Control of Phosphorylase Activity in a Muscle Glycogen Particle

III. REGULATION OF PHOSPHORYLASE PHOSPHATASE*

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SUMMARY

This study describes a new type of inhibition of phosphorylase phosphatase that strongly suggests that the activity of this enzyme is modulated during regulation of the phosphorylase system. Phosphorylase phosphatase included in a muscle protein-glycogen complex along with phosphorylase, phosphorylase kinase, and other enzymes of glycogen metabolism undergoes a reversible inhibition when phosphorylase is activated. This inhibition requires free Ca²⁺ in addition to Mg-ATP, i.e., the same conditions that trigger phosphorylase activation; it is observed only in the intact protein-glycogen complex. It is not caused by AMP or IMP generated from rapid breakdown of ATP, two nucleotides known to inhibit the phosphorylase phosphatase reaction by binding to the substrate phosphorylase α. (a) Addition of AMP or IMP to the particulate system is without effect; (b) phosphatase activity returns to normal at the end of an activation cycle of phosphorylase when concentration of IMP is maximum; and (c) the same inhibition pattern is observed when a phosphopeptide derived from phosphorylase α is used as substrate, a reaction not affected by AMP or IMP. Digestion of glycogen in the complex by α-amylase abolishes the Ca²⁺-dependent inhibition of phosphorylase phosphatase, but the activity of this enzyme is still predominantly unaffected by nucleotides. On the other hand, dissociation of the protein-glycogen complex by dilution brings about both a loss of the Ca²⁺-dependent inhibition of the phosphatase and the reappearance of its sensitivity to AMP or IMP. Phosphorylase phosphatase included in the complex acts on phosphorylase α endogenously produced by addition of Ca²⁺, Mg²⁺, and ATP 16-fold faster than on soluble phosphorylase α added to the system. Nevertheless, the phosphatase reaction on the exogenous substrate still remains unaffected by high concentrations of AMP or IMP. No evidence was obtained that during inhibition the enzyme was covalently modified, e.g., by phosphorylation; rather, it is proposed that this inhibition results from the interaction of phosphorylase phosphatase with some of the other components of the phosphorylase system.

The first paper of this series described the isolation and characterization of a protein-glycogen complex from rabbit muscle (1) and it was proposed that this complex is a structural and functional unit of the cell. In the second article (2) some aspects of phosphorylase regulation were described, especially with respect to phosphorylase kinase activation. When Ca²⁺ and Mg-ATP were added to this protein-glycogen complex, an immediate activation of phosphorylase was observed, resulting from a phosphorylase b to a conversion, followed by a rapid return to the original inactive b form. The over-all reaction was referred to as the “flash activation” of phosphorylase.

Previous evidence obtained both with the intact animal and on purified systems all indicated that control of phosphorylase activity occurred through activation of phosphorylase kinase, not inhibition of phosphorylase phosphatase (3). Extensive studies on purified phosphorylase phosphatase revealed few clues as to its possible involvement in a regulatory process as found for phosphorylase kinase or glycogen synthetase, aside from its known inhibition by AMP or IMP and its slight activation by methylxanthines, glucose, or glucose 6-P (4-7). Purified phosphorylase phosphatase is hardly affected, if at all, by cyclic 3',5'-AMP, various divalent metal ions, or chelating agents such as EDTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid, o-phenanthroline, etc.; at some stage of its purification, it is activated 2- to 3-fold by limited tryptic attack, but not by the Ca²⁺-dependent kinase activating factor (7). On the other hand, some changes in activity under the influence of Mg²⁺, ATP, and cyclic 3',5'-AMP have been reported in other systems including bovine adrenal cortex (8, 9), pigeon breast muscle (10), and dog liver (11). No similar effects have been observed with the rabbit muscle enzyme. The present article presents evidence that in the protein-glycogen complex, but not in the disrupted system, phosphorylase phosphatase displays enzymatic characteristics different from those of the purified enzyme and that its activity can be modulated in a way suggesting that it actively participates in the regulation of glycogen phosphorylase.
MATERIALS AND METHODS

Materials and methods not listed in this paper have been described in the two preceding articles (1, 2).

The phosphorylase α labeled with 32P was prepared according to Fischer et al. (12) (specific radioactivity approximately 4 × 106 cpm per mg).

Phosphorylase Phosphatase Assays

Method 1—Labeled phosphorylase (0.1 ml of a 3 mg per ml solution) was added to 0.1 ml of phosphate solution diluted in the same buffer. After 10 min at 30°, 0.1 ml of a 25 mg per ml solution of bovine serum albumin was added and the reaction stopped by addition of 0.7 ml of 10% trichloroacetic acid. The samples were centrifuged for 5 min in a clinical centrifuge and the radioactivity was measured in 0.5 ml of the supernatant in a scintillation counter. The control was made by replacing the phosphorylase solution with buffer; in this instance, the pellet was dissolved in 1.0 ml in 88% formic acid and the radioactivity determined on 0.5 ml. A unit of phosphatase activity is defined as that amount of enzyme liberating 1 n mole of 32P per min under these assay conditions.

Method 2—Samples from the reaction mixture were measured without further dilution. A suspension of 32P-labeled phosphorylase α a crystals (30 mg per ml) was dissolved by warming at 30° for a short time. The reaction was started by adding 50 μl of this solution to 0.5 ml of the phosphorylase fraction; at 20, 40, and 60 sec, 0.1 ml samples were removed, precipitated with trichloroacetic acid, and measured for release of radioactivity as before. Initial rates were determined; in both methods, release of 32P was linear with time when less than 50% of the total protein-bound radioactivity was liberated.

Method 3—A large (approximately 80 amino acid) radioactive phosphopeptide was isolated from 32P-labeled phosphorylase following CNBr degradation of this enzyme according to Saari (13). The pool from the Sephadex G-50 column was lyophilized and used as substrate without further purification. The peptide fraction was added to the enzyme solution in the same volume ratio (100 μl per ml) as used in Method 2 and at a final concentration of 30 nmoles of 32P per ml. The reaction was stopped by adding 0.1 ml samples of this reaction mixture to tubes containing 0.5 ml of a slurry of Dowex 50-XS (11+) in water (0.25 g per ml) plus 0.4 ml of 10% trichloroacetic acid. Radioactivity release was measured on the supernatant obtained after centrifugation. Linear initial rates were observed only when less than 20% of the total radioactivity was released.

Partially purified phosphorylase phosphatase was prepared by a modification of the procedure of Hurvitz (7). The precipitate obtained by centrifugation at 80,000 × g (30-pellet) was prepared according to the method of Krebs et al. (12) with the exceptions that 20 mM Tris-HCl, 50 mM β-mercaptoethanol buffer, pH 7.5, was used and the suspension was treated for 10 min at 30° with 10 μl per ml of a crystalline suspension (approximately 8 mg per ml) of salivary amylase. The amylase-treated suspension was diluted with an equal volume of the same buffer and centrifuged at 140,000 × g for 3 hours. The supernatant was then adsorbed on a column of Whatman DE-52 (approximately 60 ml of packed cellulose bed per 600 g of muscle) equilibrated with the same buffer containing 70 mM NaCl. The column was washed with the equilibration buffer until the A260 of the eluant fell below 0.2. A solution of 20 mM Tris-HCl, 50 mM β-mercaptoethanol, and 400 mM NaCl, pH 7.5, was then applied and the entire pool collected. This fraction was brought to 45% saturation with solid ammonium sulfate at 0° and centrifuged after 2 to 3 hours. The precipitate was resuspended in a small volume (approximately 10 ml) of the Tris-mercaptoethanol buffer and dialyzed against the same buffer. The solution obtained (approximately 15 mg of protein per ml) was stored at 0°; the specific activity of the phosphatase was approximately 180 units per mg of protein and represented a purification of approximately 150-fold. Examination of this fraction on disc electrophoresis by use of a protein stain and activity tests on thin slices of the gel indicated that the phosphatase comprised only a very small fraction of the total protein; the phosphorylase b content was approximately 80 μg per ml or 0.5%.

Separation of nucleotides was achieved by chromatography on thin layer cellulose plates with fluorescent indicator (Eastman). The solvent was l-amyl alcohol-formic acid-H2O (3:2:1) (14) and the time of development, 24 hours. Nucleotides were located by their quenching properties when examined under a shortwave ultraviolet lamp. For the analysis, 0.2 ml samples were added to 0.2 ml of 10% HClO4 at 0°. The suspension was neutralized with 0.2 ml of 1 N K2CO3 and, after the foaming stopped, the tubes were centrifuged. The supernatant (3 to 4 μl) was spotted on the thin layer plate and chromatographed with standard nucleotides as markers. Areas corresponding to the nucleotides were scraped off the plate directly into a scintillation vial and measured for radioactivity with toluene scintillation fluid. Under these conditions, each nucleotide ran as a discrete spot with no trailing. The method is capable of detecting nucleotide concentrations as low as approximately 20 μM.

RESULTS

Phosphorylase Phosphatase Activity During Flash Activation of Phosphorylase in Muscle Protein-Glycogen Complex—To measure phosphorylase phosphatase activity in the protein-glycogen complex during an activation cycle of phosphorylase, samples were removed at various times and 0.1 volume of a solution of 32P-labeled phosphorylase α of high specific radioactivity was added. From these samples, three aliquots were removed on time and measured for release of 32P, as described under "Materials and Methods" (Method 2) from which the initial rate of phosphatase activity was calculated. If phosphorylase phosphatase activity is followed in this fashion, a pattern emerges that is illustrated in Fig. 1. One observes an immediate approximately 80% inhibition of the phosphatase followed by reactivation of the enzyme at the end of the flash activation. This inhibition is mostly dependent on the presence of free Ca2+ since in the absence of this metal ion no phosphorylase activation occurs, no more than a 20 to 25% inhibition of the phosphatase is observed. Maximum inhibition occurs at the peak of the flash activation where maximum phosphorylase activity is produced.

It was of interest to determine whether the observed effects were dependent upon the integrity of the protein-glycogen complex or whether they could be reproduced with purified enzymes. If phosphorylase phosphatase in the 30-pellet suspension (2) is liberated from the protein-glycogen complex to which it is bound by dilution of the system, its enzymatic behavior is indeed different. This is exemplified in Fig. 2, which shows that phosphorylase phosphatase present in a 40-fold diluted 30-pellet suspension is strongly inhibited upon addition of Mg-ATP but
Fig. 1 (upper). Phosphorylase phosphatase activity during flash activation of phosphorylase in an undiluted 30-pellet suspension. Phosphorylase and phosphorylase phosphatase (Procedure 2) assays were described under “Materials and Methods.” Phosphatase activity is expressed as percentage of initial activity obtained before addition of ATP. ○ and ○, phosphorylase; ■ and □, phosphorylase phosphatase activity; ● and △, 0.3 mM free Ca\(^{2+}\); and ○ and △, excess EDTA.

FIG. 2 (lower). Phosphorylase phosphatase activity during flash activation in a protein-glycogen complex disrupted by a 40-fold dilution. Phosphorylase phosphatase assay procedure and other conditions are described under “Materials and Methods.” ▽, control without ATP; □ and ■, with 1 mM ATP; ○, excess EDTA, and △ and ▽, 0.3 mM free Ca\(^{2+}\).

Effect of AMP and IMP on Phosphorylase Phosphatase Activity—A number of experiments were devised to determine the cause of the phosphatase inhibition and the reason for differences observed between the behavior of the enzyme when included in the protein-glycogen complex and when dissociated from it. It is well known, for instance, that purified phosphorylase phosphatase is strongly inhibited by nucleotides such as AMP and IMP (4). The observed inhibitions could simply result from the production of such effectors from the breakdown of ATP. Alternatively, inhibition could result from a conformational or covalent modification of the phosphatase as observed for other components of the phosphorylase system since these added to the reaction mixture; the slight decrease in activity of the control is the result of the instability of the enzyme at pH 6.8. The decrease in phosphorylase phosphatase activity is approximately 25% slower if one omits glycogen and 90% slower if one omits phosphorylase kinase.

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Inhibition is independent of any conversion of phosphorylase b to a since it also occurs in the absence of added Ca\(^{2+}\); and the inhibition remains for at least 30 min; no phosphatase inhibition is observed in the absence of Mg-ATP.

The same inhibition pattern is observed (Fig. 3) in a system reconstituted with purified enzymes. The reaction mixture contained 25 mM sodium glycerol-3-P-1 mM EDTA buffer, pH 6.8, 1% glycogen, 1 mM ATP, 3 mM Mg\(^{2+}\), 1.5 mM Ca\(^{2+}\), 0.5 mg per ml of purified phosphorylase kinase, 0.4 mg per ml of purified phosphorylase phosphatase, and 0.5 mg per ml of phosphorylase b. Phosphatase assays were carried out according to Procedure 2. ▽, control without ATP; ■, the reconstituted system.

Fig. 4 (lower). Variation of nucleotide concentrations during phosphorylase activation in an amylase-treated supernatant solution. Conditions are described under “Materials and Methods;” nucleotides are as follows: ○, ATP; △, ADP; ●, AMP; and △, IMP.
Effect of nucleotides on phosphorylase phosphatase activity.

The phosphatase assay (Procedure 2) is described under “Materials and Methods.” Each sample contained 0.3 mM free calcium and 5 mM magnesium; inhibitions were measured 30 sec after addition of the nucleotides.

<table>
<thead>
<tr>
<th>Nucleotide (1 mM)</th>
<th>Percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30-Pellet suspension undiluted</td>
</tr>
<tr>
<td>None</td>
<td>%</td>
</tr>
<tr>
<td>ATP</td>
<td>73</td>
</tr>
<tr>
<td>ADP</td>
<td>23</td>
</tr>
<tr>
<td>AMP</td>
<td>5</td>
</tr>
<tr>
<td>IMP</td>
<td>7</td>
</tr>
</tbody>
</table>

Table 1 shows the effect of nucleotides other than ATP on the activity of the enzyme before and after dissociation. In the latter instance, and as expected, both AMP and IMP inhibited the phosphatase reaction; this inhibition was previously shown to result from an interaction of the nucleotides with the substrate, phosphorylase α, rather than with the phosphatase itself (4); $K_i$ values of approximately $5 \times 10^{-4}$ M were found for AMP and approximately $1 \times 10^{-3}$ M for IMP (7).

In the diluted 30-pellet suspension, although one sees a substantial inhibition of the phosphatase, the extent of inhibition is not as large as predicted by the above inhibition constants.

By contrast, in the undiluted system, containing the intact protein-glycogen complex, essentially no inhibition was caused by AMP or IMP (added together with Ca$^{2+}$ and Mg$^{2+}$) and the slight effect produced by ADP could be ascribed to the formation of ATP under the influence of myokinase also present. These data indicate that the reversible inhibition of phosphorylase phosphatase observed during flash activation cannot be simply ascribed to a transient production of AMP or IMP from ATP. This conclusion was confirmed by the use as substrate of a phosphopeptide derived from phosphorylase α, as will be described in a later section.  

Correlation between Phosphatase Inhibition and Nucleotides Produced during Phosphorylase Activation—The above conclusions were confirmed by determining the nucleotides generated from ATP during phosphorylase activation in the disrupted and intact protein-glycogen complex. To this effect, [8-$^{14}$C]-ATP (1 μCi per pmole) was added to an α-amylase-treated supernatant solution and to both a diluted and undiluted 30-pellet suspension; at various times, samples were removed and analyzed by thin layer chromatography as described under “Materials and Methods.” Results are illustrated in Figs. 4 and 5, respectively. In the dissociated systems, recoveries of all four nucleotides varied from 82 to 100%, as compared to only 49 and 73% in the undiluted suspension, because of the large amount of precipitate formed during deproteinization; however, no more than 2% radioactivity could be detected on the thin layer plate in areas not represented by ATP, ADP, IMP, or AMP.

Breakdown of ATP was much slower in the amylase-treated supernatant (Fig. 4) since the ATPase associated with the elements of the sarcoplasmic reticulum had been removed. In all preparations, myokinase and AMP deaminase were highly active as indicated by the low steady state levels of ADP and AMP; in all instances, there was ample IMP to completely inhibit the phosphatase as observed in the “dissociated” systems. Yet, in the particulate system, there was no correlation between IMP accumulation and enzyme inhibition. Fig. 1 shows that the enzyme is maximally inhibited within 30 sec but returns to normal after 4 to 5 min when the concentration of IMP is maximum.

Phosphorylase Phosphatase Inhibition with Phosphopeptide Derived from Phosphorylase α as Substrate—The above data indicate that in the protein-glycogen complex (as opposed to the dissociation system), inhibition of phosphorylase phosphatase results from a decrease in the activity of this enzyme, not from a reduced susceptibility of the substrate to enzymatic dephosphorylation as afforded by AMP. Most convincing evidence was obtained by the use as substrate of an 80-amino acid, $^{32}$P-labeled phosphopeptide derived from phosphorylase α by CNBr cleavage (13). This phosphopeptide is dephosphorylated at

regulatory enzymes are also present in the protein-glycogen complex.

To examine these two possibilities, inhibition of phosphatase was first studied in a protein-glycogen complex which had undergone dissociation by dilution.
25% of the rate at which phosphorylase a is attacked; however, the former reaction is totally unaffected by 1 mM AMP or IMP.

Table II clearly indicates that in the intact system (undiluted 30-pellet suspension) essentially the same inhibition pattern was observed when the phosphoprotein was added as substrate (maximum inhibition with Ca++ and Mg-ATP, little with Mg-ATP alone, and none with Mg-IMP). As in Fig. 1, the inhibition disappeared within 5 min. By contrast, in the dissociated system essentially no reduction in activity occurred confirming once more that the observed inhibitions were caused by the production of AMP or IMP.

Evidence against Covalent Modification of Phosphorylase Phosphatase during Inhibition—The most likely mechanism for the inhibition described above might entail a covalent modification (e.g. phosphorylation) of the protein. If this were the case, one would expect that the phosphatase would remain inhibited even after dissociation from the other components of the system by a process such as high dilution. To examine this possibility, the undiluted 30-pellet suspension was incubated with Ca++ and Mg-ATP; after 30 sec when phosphatase inhibition was maximum (75%) the material was rapidly diluted 100-fold into 50 mM sodium glycophosphate, 1 mM EDTA buffer, pH 6.8, and assayed. No inhibition remained as compared to a control without AMP. It can be concluded, therefore, that unless substitution is rapidly reversed (e.g. dephosphorylation) inhibition of phosphoprotein phosphatase in the intact protein-glycogen complex does not result from a covalent modification of the protein.

Effect of Glycogen Degradation on Phosphorylase Phosphatase Activity in Protein-Glycogen Complex—As shown above, dissociation of the 30-pellet suspension by dilution drastically affects the conditions under which phosphorylase phosphatase is inhibited. It was, therefore, of interest to determine the role of glycogen in maintaining the type of reversible inhibition found in the original protein-glycogen complex. To this effect, the 30-pellet suspension was digested with a-amylase and phosphatase inhibition was studied as a function of glycogen removal and restoration.

As seen in Table IIIA, removal of glycogen resulted in a marked loss in the inhibition caused by Ca++ and Mg-ATP, and an increase in the inhibition caused by IMP. However, this inhibition is still much lower than that observed in the complex disrupted by dilution (Table IIIB). Readdition of glycogen to the undiluted amylase-treated suspension did not restore the original inhibition pattern (Table IIIA). In contrast, hydrolysis of glycogen reduces the flash activation of phosphorylase by approximately 2-fold and this effect is completely reversed by readdition of glycogen (2). Therefore, phosphorylase kinase behaves differently than phosphorylase phosphatase in that its original enzymatic characteristics are restored by readdition of glycogen.

Readdition of glycogen (2%) to the protein-glycogen complex disrupted by dilution did not reverse the IMP inhibition of phosphorylase phosphatase (Table IIIIB). This indicates that appearance of the IMP sensitivity upon dilution is not simply caused by a lowering of the glycogen concentration, but perhaps depends on protein-protein interaction.

Comparison between Phosphorylase Phosphatase Activity on Endogenous and on Exogenous Phosphorylase a—The data presented thus far indicate that phosphorylase phosphatase is associated with a protein-glycogen complex in which its enzymatic characteristics are different from those of the dissociated enzyme. One must, therefore, ask what effect this particulate system has on the interaction of phosphorylase phosphatase with soluble phosphorylase a as compared to endogenous phosphorylase a present in the protein-glycogen complex. In the 30-pellet suspension, one can measure phosphorylase phosphatase activity either by addition of 32P-labeled phosphorylase a (see "Materials and Methods") or lose of endogenous phosphorylase a activity produced in the 30-pellet suspension during flash activation. This latter procedure gives an accurate measure of phosphorylase phosphatase activity only after ATP has been depleted and the phosphorylase kinase has become inactive. Comparison between these two procedures in a 20-fold diluted

### Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Free Calcium (0.2 mM)</th>
<th>Nucleotide (1.0 mM)</th>
<th>Percentage of Phosphatase Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intact system</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-Pellet suspension</td>
<td>+</td>
<td>ATP</td>
<td>0</td>
</tr>
<tr>
<td>30-Pellet suspension</td>
<td>+</td>
<td>ATP</td>
<td>9</td>
</tr>
<tr>
<td>30-Pellet suspension</td>
<td>+</td>
<td>IMP</td>
<td>0</td>
</tr>
<tr>
<td><strong>Dissociated system</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-Pellet suspension</td>
<td>+</td>
<td>ATP</td>
<td>0</td>
</tr>
<tr>
<td>30-Pellet suspension</td>
<td>+</td>
<td>ATP</td>
<td>11</td>
</tr>
<tr>
<td>30-Pellet suspension</td>
<td>+</td>
<td>IMP</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table III

**Effect of glycogen on phosphorylase phosphatase activity in the 30-pellet suspensions.** The phosphatase was assayed according to Method 3. Each sample contained 0.3 mM free Ca++, 5 mM Mg++, and inhibitions were measured 30 sec after addition of the nucleotides. For a-amylase treatment, 8 μg per ml of crystalline salivary amylase were added. Glycogen was readded to a final concentration of 2%.

<table>
<thead>
<tr>
<th>Sample</th>
<th>α-Amylase treatment</th>
<th>Nucleotide (1.0 mM)</th>
<th>Percentage of phosphatase inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact system</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-Pellet suspension</td>
<td>+</td>
<td>ATP</td>
<td>73</td>
</tr>
<tr>
<td>30-Pellet suspension</td>
<td>+</td>
<td>ATP</td>
<td>9</td>
</tr>
<tr>
<td>30-Pellet suspension + readded glycogen</td>
<td>+</td>
<td>ATP</td>
<td>30</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dissociated system</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluted 30-pellet suspension</td>
<td>+</td>
<td>IMP</td>
<td>62</td>
</tr>
<tr>
<td>Diluted 30-pellet suspension + glycogen</td>
<td>+</td>
<td>IMP</td>
<td>57</td>
</tr>
</tbody>
</table>
30-pellet suspension shows indeed that a discrepancy exists: a rate of 0.16 unit per ml was found with externally added phosphorylase a as compared to 0.80 unit per ml on phosphorylase a included in the complex. Each of these measurements was made 10 min after flash activation had been initiated, and when the phosphatase was maximally inhibited; the rates were corrected for dilution of the specific radioactivity and normalized to the same concentrations of phosphorylase a since in neither case was the substrate saturating. One sees, therefore, that even in this dissociated system, phosphatase activity was still approximately 5-fold greater when measured on endogenous substrate than when exogenous substrate was used. When similar measurements were carried out on the undiluted 30-pellet suspension, a 16-fold difference in activity was observed similar measurements were carried out on the undiluted 30-subs rate than when crotonate substrate was used. When liberated nutil-F in the undiluted 30-pellet suspension (no nucleotide ened in this dissociated system, phosphatase activity was still the case was the substrate saturating. One sees, therefore, that difference is observed between inhibition of phosphorylase phosphatase at the same concentration of phosphorylase a since in neither case was the phosphatase being included in the complex. Additional evidence that phosphorylase phosphatase is being liberated upon dilution was obtained as follows. Phosphatase activity in the undiluted 30-pellet suspension (no nucleotide added) was 1.5 units per ml as compared to 0.76 unit per ml after 20-fold dilution. Therefore, dilution caused a 10-fold increase in specific activity.

**Discussion**

Muscle contraction and appearance of phosphorylase a activity are synchronized by the release of Ca^{2+} from the sarcoplasmic reticulum (2). In the previous publication (2) it was shown that activation of phosphorylase kinase by Ca^{2+} is the main factor that triggers flash activation of phosphorylase in a protein-glycogen particle. One would expect that phosphorylase-phosphatase also participates in this control of phosphorylase activation for two reasons. (a) If phosphorylase kinase and phosphatase were both active at the same time, a wasteful recycling of phosphorylase a and b would occur, and of more importance, (b) the two enzymes would be acting in opposition to each other, thus interfering with the phosphatase b to a conversion. This antagonism decreases the efficiency of the process.

Measurements of phosphorylase phosphatase activity in muscle indicates that the activity of this enzyme does not change during contraction (3). The changes in the concentrations of various effectors, e.g. AMP and glucose-6-P, have been postulated as important factors in regulating phosphorylase phosphatase (4-7). Determination of the AMP concentration in the intact tissue indicates, however, that this level is approximately 100 times higher than the K<sub>s</sub> (7), therefore resulting in complete inhibition of phosphatase. Furthermore, the concentration of AMP does not fluctuate in a manner that would allow a physiologically meaningful regulation of the enzyme (15).

In this work with the protein-glycogen complex it was demonstrated that phosphorylase phosphatase was reversibly inhibited in synchrony with phosphorylase kinase activation. The factor which links phosphatase inhibition with kinase activation is calcium. The coordination of these two processes by this metal ion and the observation that it occurs at the same concentration that triggers muscle contraction indicates its physiological significance.

The mechanism of phosphorylase phosphatase inhibition is not attributed to nucleotides since there was a striking absence of any AMP effect on the phosphatase reaction in the protein-glycogen complex, which was further confirmed with use of a phosphopeptide as substrate. A likely mechanism for phosphorylase phosphatase inhibition is the covalent modification of the enzyme, since control by phosphorylation occurs with phosphorylase, phosphorylase kinase, and glycogen synthetase. Although a stable covalently modified enzyme was not detected in the 30-pellet fraction or in purified systems, a possibility exists that covalent modification is still involved. Other components of the system might interact with a modified phosphatase, or these components might themselves require modification in order to inhibit phosphatase; both of these cases might require high concentrations of proteins in order to be observed and thus would not be seen after high dilution.

Further evidence for the importance of a high protein concentration is the observation that even when glycogen is hydrolyzed, the other components of the 30-pellet fraction protect phosphorylase phosphatase from AMP inhibition. However, glycogen is necessary in order to observe the characteristic Ca^{2+}-dependent phosphatase inhibition pattern.

The marked increase in specific activity upon dilution and the different rates observed with endogenous and exogenous substrate both show that phosphorylase phosphatase is indeed buried in the protein-glycogen complex. Since the enzyme exists in such close association with the other components, this may well allow direct protein-protein interactions to function in the phosphatase control. Other support for this phenomenon was found by Hers et al. (16) who has shown an interaction of phosphorylase a with glycogen synthetase in such a manner as to regulate glycogen synthetase activity.

Although added phosphorylase a appears not to equilibrate with internal phosphorylase a, there is still a complete loss in the sensitivity of this added substrate to AMP. A possible explanation of this effect might be that the interaction of added phosphorylase a with some components in the complex changes the conformation of this substrate so that it is no longer effected by AMP. This would also support the hypothesis that protein-protein interactions participate in the over-all phosphorylase system control.

**References**

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