletal muscle phosphorylase *b* has a requirement for a nucleotide activator (13). The activator site is located some 30 Å away from the catalytic site. This requirement is met most proficiently by AMP (14), although it can also be satisfied by IMP under appropriate conditions (*e.g.* Refs. 12 and 15).

The presence of a second nucleotide site on phosphorylase has been postulated by Wang *et al.* (3). Recently, the location of this site on phosphorylase *a* has been provided by crystallographic studies and its kinetic characteristics were determined (4). Nucleoside binding at this site is competitive with glucose-1-P, which binds 10 Å away. Of the compounds capable of inhibiting phosphorylase by binding to this inhibitor site, the more hydrophobic compounds such as adenosine, caffeine, and riboflavin are more effective than the more hydrophilic nucleotides. Soman and Philip have shown that the ability of aromatic compounds to inhibit phosphorylase is related to their hydrophobicity (11). These aromatic compounds have also been found to be competitive with glucose-1-P.[2]

We have found that organic solvents could be used to further study the nature of the binding of nucleotides, nucleosides, and their analogs at the two sites on phosphorylase and possible controls on activity by differential binding at these sites. The ability of 1,2-dimethoxyethane to replace nucleotides in the activation of phosphorylase *b* was surprising since prior to this the activation process had been shown to be quite specific for nucleotides. Dreyfus *et al.* (16) have also recently reported the ability of alcohols to activate phosphorylase *b* in the absence of nucleotides. The ability of 1,2-dimethoxyethane to replace the nucleotide activation as well as the higher V_{max} and lower K_m for glucose-1-P exhibited for AMP-activated phosphorylase *b* in its presence suggests that 1,2-dimethoxyethane produces a phosphorylase *b* molecule with activity characteristics similar to phosphorylase *a*. This suggestion is also consistent with the observation that activation by 1,2-dimethoxyethane is dependent upon the enzyme's activity state; *i.e.* phosphorylase *b* in the absence of AMP exhibits the most activation by the organic solvent and phosphorylase *a* in the presence of AMP shows the least. 1,2-Dimethoxyethane activation of phosphorylase *b* plus AMP and phosphorylase *a* minus AMP is between these extremes.

The effect of 1,2-dimethoxyethane on phosphorylase could be directly on the conformation of the molecule or could be an indirect effect on the binding interaction with the nucleotide or nucleotide analog. A direct effect on the activator site is suggested by the ability of 1,2-dimethoxyethane to activate phosphorylase *b* in the absence of AMP. An indirect effect involving this site might be the facilitation of a charge interaction. Mott and Bieber have suggested that the 5′-phosphate portion of the nucleotide is important in the activation process (17). A charge interaction involving this portion of the molecule might be expected to be strengthened by lowering the dielectric constant of the medium (18). Further experiments are needed to evaluate the relative importance of these two factors in the lowering of the K_a for AMP by the solvent.

An indirect effect might also be important in the higher K_i for caffeine exhibited in the presence of 1,2-dimethoxyethane. The inclusion of an organic solvent increases the hydrophobicity of the medium so that the affinity of a hydrophobic compound for an apolar site on the enzyme might be expected to decrease. The hydrophobic dependency for binding at this site has been mentioned. The ability of organic solvents to "solvate" hydrophobic substrates or inhibitors from their binding site has been previously described for α-chymotrypsin (19).

Nucleotide activation of rabbit liver phosphorylase exhibits properties markedly different from the muscle isozyme. The liver enzyme is only slightly active in the presence of AMP (20). Further activation requires the presence of high salt concentrations (20) or high concentrations of glucose-1-P (21). These conditions are similar to those found necessary for best activation of muscle phosphorylase *b* in the presence of IMP (12). The presence of the nucleoside inhibitor site on the liver isozyme has been reported by Kasvinsky *et al.* (4), although no information is available on the relative affinity of nucleotides for the two sites.

Our results on nucleotide activation of liver phosphorylase *b* in the presence of 1,2-dimethoxyethane suggest that, in contrast to the situation observed with muscle phosphorylase, AMP may have an affinity for the nucleoside inhibitor site that is comparable to or greater than its affinity for the activator site. Only after AMP inhibition at the inhibitor site is reduced by the solvent can activation by the nucleotide be seen. This is consistent with the observation that the low activity with liver phosphorylase *b* is due to a high K_m for glucose-1-P.

Our results also suggest that 1,2-dimethoxyethane may be useful in determining total phosphorylase levels in crude liver extracts. The current assay utilizing high levels of fluoride or sulfate (21) is inadequate since these anions inhibit liver phosphorylase *a* activity while giving only partial activation of phosphorylase *b*. We have found that 10% 1,2-dimethoxyethane (v/v) does not affect liver phosphorylase *a* activity and activates liver phosphorylase *b* in the presence of AMP or IMP to a level comparable to that of the *a* form. These results suggest that 1,2-dimethoxyethane may be useful in more accurately determining activities for the different forms of liver phosphorylase using the low glucose-1-P assay described under "Materials and Methods."

[2] J. Miller and D. J. Graves, unpublished observations.

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J. Biol. Chem. 1979, 254:3166-3169.