ADENOSINE 5'-O(3-THIOTRIPHOSPHATE) IN THE CONTROL OF PHOSPHORYLASE ACTIVITY†

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ABSTRACT: Rabbit muscle phosphorylase b (EC 2.4.1.1) is converted to a thio-analog of phosphorylase a by phosphorylase kinase, Mg^{2+} and adenosine 5'-O (3-thiotriphosphate)(ATPγS)[1]. Conversion proceeds at one-fifth the rate obtained with ATP though the extent of reaction and final level of activation of the enzyme are the same. However, the thiophosphorylase a produced is resistant to phosphorylase phosphatase and, therefore, behaves as a competitive inhibitor with a K_I of 3 μM, similar to the K_M obtained with normal phosphorylase a. ATPγS can also be utilized by protein kinase in the activation of phosphorylase kinase at a rate similar to that obtained with ATP. It is hydrolyzed at 5 to 10 times the normal rate by the sarcoplasmic reticulum ATPase. When added to a muscle glycogen-particulate complex in the presence of Ca^{2+} and Mg^{2+}, ATPγS triggers an activation of phosphorylase with simultaneous inhibition of phosphorylase phosphatase as previously observed with ATP.

INTRODUCTION

Since their recent discovery (1) thiophosphate analogs of nucleotides have attracted considerable interest in studies related to enzyme mechanism or regulation (2-10). Most prominent among these is adenosine 5'-O(3-thiotriphosphate) (ATPγS)[1] because of the general importance of ATP in biological reactions (5). As compared to ATP, ATPγS is hydrolyzed very slowly by alkaline phosphatase (3,8-10) or myosin ATPase (7). Likewise, the γ-thiophosphoryl group can be transfered to various nucleoside diphosphates by adenylate kinase

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1 Abbreviations: Adenosine 5'-O(3-thiotriphosphate), ATPγS.

or other nucleoside diphosphate kinases though, again, very slowly as compared to the normal triphosphates (6).

The regulation of glycogen synthesis and breakdown depends on the successive phosphorylation and dephosphorylation of several enzymes. While conversion of rabbit muscle phosphorylase b to a results predominantly from phosphorylase kinase activation (11; for review, see 12), a synchronous modulation of phosphorylase phosphatase activity was described in a muscle glycogen-particulate complex (13), raising the possibility that some regulation might occur at this step also. On the other hand, there are major difficulties in analyzing reactions in such complex systems mainly because of the near impossibility of dissociating the two competing events, namely, kinase activation from phosphatase inhibition. In the present communication, this question is reexamined with the use of ATPγS instead of ATP, following a suggestion made by Dr. Fritz Eckstein, from the Max Planck Institut für Medizinische Chemie, Göttingen, Germany.

MATERIALS AND METHODS

Adenosine 5'-O-(3-thiotriphosphate) was a generous gift from Boehringer Co. Phosphorylase b and a (14), phosphorylase kinase (15) and phosphatase (16), protein kinase (17), and the muscle glycogen-particulate complex (18) were prepared as previously described. Phosphorylase and phosphorylase kinase were assayed according to Hedrick and Fischer (19) and Krebs et al. (20), respectively, and phosphorylase phosphatase either by Method II of Haschke et al. (13) or by measuring residual phosphorylase a activity in the absence of AMP. Activation of phosphorylase b in the glycogen complex was followed as described by Heilmeyer et al. (21) except that samples were diluted 300-fold in the same buffer containing 0.1 M NaF. Dogfish sarcoplasmic reticulum was prepared according to MacLennan (22) and its ATPase activity was determined at 37° in 0.05 M Tris buffer, pH 7.5, containing 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM Ca acetate and either 2 mM ATP or ATPγS; 0.1 ml aliquots were removed, precipitated with 0.9 ml 10% trichloroacetic acid and the ADP generated was determined in

a coupled system involving pyruvate kinase and lactic dehydrogenase (23).

RESULTS

Conversion of phosphorylase b to a by phosphorylase kinase in the presence of Mg^{2+} and ATPγS is illustrated in Fig. 1A. The initial rate of reaction is

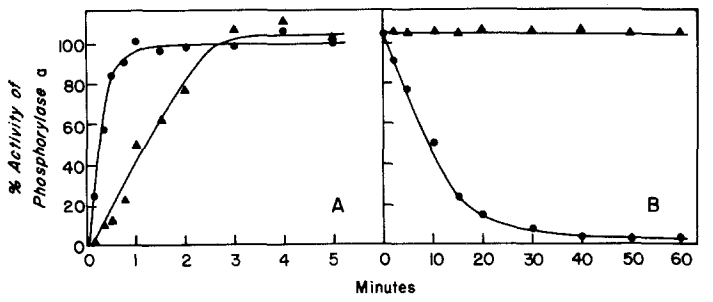

FIGURE 1: A, Conversion of phosphorylase b to a (21 mg protein/ml) by phosphorylase kinase (4 µg/ml) at pH 8.6, 30° in the presence of 5 mM Mg^{2+} and 1 mM each of ATP (●) or ATPγS (▲). B, Phosphorylase a to b conversion of 3 mg/ml each of crystalline phosphorylase a (●) or thiophosphorylase a (▲) by phosphorylase phosphatase (12 µg/ml) at pH 7.5, 30°. Conditions and procedures are described under Methods.

4-5 times slower than observed with ATP but the extent of reaction is the same. The thiophosphorylase a produced crystallizes readily in the cold at pH 7; it has the same specific activity and sedimentation coefficient ($s_{20,w}$ ca. 13.6S) as normal phosphorylase a indicating no gross change in tertiary or quaternary structure.

On the other hand, thiophosphorylase a is a very poor substrate for phosphorylase phosphatase (if it is at all attacked), with a rate of dephosphorylation well below 1% of the control, as illustrated in Fig. 1B; increasing the phosphatase concentration 10-fold did not help. The lack of reaction could not be attributed to inhibition by excess free ATPγS since thiophosphorylase a was routinely crystallized three times before use.

As expected, thiophosphorylase a is a competitive inhibitor of phosphory-

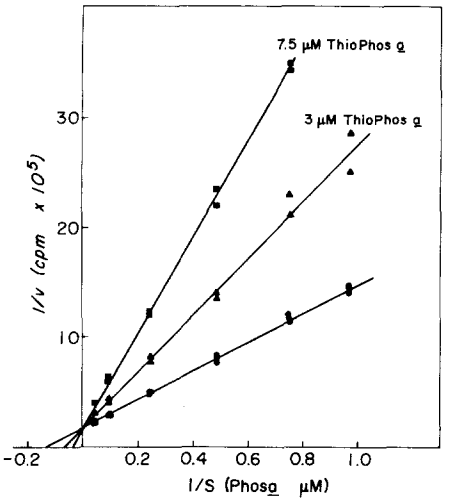

FIGURE 2: Lineweaver-Burk plot of phosphatase reaction in the presence or absence of thiophosphorylase a (see Methods). Control with phosphorylase alone (●).

lase phosphatase (Fig. 2) with a K_I of 3 μM, an affinity similar to that displayed by phosphorylase a (k_M = 5 μM, Detwiler, Gratecos and Fischer, unpublished results). Prolonged incubation of the phosphatase alone or in the presence of thiophosphorylase a did not alter the level of inhibition suggesting no direct effect of the analog on the phosphatase itself.

Nucleotides are known to inhibit the a to b conversion of phosphorylase, not by interacting with the phosphatase but with the protein substrate (24). Table I shows that ATPγS brings about an even stronger inhibition of the phosphatase than ATP, whether or not thiophosphorylase a is also present.

The cyclic AMP-dependent protein kinase from rabbit muscle (17) can also utilize ATPγS for the phosphorylation and activation of phosphorylase kinase, with a rate of reaction and extent of activation essentially identical to those observed with ATP. Hexokinase and phosphofructokinase were essentially inactive but, surprisingly, dogfish muscle sarcoplasmic reticulum ATPase (see Methods) hydrolyzed ATPγS 5 to 10 times faster than ATP.

Role of ATPγS on the Control of Phosphorylase Activity in a Muscle

TABLE I

INHIBITION OF PHOSPHORYLASE PHOSPHATASE

BY ATP AND ATPγS

	% Inhibition by Nucleotides		
PROTEIN SUBSTRATE	None (control)	ATP (1 mM)	ATPγS (1 mM)
Phosphorylase a (4 μM)	0	37	73
Phosphorylase a plus 7.5 μM thiophos. a	38	58	76

Protein-Glycogen Complex. A glycogen-particulate complex was described from rabbit muscle (18) which contains most of the enzymes of glycogen metabolism plus elements of the sarcoplasmic reticulum. In this complex, phosphorylase and phosphorylase kinase are essentially inactive while phosphorylase phosphatase is partially active. Addition of Ca^{2+}, Mg^{2+} and ATP triggers a fast activation of phosphorylase (b to a conversion) followed by a slower return to the initial level when all the ATP has been consumed (21). During this transient activation process, phosphorylase phosphatase undergoes a reversible, 85% inhibition (13) synchronous with phosphorylase activation (Fig. 3A).

A similar synchronous inhibition of phosphorylase phosphatase was observed when the reaction was carried out in the presence of ATPγS. This conversion of phosphorylase b to a proceeded at a slower rate, as expected: only ca. 20% of the a form could be generated before all the ATPγS was hydrolyzed by elements of the sarcoplasmic reticulum present in the preparation. On the other hand, since thiophosphorylase a is resistant to the phosphatase, it could not be converted back to the inactive b form and remained as such in the

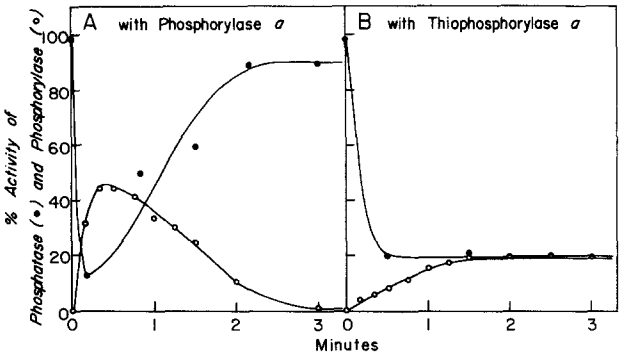

FIGURE 3: Changes in phosphorylase and phosphorylase phosphatase activities in a glycogen-protein complex (13) containing 5 mM Mg^{2+} and 1 mM Ca^{2+} at pH 30°. The reaction was started by adding either 1 mM ATP (A) or 1 mM ATPγS (B). Conditions are described under Methods.

preparation. As can be seen in Fig. 3B, and simultaneously with this activation process, phosphorylase phosphatase underwent an 80% inhibition and remained in this inhibited form during the entire course of the reaction. Again, this inhibition could not be attributed to excess ATPγS since the analog is hydrolyzed within a minute by the sarcoplasmic reticulum ATPase, as described above.

Finally, when the glycogen complex was disrupted by α-amylase and elements of the sarcoplasmic reticulum were removed by centrifugation, conversion of phosphorylase b to a in the presence of Ca^{2+}, Mg^{2+} and ATPγS proceeded to completion. Here again, phosphorylase phosphatase became strongly inhibited and remained as such for the duration of the experiments.

DISCUSSION

Both the cyclic AMP-dependent protein kinase and phosphorylase kinase from rabbit muscle are capable of transferring γ-thiophosphoryl groups from ATPγS to their respective substrates. The rates observed here were significantly higher than those reported for the transfer of the thiophosphoryl groups by dinucleoside diphosphokinases. Whether this unequal ability to transfer thiophosphoryl groups reflects a basic mechanistic or structural difference among phosphokinases is not known.

No clear explanation has been offered as yet for the transient inhibition of phosphorylase phosphatase observed in synchrony with phosphorylase activation following addition of Ca^{2+}, Mg^{2+} and ATP to a muscle glycogen complex. The fact that a similar inhibition was observed with ATPγS is of distinct interest. It would appear that this inhibition is directly related to the activation of either phosphorylase or phosphorylase kinase; since both thioenzymes are resistant to the action of their respective phosphatases, they remained in their active states during the entire course of the reaction. This, in turn, maintained phosphorylase phosphatase in its inactive state. Complex formation between either or both of these enzymes and the phosphatase could be postulated. Phosphorylase phosphatase displays similar affinities for both thio and normal phosphorylase a. However, the kinetic measurements were carried out on a purified system; it is conceivable that, in the glycogen-particulate complex which contains many interacting components, quite different values might be obtained.

Thiophosphate derivatives that are resistant to the action of phosphatases should prove to be extremely helpful in the study of metabolic interconversions that depend on the phosphorylation and dephosphorylation of regulatory enzymes or proteins. There have been several recent reports of covalent modifications of structural muscle proteins by phosphorylase kinase or protein kinase (25-28); however, most experiments were carried out in cell-free systems. Should such phosphorylation reactions be transient and difficult to detect in intact muscle fibers, thiophosphate derivatives might provide the right answer.

Finally, since thiophosphorylase a displays a high affinity for, but is resistant to, phosphorylase phosphatase, it might serve as an ideal support for the purification of this enzyme by affinity chromatography. The same should apply to other protein phosphatases.

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