A Role for Pyridoxal Phosphate in the Control of Dephosphorylation of Phosphorylase a*

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The control of the dephosphorylation of phosphorylase a by glucose or caffeine, but not by AMP requires the coenzyme, pyridoxal phosphate. The dephosphorylation of apophosphorylase a and reconstituted phosphorylase a derivatives prepared from analogs with less than two ionizable hydrogens at the 5′ position cannot be activated by glucose or caffeine. No dissociation of these inactive forms could be detected in the presence of glucose or caffeine in the ultracentrifuge, indicating that the coenzyme has an important effect on the binding, or structural changes, or both, induced by glucose or caffeine. The dephosphorylation of active phosphorylase a derivatives, pyridoxal-phosphorylase a in the presence of inorganic phosphate, or enzyme forms generated with 5′ side chains containing phosphate groups can be activated by glucose or caffeine. Dephosphorylation of phosphorylase a reconstituted with α-methylpyridoxal phosphate can be stimulated by caffeine but not by glucose. The phosphate groups of the analogs have higher pKₐ values than does the phosphoryl portion of pyridoxal phosphate; phosphorylase reconstituted with these derivatives requires a higher pH for maximum stimulation by glucose or caffeine than does the native phosphorylase a. Ultracentrifugal experiments on active phosphorylase a derivatives do not show an exact correlation between effects of glucose or caffeine on the dimer-tetramer forms of phosphorylase a and activation. The results suggest that the ionic state of the 5′ position of pyridoxal phosphate is particularly important for the regulation of the dephosphorylation of phosphorylase a by glucose or caffeine.

All α-glucan phosphorylases contain firmly bound pyridoxal phosphate and require this coenzyme for catalytic activity (1–3). The exact role of pyridoxal phosphate in catalysis is not known, but clearly it does not function as it does in other vitamin B₆-containing enzymes (3, 4). Amino acid sequence analysis of pyridoxal phosphate binding sites of phosphorylases from rabbit, potato, yeast, and Escherichia coli (5–8) show that this region of the protein is highly conserved, a fact that is consistent with an involvement of the coenzyme in an important function.

One approach taken to evaluate the role of the coenzyme is to study the properties of the apoenzyme and phosphorylases reconstituted with various pyridoxal phosphate analogs. Physical studies have shown that the structures of apophosphorylases b and a are different from their holoenzymes (9–11), and although major structural changes occur upon the binding of various pyridoxal phosphate analogs (3, 10), differences still exist in the conformation of these reconstituted phosphorylases from the native enzyme (11). Graves et al. (12, 13) used enzymes to probe conformational differences in apophosphorylase, pyridoxal-reconstituted phosphorylase, and native phosphorylase and concluded that the coenzyme affects enzymatic reactions involving the NH₂-terminal domain, e.g. phosphorylation and dephosphorylation and limited proteolysis by trypsin. Results from measurements of catalytic activity of various reconstituted phosphorylases suggest that the phosphoryl group of the coenzyme is particularly important, and suggestions have been made that it might participate in a proton shuttle (11, 14, 15).

Recent studies using x-ray crystallography (16, 17) and pyridoxal-reconstituted phosphorylase (18) show that the coenzyme is in close proximity to the binding site for the substrate, glucose 1-phosphate. X-ray crystallography has also shown that a nucleoside binding site is adjacent to the glucose or glucose 1-phosphate site (19) and is some 30 Å away from the AMP binding site, which in turn is near the phosphorylatable serine (20). Because our earlier results (12, 13) suggested that the influence of effectors on dephosphorylation of phosphorylase a depended on the coenzyme, and because information is now available about effector binding sites in phosphorylase, we have made a comprehensive study of the action of certain effectors. Our results with phosphorylases reconstituted with different pyridoxal phosphate analogs suggest that the phosphoryl group of the coenzyme is important for effectos of glucose and caffeine on phosphorylase a structure and for dephosphorylation. Effects of AMP on dephosphorylation, however, are seen in the absence of coenzyme.

MATERIALS AND METHODS

Rabbit muscle phosphorylase b was prepared according to the method of Fischer and Krebs (21). Phosphorylase a was made by the procedure of Krebs (22) using [γ-32P]ATP (23) with phosphorylase kinase (24). Phosphoprotein phosphatase was purified according to the method of Brandt et al. (25). The final step of purification, the hexadecylammonium chromatography, was not included.

Phosphorylase a was prepared from phosphorylase a by a modification of Shaltiel’s method (10). The major changes were the inclusion of 10% 1,2-dimethoxyethane in the deforming buffer and a higher incubation temperature (about 22°C). Complete details of the modification will be published elsewhere. The extent of resolution was tested by preincubating the apophosphorylase a with or without a 5-fold excess pyridoxal phosphate at 30°C for 15 min. The resulting phosphorylase activity was measured by the method of Illingworth and Cori (26). The apophosphorylase a thus obtained was better than...
99.5% resolved and could be reconstituted with pyridoxal phosphate to at least 60 IU/mg in the presence of 1 mM AMP. Apophosphorylase α reconstituted with pyridoxal phosphate had the same properties as native phosphorylase α, as determined by sedimentation characteristics, enzymatic activities, and as a substrate for phosphoprotein phosphatase.

A methylypyridoxal phosphate (1-methyl-1-(4-formyl-3-hydroxy-2-methyl-5-pyridyl)methyl-phosphoric acid) was a generous gift from Dr. Walter Korzyntyk, Roswell Park Memorial Institute, Buffalo, N.Y. The phosphonoethanol analog of pyridoxal phosphate (2-(4-formyl-3-hydroxy-2-methyl-5-pyridyl)methyl phosphonic acid) and the phosphonoethanol analog (2-(4-formyl-3-hydroxy-2-methyl-5-pyridyl)methyl phosphonic acid) were prepared by Dr. Retsu Miura according to Huliar (27). 5-Deoxypyridoxal and the carboxyethenyl analog of pyridoxal phosphate (2-(4-formyl-3-hydroxy-2-methyl-5-pyridyl)ethenyl carboxylic acid) were synthesized by the methods of Iswara, and Miura and Metzler, respectively (28, 29). The phosphonomethyl analog of pyridoxal phosphate (1-(4-formyl-3-hydroxy-2-methyl-5-pyridyl)methyl phosphonic acid) and pyridoxal phosphate methyl ester (methyl 1-(4-formyl-3-hydroxy-2-methyl-5-pyridyl)methyl-phosphoric acid) were made by C. N. Han (30). Phosphorylase α reconstituted with pyridoxal phosphate analogs was prepared by incubating 3 to 4 mg/ml of apophosphorylase α with a 25-fold excess of analogs in 40 mM β-glycerol phosphate, 30 mM mercaptoethanol, pH 6.8. After 30 min at 30°C, the reconstituted enzyme was precipitated by adding an equal volume of neutralized saturated ammonium sulfate solution. Then the reconstituted enzyme was dialyzed extensively against 50 mM Tris, 30 mM mercaptoethanol, pH 7.5. Pyridoxal phosphate-reconstituted phosphorylase α was prepared with only 5-fold excess of pyridoxal phosphate over the apophosphorylase α. The extent of reconstitution by all the analogs was tested by assaying activity with or without pyridoxal phosphate and was found to be 100%.

Concentrations of phosphorylase were determined either by radioactivity or spectrophotometrically at 280 nm assuming an absorbance of 13.0 for a 1% protein solution (13).

The rate of dephosphorylation of [32P]phosphorylase α or pyridoxal phosphate analog-reconstituted [32P]phosphorylase α was measured by the same procedure used by Parrish and Graves (31). Inorganic [32P]phosphate released was counted by Cerenkov radiation (32).

Sedimentation velocity experiments were done with a Beckman Spinco model E ultracentrifuge. The movement of protein boundaries was followed by schlieren optics. The schlieren patterns were analyzed with a microcomparator. *S*_{20,w} values were calculated by a linear regression program of an HP-25 calculator. 1,2-Dimethoxyethane was obtained from Eastman Kodak. Grade I imidazole from Sigma was recrystallized three times from reagent grade acetone before use. Shellfish glycogen from Sigma was further purified according to the method of Anderson and Graves (33).

### RESULTS

In 1951, Sutherland (34) found that caffeine and AMP affected the dephosphorylation of phosphorylase α by phosphoprotein phosphatase. Using an alternative substrate, Nolan et al. found that the effects of caffeine and AMP were due to their interactions with the substrate, and not with the phosphatase (35). To determine the role of pyridoxal phosphate in the influence of AMP and caffeine on enzymatic dephosphorylation, effects of these modifiers were compared on native phosphorylase α, apophosphorylase α, and phosphorylase α reconstituted with pyridoxal.

As shown in Fig. 1a, the rate of dephosphorylation of native phosphorylase α by phosphoprotein phosphatase is increased 2.2-fold by 3 mM caffeine and is inhibited 95% with 3 mM AMP. Three different concentrations (0.2, 1, 3 mM) of caffeine and AMP were tried. Only the data for effectors at 3 mM are shown in Fig. 1 because this concentration gave the maximum effect. Three different protein substrate concentrations (3, 1, 0.2 mg/ml) were also tried because the quaternary structure of phosphorylase α changes with enzyme concentration, i.e., dissociation of a tetramer to a dimer occurs upon dilution (36). The percentage change in rate of dephosphorylation caused by the effectors, however, was found independent of protein concentration. Fig. 1b indicates that the rate of dephosphorylation of apophosphorylase α is not affected by caffeine, but is still inhibited 70% by 3 mM AMP. The same results are seen for the dephosphorylation of pyridoxal-reconstituted phosphorylase α as shown in Fig. 1c. If 6 mM phosphate is included in the test solution, the rate for dephosphorylation of pyridoxal-reconstituted phosphorylase α is increased 1.8-fold by 3 mM caffeine. It was suggested previously that phosphate binds noncovalently at the site normally occupied by the 5'-phosphate of pyridoxal phosphate in phosphorylase α (13, 18). Thus it seems that the coenzyme is necessary for the response to caffeine and that the phosphoryl portion of the coenzyme is particularly important.

A shift of the dimer-tetramer equilibrium of phosphorylase α toward the dimer by caffeine has been reported by Bot et al. (37). With the conditions used for dephosphorylation, phosphorylase α is dissociated by 3 mM caffeine to a form of

### Table I

| Sample                       | Sedimentation constants |
|------------------------------|-------------------------|
|                              | Dimer/Tetramer          |
| Phosphorylase α              | None/14.1               |
| Phosphorylase α + 3 mM caffeine | 8.8/None               |
| Apophosphorylase α           | 6.9/16.0                |
| Apophosphorylase α + 3 mM caffeine | 6.6/16.0               |
| Pyridoxal phosphorylase α    | None/13.6               |
| Pyridoxal phosphorylase α + 3 mM caffeine | None/12.9             |
| Pyridoxal phosphorylase α + 6 mM phosphate | None/12.7           |
| Pyridoxal phosphorylase α + 6 mM phosphate + 3 mM caffeine | None/12.5            |
| Pyridoxal phosphorylase α + 30 mM caffeine | 9.1/None             |

*Fig. 1. The effect of caffeine and AMP on the dephosphorylation of different forms of 32P-phosphorylase α by phosphoprotein phosphatase. Three milligrams per ml of native phosphorylase α (a), or apophosphorylase α (b), or pyridoxal-reconstituted phosphorylase α (c), or pyridoxal-reconstituted phosphorylase α + 6 mM phosphate (d) was used as substrate. Final volume of reaction mixture was 0.5 ml, with 50 mM Tris, 30 mM mercaptoethanol, pH 7.5, as buffer containing (□) 3 mM caffeine, (△) 3 mM AMP, or (○) no addition. The reaction was initiated with 0.1 unit of phosphoprotein phosphatase at 30°C (25). At different intervals, 100 μl of the reaction mixture was removed and stopped by 200 μl of 30% trichloroacetic acid and 100 μl of 20 mg/ml of bovine serum albumin. After filtering through glass wool, an aliquot was taken and counted as described under "Materials and Methods."*
We will provide further evidence to show that dissociation of phosphorylase correlated with changes in the dimer-tetramer equilibrium. The ultracentrifugal results indicate that caffeine can bind to pyridoxal-phosphorylase, yet the structural change necessary for enhancement of the phosphatase reaction does not occur. In the presence of phosphite, the pyridoxal-reconstituted enzyme shows a stimulation of the phosphatase reaction 1.8-fold.

Pyridoxal phosphate a in the presence of 6 mM caffeine (Table I). The dephosphorylation of pyridoxal-phosphorylase a did not change the ultracentrifuge pattern similarly as a mixture of monomer, dimer, and tetramer (10). Caffeine at 3 mM did not change the ultracentrifugation pattern of apophosphorylase a. No dissociation of apophosphorylase a could be detected even at 30 mM caffeine. Pyridoxal-phosphorylase a is not dissociated to any appreciable amount by 3 mM caffeine, but it is fully dissociated to the dimer by 30 mM caffeine (Table I). The dephosphorylation of pyridoxal-phosphorylase a, however, is not activated by 30 mM caffeine. Pyridoxal-phosphorylase a in the presence of 6 mM phosphate was not dissociated by 3 mM caffeine, although as previously mentioned the phosphatase reaction is stimulated 1.8-fold under these conditions. The ultracentrifugal results indicate that caffeine can bind to pyridoxal-phosphorylase, yet the structural change necessary for enhancement of the phosphatase reaction does not occur. In the presence of phosphate, the pyridoxal-reconstituted enzyme shows a stimulation of the phosphatase reaction, although this stimulation cannot be correlated with changes in the dimer-tetramer equilibrium. We will provide further evidence to show that dissociation of phosphorylase a is not sufficient to explain activation.

Because our present results and an earlier study (13) suggested that the phosphoryl portion of the coenzyme is important for response to caffeine and glucose, we examined carefully the effects of modification of the 5' position of the coenzyme on phosphorylase a structure and enzymatic dephosphorylation. Seven pyridoxal phosphate analogs were used to reconstitute apophosphorylase a. The rates of dephosphorylation of these enzymes under various conditions are listed in Table II. The highest rate of dephosphorylation in the absence of effectors is seen with native phosphorylase a. Although kinetic parameters have not been established ($K_m$ and $V_{max}$ values) for the different reconstituted phosphorylases, it is clear that all phosphorylases reconstituted with analogs are poorer substrates. Also shown in Table II are activities of the various phosphorylases. These had been reported earlier with phosphorylase b (3, 10, 38). None of the enzyme forms without activity could be stimulated by glucose or caffeine in the enzymatic dephosphorylation test. All active forms except phosphorylase a reconstituted with a-methylpyridoxal phosphate were stimulated by caffeine and glucose. With the a-methylpyridoxal phosphate-reconstituted enzyme, no stimulation of the dephosphorylation was seen by glucose at the concentration used, although stimulation by caffeine was similar to that of the native enzyme.

From 31P-NMR studies of phosphorylase (39) and the effects of phosphate analogs on the activity of pyridoxal-reconstituted phosphorylase (18), it was suggested that the state of ionization of the phosphoryl portion of the pyridoxal phosphate is important for catalysis. All the pyridoxal phosphate analogs used here, that gave activity upon reconstitution with apophosphorylase a, have higher $pK_{a}$ values for the phosphoryl portion than does pyridoxal phosphate. The $pK_{a}$ values are 6.20 (40), 6.76 (30), 7.0 to 7.35 (38), and 7.1 for pyridoxal phosphate, phosphonomethyl, phosphonoethyl, and phosphonoethenyl analogs of pyridoxal phosphate, respectively. Vidgoft et al. (38) showed earlier that the pH optimum was shifted to a higher pH for phosphorylase reconstituted with the phosphonoethenyl analog of pyridoxal phosphate. The effect of pH on the activation of enzymatic dephosphorylation by caffeine was studied to determine whether the higher $pK_{a}$ values of coenzyme analogs also caused a shift in the pH curve. As shown in Fig. 2, caffeine gives the maximum amount
of activation to the pyridoxic phosphate reconstituted phosphorylase \( \alpha \) from pH 6.0 to 6.4 and gradually loses the effect at higher pH. For phosphorylase \( \alpha \) reconstituted with phosphonooethyl or phosphonoethenyl analogs of pyridoxic phosphate, the maximum stimulation by caffeine is around pH 7.6 and pH 8.2, respectively. Thus the results show generally that a shift in the pH optimum to the alkaline side occurs with increasing \( k_v \) values.

Sedimentation velocity measurements were done on all the phosphorylases reconstituted with pyridoxic phosphate analogs reported in Table II to determine whether activation was correlated with the dissociation of phosphorylase \( \alpha \). All of the analog-reconstituted phosphorylases that did not show any response to caffeine or glucose in the enzymatic dephosphorylation were also not dissociated by these effectors. These forms include phosphorylase \( \alpha \) reconstituted with pyridoxic phosphate methyl ester, deoxypyridoxic, or the carboxyethenyl analog of pyridoxic phosphate. All of these proteins have \( s_{20, w} \) values of about 13 S with or without 3 mM caffeine or 10 mM glucose. Ultracentrifugation also was done in high salt solutions since it was shown previously that dissociation of native phosphorylase \( \alpha \) occurs in the presence of NaCl (41). As shown in Fig. 3, the phosphorylase \( \alpha \) reconstituted with the carboxyethyl analog was not dissociated by 2.5 mM NaCl as compared with the native phosphorylase \( \alpha \). The former sedimented as a single component with an \( s_{20, w} = 13 \) S whereas the native enzyme showed a broad peak characteristic of the equilibrium between dimeric and tetrameric forms. The sedimentation pattern of phosphorylase \( \alpha \) reconstituted with pyridoxic phosphate methyl ester in 2.5 mM NaCl showed some asymmetry indicating some dissociation but far less than that of the native enzyme (data not shown). These results suggest that the 5' side chain group of the coenzyme has an important influence on phosphorylase \( \alpha \) structure.

The sedimentation values for reconstituted phosphorylases whose dephosphorylations were stimulated by caffeine or glucose are listed in Table III. Caffeine at 3 mM dissociates phosphorylase \( \alpha \) reconstituted with the phosphonoethyl analog of pyridoxic phosphate to the same extent as that of native phosphorylase \( \alpha \) (Table III, Fig. 4a). Phosphorylase \( \alpha \) reconstituted with the phosphonooethyl analog is also dissociated but less effectively (Fig. 4c). Caffeine at 3 mM, however, only dissociates slightly the \( \alpha \)-methylpyridoxic phosphate-reconstituted phosphorylase \( \alpha \) as revealed by a slight broadening of the peak and a change in sedimentation constant, \( s_{20, w} \) from 13.0 to 12.5 S (results not shown). For phosphorylase \( \alpha \) reconstituted with the phosphonomethyl analog, caffeine at 3 mM does not show any shift from tetramer to dimer (Table III). Glucose at 10 mM, on the other hand, cannot dissociate any of the reconstituted phosphorylases in Table III to the extent observed for phosphorylase \( \alpha \) reconstituted with pyridoxic phosphate. The sedimentation pattern of phosphorylase \( \alpha \) reconstituted with the phosphonoethyl analog is broadened (4b) but little or no effect is seen with phosphorylase \( \alpha \) reconstituted with the phosphonoethyl analog (4d). Also glucose has little or no effect on the ultracentrifugal characteristics of phosphorylases reconstituted with \( \alpha \)-methylpyridoxic phosphate or the phosphonomethyl analog. These results and those of Table II suggest that the extent of activation of the phosphatase reaction and the dissociation are not exactly correlated.

One of the possible reasons that glucose at 10 mM did not dissociate reconstituted phosphorylases with the phosphonoethyl or phosphonoethyl analogs is that these phosphorylases do not have as high an affinity for glucose as does the native phosphorylase \( \alpha \). As shown in Table IV the \( K_v \) values of glucose for these two reconstituted phosphorylases are

![Fig. 2. Effect of pH on the activation of the dephosphorylation of phosphorylase \( \alpha \) derivatives by caffeine. The dephosphorylation of \( ^3\text{P}\)-phosphorylase \( \alpha \) reconstituted with (A) pyridoxic phosphate, (B) phosphonoethyl, (C) phosphonoethyl, (D) phosphonomethyl analogs of pyridoxic phosphate at different pH values in the presence of 3 mM caffeine is shown. Procedure is same as that described in the legend for Fig. 1, except that 100 mM imidazole, 100 mM Tris, 30 mM mercaptoethanol buffer is used. The activation by 2 mM caffeine at different pH values is plotted against pH.](http://www.jbc.org/)

![Fig. 3. Effect of NaCl on the ultracentrifugal patterns of phosphorylase \( \alpha \), reconstituted with pyridoxic phosphate and its carboxyethyl analog. Top, pyridoxic phosphate reconstituted phosphorylase \( \alpha \) with 2.5 mM NaCl; bottom, carboxyethyl analog-reconstituted phosphorylase \( \alpha \) with 2.5 mM NaCl. Protein concentrations were 3 mg/ml in 50 mM Tris, 30 mM mercaptoethanol, pH 7.5. Temperature of runs is 22 ± 0.5°C. The picture was taken 48 min after attaining full speed of 32,000 rpm.](http://www.jbc.org/)

| Pyridoxic phosphate analogs used to reconstitute apophosphorylase \( \alpha \) | Caffeine (3 mM) | Glucose (10 mM) | Sedimentation constant |
| --- | --- | --- | --- |
| \( \alpha \)-Methylpyridoxic phosphate | – | – | 13.0 |
| Phosphonoethyl analog | – | – | 13.3 |
| Phosphonomethyl analog | – | – | 12.3 |
| Phosphonoethyl analog | 20 mM | – | 10.0 |
| Phosphonomethyl analog | – | – | 13.0 |
| Glucose (10 mM) | – | – | 12.3 |

All protein concentrations are 3 mg/ml in 50 mM Tris, 30 mM mercaptoethanol, pH 7.5. Temperature of runs is 22 ± 0.5°C.
lowered utilizing the filter namer assav of Thomas et al. (42). The enzymatic dephosphorylation of phosphorylase a. The muscle phosphorylase a from a tetramer to a dimer (37, 50).

FIG. 4. Effect of glucose and caffeine on the ultracentrifugal patterns of phosphorylase a derivatives. (a) top, phosphonoethenyl analog; bottom, phosphonoethyl analog and 3 mM caffeine; (b) top, phosphonoethyl analog; bottom, phosphonoethenyl analog and 10 mM glucose; (c) top, phosphonoethenyl analog; bottom, phosphonoethyl analog and 3 mM caffeine; (d) top, phosphonoethyl analog; bottom, phosphonoethenyl analog and 10 mM glucose. All protein concentrations were 3 mg/ml in 50 mM Tris, 30 mM mercaptoethanol, pH 7.5, at 22°C. The pictures were taken 39 min (a), 30 min (b), 44 min (c), and 34 min (d), after attaining full speed of 52,000 rpm.

TABLE IV

Comparison of $K_v$ values of glucose and caffeine for active phosphorylases reconstituted with pyridoxal analogs

| Analogs used to reconstitute apophosphorylase a | $K_v$ for glucose | $K_v$ for caffeine |
|-----------------------------------------------|------------------|-------------------|
| Pyridoxal phosphate                            | 6                | 0.32              |
| Phosphonoethenyl analog                        | 30               | 0.65              |
| Phosphonoethyl analog                          | 12               | 0.65              |

higher than those of the native enzyme especially that of the phosphorylase a reconstituted with the phosphonoethenyl analog which is 5-fold higher. $K_v$ values for caffeine are elevated 2-fold for these reconstituted phosphorylases. These results suggest that the 5' side chain function of the coenzyme affects the structural integrity of the binding sites for glucose and caffeine and could explain the differences seen in the ultracentrifuge for pyridoxal phosphate-reconstituted and analog-reconstituted phosphorylases in the presence of glucose.

DISCUSSION

An important control mechanism in glycogen degradation is the enzymatic dephosphorylation of phosphorylase a. The activators, glucose (43-45) and caffeine (35), and the inhibitor, AMP (35), affect dephosphorylation by binding to the substrate, phosphorylase a. Our earlier results suggested that the coenzyme, pyridoxal phosphate was important for the effect of glucose (13). This study shows that the coenzyme is also important for activation by caffeine, but it is not essential for AMP inhibition.

Because AMP binds extremely well to apophosphorylase (46), it is not surprising that it affects enzymatic dephosphorylation. AMP binds some 30 A from the coenzyme site near the phosphorylatable serine while glucose and caffeine bind to two distinct sites in close proximity to the pyridoxal phosphate site (47). Conceivably the coenzyme could be important for the structural integrity of the two sites near it, and the lack of effect of glucose and caffeine on enzymatic dephosphorylation and on the ultracentrifugal characteristics of apophosphorylase a could be due to poor binding of these effectors.

We have found that the phosphonyl group of the coenzyme is particularly important for the effects of glucose and caffeine. This was first indicated by the fact that pyridoxal, itself, was not sufficient to give an enzyme form whose dephosphorylation was activated by glucose (13) or by caffeine. However, both glucose and caffeine can affect pyridoxal phosphate in the presence of inorganic phosphate, a phosphate analog believed to bind at the site where the phosphate group of the natural coenzyme resides (18). Second, only phosphorylases reconstituted with analogs containing two ionizable hydrogens on the 5' side chain showed activation of the phosphate reaction by glucose or caffeine. In addition, phosphorylase reconstituted with the carboxyethyl analog of pyridoxal phosphate or pyridoxal phosphate methyl ester showed an altered response to NaCl, an agent that readily promotes dissociation of the tetramer of phosphorylase a to the dimer (41), indicating that important structural differences exist between the native enzyme and these phosphorylases. The charge state of the phosphonyl function is likely important for response to glucose or caffeine. Analog containing phosphonyl functions possess higher $pK_v$ values than does the phosphate group of pyridoxal phosphate and require a higher pH value for maximum response to effectors. These results suggest that a diaminic form of the coenzyme is important for control of dephosphorylation, as we suggested earlier for phosphorylase activity (39).

A relationship seems to exist between the activity of glycogen phosphorylase and the response to glucose or caffeine. All analogs that are inactive show no response to glucose or caffeine. There is no information available that shows whether glucose or caffeine can bind to these inactive enzyme forms. All active derivatives show a response to 10 mM glucose or 3 mM caffeine except for phosphorylase a reconstituted with a-methylpyridoxal phosphate. Differences exist in response to these effectors (Table IV), and a study is presently being done to determine the exact relationship between binding and activation. Our results suggest that the involvement of pyridoxal phosphate in catalysis is related to its role in the enzymatic dephosphorylation of phosphorylase a in response to glucose and caffeine. These two events could occur through a common mechanism, perhaps through a proton shuttle, a conformational event, or another process. Some phosphorylases do not undergo covalent modification, and the coenzyme is evidently necessary just for activity (48, 49). Animal phosphorylases, however, require pyridoxal phosphate for activity as well as for the control of enzymatic covalent modification as shown in this study.

Caffeine or glucose is known to cause a dissociation of muscle phosphorylase a from a tetramer to dimer (37, 50).
The relationship between dissociation and activation of the phosphatase reaction has been studied by several investigators (37, 43, 45, 51, 52). Bot and his associates (37, 51, 52) suggest that phosphoprotein phosphatase can dephosphorylate only the dimeric form. Our results with analogs of pyridoxal phosphate show that some dissociation occurs with analogs in response to glucose or caffeine. Phosphorylase a reconstituted with phosphate show that some dissociation occurs with analogs in shows very little dissociation by glucose while a large effect occurs with native phosphorylase a. The enzymatic dephosphorylation of this phosphonate analog under this same condition, pH 7.5, is activated 6-fold, but native phosphorylase is demonstrated that glycogen metabolism in different physiological conditions although early studies did not detect anapophosphorylase in a vitamin B, deficiency state.

The extensive study of Hers and his associates has demonstrated that glycogen metabolism in vivo is altered by glucose through its interaction with phosphorylase a (53-56). This binding promotes dephosphorylation of phosphorylase a, which is then followed by the dephosphorylation of glycogen synthase D and glycogen synthesis. Kasvinsky et al. (47) have shown that binding of AMP to phosphorylase a induces a conformational change in the NH2-terminal portion of the enzyme which moves inward toward a fold in the enzyme, making the serine-14-phosphate unavailable to phosphorylase b as a function of pH, but not much information to show whether a change in amount or type of binding of the enzyme, making the serine-14-phosphate unavailable to phosphorylase a.

The enzymatic dephosphorylation occurs through its interaction with phosphorylase a, making the serine-14-phosphate unavailable to phosphorylase a (39). pH changes or other factors might change the interaction of the 5’-phosphoryl group of the coenzyme. Further experimentation is necessary to show whether a change in amount or type of binding of pyridoxal phosphate in phosphorylase is important in the physiological response to glucose and other effectors.

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