Rabbit Muscle Phosphorylase Phosphatase. 2. Kinetic Properties and Behavior in Glycogen Particles†

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ABSTRACT: A possible regulation of phosphorylase phosphatase has been evaluated by study of the properties of the enzyme in glycogen particles where reversible inhibition has been reported (Haschke, R. H., Heilmeyer, L. M. G., Jr., Meyer, F., and Fischer, E. H. (1970), *J. Biol. Chem. 245,* 6657). In addition, the effects of nucleotides and other metabolites on the activity of the purified enzyme have been investigated. In spite of the uncertainties inherent in measuring enzyme activities in concentrated solutions, phosphorylase phosphatase activity appears to be controlled by a variety of factors. Some involve the intrinsic characteristics of the enzyme, others, interaction of the phosphatase with elements of the protein–glycogen complex, or changes in conformation or state of aggregation of the substrate. A 20-fold increase in V_{max} and decrease in K_m observed for the phosphatase upon dilution of the glycogen particles also occurs with the purified enzyme, suggesting that, at least in part, they must reflect some properties of the enzyme itself. In contrast, the phosphatase is insensitive to AMP in the undiluted protein–glycogen complex while this nucleotide inhibits the enzyme by 95% after dilution. The same inhibition is observed for the purified enzyme at any concentration. Thus, AMP insensitivity must be ascribed to some specific interaction of either the phosphatase or phosphorylase *a* with certain elements of the complex. Likewise, the reversible inhibition of the phosphatase bound to the particles observed when phosphorylase becomes maximally activated has been confirmed by measuring the turnover of endogenous phosphorylase *a* under steady-state conditions, again suggesting that interactions among the components of the system are involved. Inhibition of the enzyme by metabolites falls into several categories, including competitive and noncompetitive for phosphorylase *b* and P_i, the two products of the reaction, respectively; mixed for AMP, GMP, UDPG, and UTP; and by modification of substrate for AMP and ATP. Glucose and glucose 6-phosphate, which bring about an increase in V_{max} for the reaction with little change in K_m, also appear to act by altering the conformation or state of aggregation of the substrate.

Glycogen phosphorylase is covalently regulated by two enzymes, phosphorylase kinase which catalyzes the *b* to *a* conversion, and phosphorylase phosphatase which brings about the reverse reaction. In a kinetic analysis of phosphorylase interconversion following electrical stimulation of intact frog muscle, Danforth et al. (1962) proposed that the major regulation occurred through control of phosphorylase kinase activity with no evidence for regulation of the phosphatase. Thus, the simplest mechanism would be for the phosphatase to always be active without further control. Such a mechanism is theoretically feasible because potential kinase activity exceeds phosphatase activity by more than 1000-fold as calculated on the basis of the approximate concentrations and activities of the two enzymes involved (Fischer et al., 1971; Gratecos et al., 1977).

On the other hand, a more responsive mechanism could result from the simultaneous regulation, in an inverse manner, of both the phosphatase and the kinase, and there have been reports suggesting such a control. For example, regulation of the rate of dephosphorylation of phosphorylase *a* by modification of the substrate has been proposed (Nolan et al., 1964; Bot and Dosa, 1971; de Barsy et al., 1972; Bailey and Whelan, 1972; Martensen et al., 1974). Haschke et al. (1970) reported a reversible inhibition of the phosphatase in a protein–glycogen–particulate complex obtained from rabbit skeletal muscle. This preparation, which is believed to represent a functional unit of the cell, contains glycogen, phosphorylase, phosphorylase kinase, phosphorylase phosphatase, and other enzymes of glycogen metabolism as well as an active ATPase associated with elements of the sarcoplasmic reticulum (Meyer et al., 1970; Heilmeyer et al., 1970; Haschke et al., 1970; for a review, see Busby and Radda, 1976). Addition of Ca^{2+}, Mg^{2+}, and ATP to such a complex triggers a fast conversion of phosphorylase *b* to *a* followed by a slower return to the initial level when all the ATP has been consumed. During this cycle (referred to as "flash activation"), the phosphatase undergoes a reversible inhibition that is maximum when phosphorylase *a* reaches its peak. While this suggests a control of the phosphatase, the mechanism of this inhibition is not known. It could be attributed neither to a direct covalent modification of the enzyme, nor to a transient formation of nucleotides such as AMP or IMP; it is not clear either whether the observed changes in phosphatase activity represent a physiological event. Evidence suggesting interconvertible forms of phosphorylase phosphatase has been presented in other organs and species such as the adrenal cortex (Riley and Haynes, 1963; Merlevede and Riley, 1966), pigeon breast muscle (Torres and Chelala, 1970a,b), and *Neurospora crassa* (Tellez de Inon and Torres, 1973). Two protein inhibitors of phosphorylase phosphatase have been described (Huang and Glinsmann 1975, 1976). Nevertheless, in contrast to several other enzymes of glycogen metabolism such as phosphorylase, phosphorylase kinase, and glycogen synthase, for which a regulation of phosphorylation–dephosphorylation has been clearly established, no direct

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received May 18, 1977.* This work was supported by grants from the National Institutes of Health, Public Health Service (AM 07902), the National Science Foundation (GB 3249), and the Muscular Dystrophy Association, Inc.

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TABLE I: Effect of AMP on Activity of Phosphorylase Phosphatase in a Protein-Glycogen Complex.[a]

	Phosphatase act. (nmol of P_i released min^{-1} mL^{-1})	
	−AMP	+AMP
Undiluted complex	4.9	5.1
Tenfold diluted complex	1.9	0.1
Reconcentrated complex	6.6	6.6

[a] The protein-glycogen complex was diluted with 50 mM sodium glycerophosphate (pH 6.8) containing 0.5 mM EDTA and 50 mM 2-mercaptoethanol. The diluted complex was reconcentrated by centrifugation at 80 000*g* for 90 min and the pellet resuspended in the original volume of buffer. Phosphatase was assayed by Method II of Haschke et al. (1970).

covalent modification of the phosphatase itself has been demonstrated.

This article describes some of the experiments carried out to gain a better understanding of the properties and control of phosphorylase phosphatase in the protein-glycogen complex described above. Possible effectors have been investigated on purified phosphatase and a possible regulation by modification of the substrate itself is discussed.

Materials and Methods

Glycogen particles were prepared according to Meyer et al. (1970). Phosphorylase phosphatase was purified through the poly(L-lysine)-Sepharose step according to Gratecos et al. (1977); other methods will be found in this last reference.

For flash activation of phosphorylase (Heilmeyer et al., 1970), the reaction mixture consisted of 465 μL of the glycogen-protein suspension in 50 mM sodium glycerophosphate (pH 6.8) containing 0.5 mM EDTA,[1] 25 μL of 0.1 M $MgCl_2$, and 5 μL of 0.1 M ATP; at various times phosphorylase and phosphatase activities were measured. For phosphorylase *a* activity, samples were diluted 100- to 500-fold in cold 0.1 M sodium glycerophosphate (pH 6.8), containing 40 mM 2-mercaptoethanol, 1 mg/mL bovine serum albumin, 1 mM EDTA, and 0.1 M sodium fluoride. Activity was measured without AMP and expressed as percent of total phosphorylase activity obtained with 1 mM AMP.

Measurements of Phosphorylase Phosphatase Activity. Phosphatase activity in the glycogen particles can be measured in three ways. First (method A), it can be estimated by the rate of phosphorylase *a* to *b* conversion (down portion of the curve during "flash activation"). This assay measures the activity of endogenous phosphatase on endogenous phosphorylase. Second (method B), phosphatase activity can be measured according to standard assay conditions (release of ^{32}P from purified labeled phosphorylase *a* added in excess after appropriate dilution of the phosphatase; Gratecos et al., 1977). This gives a measure of maximum phosphatase activity present. Finally (method C), [^{32}P]phosphorylase *a* can be added directly to the glycogen particles according to Method II of Haschke et al. (1970). In this instance, samples (0.5 mL) of the reaction mixture are added at 30 °C to 50 μL of a 30 mg/mL solution of [^{32}P]phosphorylase *a*; initial rates are determined by precipitation of 0.1-mL aliquots taken after 10, 20, and 30 s with 10% trichloroacetic acid and measuring the $^{32}P_i$ released. Method C provides an estimate of endogenous phosphatase activity measured under concentrated conditions on exogenous (purified) substrate.

[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

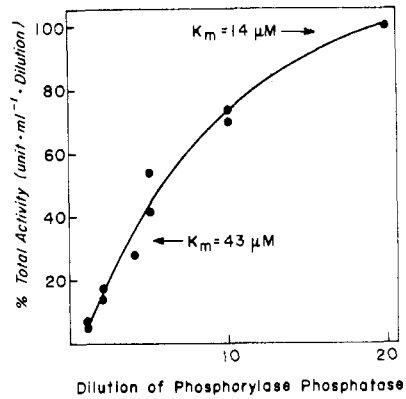

FIGURE 1: Effect of dilution on the activity and K_m of purified phosphorylase phosphatase in concentrated solutions. A solution of phosphatase (440 units/mL; ca. 15 mg/mL protein total) in 50 mM Tris-HCl (pH 7.5), containing 50 mM 2-mercaptoethanol, was diluted and assayed as described under Materials and Methods. Data from two experiments are expressed as percent activity measured after a 20-fold dilution. K_m values were calculated from Lineweaver-Burk plots with substrate concentrations ranging from 7 to 100 μM (not illustrated).

Protein-bound ^{32}P was determined by precipitating aliquots from the above reaction mixture with 9 vol of 10% trichloroacetic acid; the precipitates were twice dissolved in 1 mL of 0.1 M NaOH and reprecipitated with 1.5 mL of 15% trichloroacetic acid. They were finally dissolved in 1 mL of 88% formic acid and the solutions were counted for radioactivity.

Results

Enzymatic Properties of the Glycogen-Protein Complex. Phosphorylase phosphatase displays altered properties when its integration in the glycogen-protein complex is disrupted by dilution. For example, the insensitivity of the bound enzyme to 1 mM AMP (a powerful inhibitor of the isolated enzyme) is lost upon dilution; its activity is increased tenfold and its K_m decreased 15 times. Several hypotheses can be proposed to account for this behavior such as a concentration-dependent interaction between the phosphatase and other components of the complex (Haschke et al., 1970, 1971), the presence of inhibitors, changes in the intrinsic properties of the enzyme itself, etc. (Busby and Radda, 1976).

As shown in Table I, the insensitivity of the bound enzyme to AMP that is lost by dissociation of the complex through dilution is restored by reconcentration following centrifugation. These data cannot be attributed to the presence of soluble inhibitors in the undiluted suspension, even though a fraction obtained after heating the original suspension at 100 °C for 5 min contained low molecular weight material which strongly inhibited purified phosphatase solutions. Two such protein inhibitors have been recently partially characterized (Huang and Glinsmann, 1975, 1976; Cohen et al., 1977). However, no effect of this particular inhibitor fraction could de demonstrated on the properties of the enzyme altered by dilution of the complex; that is, it neither prevented the increase in specific activity nor did it suppress the AMP inhibition.

Figure 1 illustrates the changes in activity observed when a 15 mg/mL solution of purified phosphorylase phosphatase of the same concentration as found in the glycogen particles is diluted. At the original concentration, a low percentage of activity is expressed, as in the glycogen particle; activity increases ca. 20-fold upon dilution. The K_m value also decreases with dilution. It should be noted, however, that the change in activity is not entirely due to a lowering of K_m since V_{max} also increases. Thus, the variations in the kinetic parameters upon disruption of the complex must be due to changes in the in-

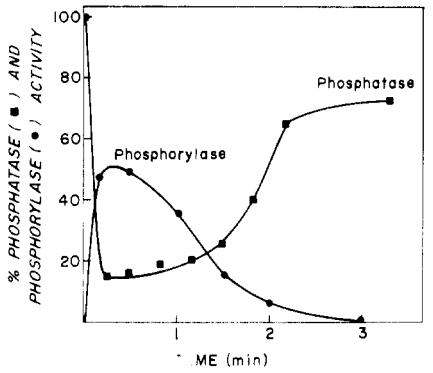

FIGURE 2: Phosphorylase phosphatase activity during transient activation of phosphorylase in an undiluted suspension of the protein-glycogen complex. The activation was initiated by addition of Mg^{2+}, ATP, and Ca^{2+} to final concentration of 5, 1, and 1 mM, respectively; phosphorylase and phosphatase activities were measured as described under Materials and Methods. Activities are expressed as percentage of the value obtained before addition of ATP: (●) phosphorylase; (■) phosphatase.

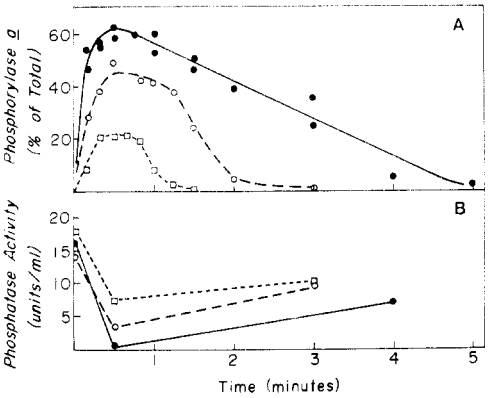

FIGURE 3: (A) Effect of addition of purified phosphatase on the transient activation of phosphorylase in the protein-glycogen complex. Experiments were carried out as described in Figure 2. Control without added phosphatase (●) or with 280 units/mL (○) or 535 units/mL (□) of added phosphatase. The suspension contained initially 160 units/mL of endogenous phosphatase as determined by the standard assay (method B) after a 400-fold dilution. (B) Phosphatase activity as measured by release of ^{32}P from ^{32}P-labeled phosphorylase *a* added to the system (method C).

trinsic properties of the enzyme itself. By contrast, even the most concentrated solutions of the *purified* phosphatase are just as much inhibited by 1 mM AMP (95%) as the highly diluted solutions, indicating that, in this instance, a different mechanism must be operative.

Determination of Phosphatase Activity in the Protein-Glycogen Complex. Figure 2 depicts a transient activation of phosphorylase present in the glycogen particles, as described in the introductory statement. The rate of phosphorylase *a* to *b* conversion by the endogenous phosphatase represents a minimum estimate of phosphorylase activity (minimum because there might be some residual ATP allowing the kinase reaction to proceed, and because the phosphatase might be partially inhibited). This type of measurement reveals that about 70% of the phosphatase activity (as determined by method B) is expressed, while an assay using added [^{32}P]-phosphorylase to the undiluted protein-glycogen complex (method C) reveals only 5 to 15% of this activity. The difference, together with the AMP insensitivity in the undiluted complex, could suggest some type of compartmentation within the particulate preparation.

To test this hypothesis, enough purified phosphatase was added to the complex to approximately double or triple the total potential endogenous activity (Figure 3A). As can be seen, both the steady-state level of phosphorylase *a*, and the rate at which this steady state is reached, are decreased. While both effects could be due to a higher ratio of phosphatase to kinase, some direct interaction between these two enzymes resulting in kinase inhibition cannot be excluded. The addition of phosphatase increases the rate of conversion of endogenous phosphorylase *a* to *b* demonstrating that added phosphatase equilibrates readily with the endogenous enzyme. By contrast (Figure 3B), when the phosphatase was tested by method C, approximately the same activity was detected in all three instances. This again supports the view that the phosphatase cannot be reliably measured in concentrated solutions by a direct assay involving endogenous substrate.

At first glance, the above results would appear to cast some doubts on the interpretation of the phosphatase inhibition illustrated in Figure 2, since activity was measured with added substrate. To dispel these doubts, attempts were made to measure the interconversion of endogenous phosphorylase during the steady-state phase of the "flash activation" by use of [γ-^{32}P]ATP (Figure 4). The duration of this steady state is, of course, dependent on the amount of ATP present. That

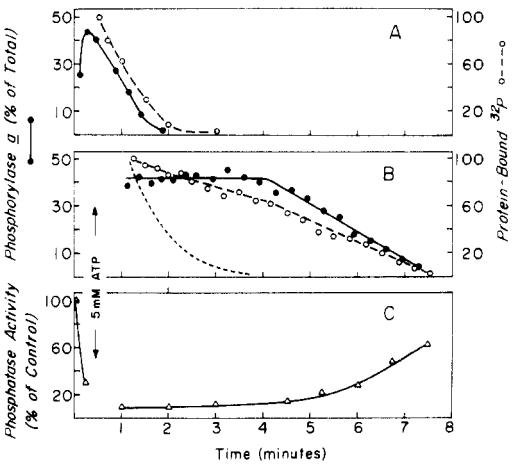

FIGURE 4: Comparison of phosphorylase *a* turnover with phosphatase activity on endogenous and exogenous substrate during "flash activation". Experimental conditions and procedures are described under Materials and Methods. Protein-bound ^{32}P (○) is expressed as percent of the value found in the first measurement (30 or 45 s). In A, activation of phosphorylase was initiated with 0.5 mM [γ-^{32}P]ATP (sp act. ca. 5×10^6 cpm/μmol). In B and C, activation was initiated with 0.5 mM [γ-^{32}P]ATP followed by 5 mM unlabeled ATP after 25 s. This addition represents a large dilution of radioactivity since most of the labeled ATP initially present is destroyed within 10 to 15 s by the sarcoplasmic ATPase. Diagram C represents phosphatase activity measured as described under Materials and Methods. The dotted line in B represents the theoretical, first-order rate of decrease in protein-bound ^{32}P assumming that phosphatase was fully active.

is, when small amounts of ATP (0.5–1 mM) are added, phosphorylase activation is very brief (Figure 4A) since all the ATP is consumed within seconds by the endogenous ATPase. By contrast, addition of a large amount of ATP (4–5 mM) prolongs the steady-state phase for several minutes (Figure 4B).

Since phosphorylase is the main protein in the complex that contributes significantly to ^{32}P uptake, measurements of protein-bound radioactivity will provide a reliable estimate of the concentration of phosphorylase *a* present. After 25 s, an excess of unlabeled ATP is added, allowing for the establishment of a new plateau of phosphorylase *a* which will include, in part, the protein labeled in the initial phase of the reaction (Figure 4B). The steady-state level represents the balance

TABLE II: Effect of Nucleotides and Metabolites on Phosphorylase Phosphatase Activity.[a]

Effectors[b]	% of control 0.25 mM	% of control 2.5 mM	Type of inhibition[c]
Inhibitors			
AMP[d]	5	0	Substrate
ADP	—	20	
ATP	82	23	Substrate
UMP	42	17	
UDP	75	31	
UTP	93	36	Mixed
UDPG	80	20	Mixed
GMP	21	23	Mixed
GDP	58	29	
GTP	65	20	
IMP	19	13	Mixed
Phosphate (50 mM)	30		Noncompetitive
Phosphorylase *b* (0.1 mM)	60		Competitive
Activators			
Glucose (50 mM)	255		
Glc-6-P (0.25 mM)	155		
(2.5 mM)	141		

[a] Purified phosphatase was assayed under standard conditions as described by Gratecos et al. (1974). Metabolites were added to the substrate before starting the reaction. [b] Other effectors that were without effect when tested at 2.5 mM: glucose 1-phosphate, fructose 6-phosphate, creatinine, creatinine phosphate, citrate, xanthine, and hypoxanthine. [c] Based on double reciprocal plots as depicted in Figure 5. [d] 60% inhibition at 0.01 mM AMP.

between kinase and phosphatase activities. If the phosphatase maintained its full activity toward the endogenous substrate, phosphorylase turnover would result in a decrease in protein-bound ^{32}P with a first-order rate constant equal to that obtained for the phosphorylase *a* to *b* conversion depicted in Figure 4A; this theoretical rate is shown by the dotted line in Figure 4B. On the other hand, if the phosphatase were completely inhibited as suggested by the direct assay illustrated in Figure 4C, there would be no turnover and the curve for protein-bound ^{32}P would parallel that obtained for total phosphorylase *a* activity. While the experimental values are intermediate between these two extremes, it is clear that release of ^{32}P from phosphorylase *a* has been considerably retarded. These data indicate that when phosphorylase *a* is transiently produced, the phosphatase is indeed inhibited by about 85%, in good agreement with the 90% inhibition observed in the direct assay in Figure 4C.

Effect of Metabolites on Phosphatase Activity. Since phosphorylase *a* is under complex allosteric regulation, in particular by nucleotides, sugars, and sugar esters, the effects of various metabolites on phosphatase activity have been investigated, as summarized in Table II. These data were compiled from a number of experiments carried out under a variety of conditions so that quantitative comparisons among these should not be made. Nevertheless, several generalities emerge. Except for AMP which acts in the micromolar range, all nucleotides tested are inhibitory, with 50% inhibition being generally observed for a concentration in the millimolar range. Mononucleotides are more inhibitory than either di- or trinucleotides. Glucose and glucose 6-phosphate stimulate the phosphatase, while other hexose phosphates have little effect. Phosphorylase *b* and inorganic phosphate, the two products of the reaction, are also inhibitory. In these ranges of concentration, no inhibition was afforded by glucose 1-phosphate,

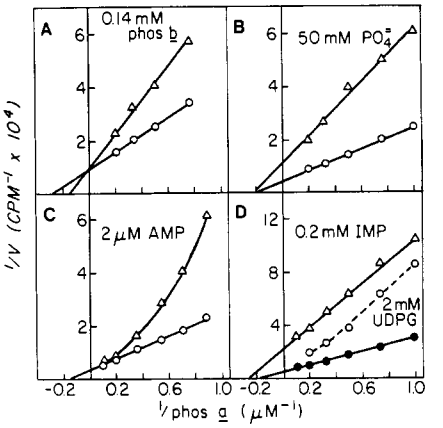

FIGURE 5: Lineweaver-Burk plots of the phosphorylase phosphatase reaction in the presence of various inhibitors. Assays were carried out under standard conditions as described by Gratecos et al. (1977). Rates are relative since enzyme activity, time of incubation, and specific radioactivity of [^{32}P]phosphorylase *a* are not identical in all experiments.

divalent cations, or EDTA, which inhibit the enzyme under other sets of conditions (Martensen et al., 1974; see also, Busby and Radda, 1976).

The nature of the inhibitions was further examined by Lineweaver-Burk plots. Four distinct types (competitive, noncompetitive, modification of the substrate, and mixed inhibition) were observed (Figure 5); all the inhibitors investigated fell within one of these categories (see also Table II). Phosphorylase *b* and P_i behave as typical competitive and noncompetitive inhibitors, respectively. The distinctive effect of AMP results from a modification of the substrate, phosphorylase *a*, rather than the phosphatase as shown previously by different approaches (Nolan et al., 1964; Martensen et al., 1974; Martensen and Graves, 1974; Busby and Radda, 1976). ATP gives plots similar to those observed for AMP but in the millimolar rather than in the micromolar range. GMP and UMP are also strong inhibitors with a kinetic behavior similar to that displayed by IMP and different than that for AMP, bringing about changes in both V_{max} and K_m. Some of these kinetic parameters are listed in Table III.

Phosphorylase phosphatase displays an affinity for phosphorylase *b* ca. 30 times lower than for phosphorylase *a*, but product inhibition is significantly increased (threefold) when phosphorylase *b* is converted to its active conformation by AMP. This result may not be surprising since it has been proposed that the AMP-phosphorylase *b* complex is in a conformation and state of aggregation similar, if not identical, to that of phosphorylase *a* (Wang and Black, 1968; Buc et al., 1976; Vandenbunder et al., 1976).

Discussion

The mechanism by which phosphorylase phosphatase activity is regulated under physiological conditions still represents a challenging question. The protein-glycogen preparation from rabbit muscle that has been used to demonstrate a reversible inhibition of phosphorylase phosphatase is a compromise between an experimentally difficult in vivo system and a more defined soluble in vitro preparation (for a review, see Busby and Radda, 1976). While it offers many advantages, it has its own complexities and there are major difficulties in attempting to evaluate enzyme activities in such a particulate system. The very high local concentration of proteins and polysaccharides, as well as the presence of other subcellular elements, might allow for multiple interactions. For ideal kinetic measurements, the suspension would have to be diluted 100- to 1000-fold, but,

TABLE III: Kinetic Parameters for the Phosphorylase Phosphatase Reaction.[a]

	V_{max} (%)	K_m (μM)	K_i(comp) (μM)
Phosphorylase *a* (tetramer)	100	5.5	
Phosphorylase *a* + glucose (dimer)	290	5.0	
Phosphorylase *a* + Glc-6-P	195	8.7	
Phosphorylase *a* + AMP	5	7.5	
Phosphorylase *b*			144
Phosphorylase *b* + AMP			54

[a] Kinetic parameters were calculated from experiments such as shown in Figure 5. Glucose (50 mM), AMP (5 mM), glucose 6-phosphate (2 mM or 5 mM), or phosphorylase *b* was mixed with phosphorylase *a* before starting the reaction by addition of the phosphatase.

under these conditions, the bound phosphatase loses several of its intrinsic properties such as its reversible inhibition during "flash activation", its insensitivity to AMP, and low K_m and V_{max}. Some of the experiments reported herein were devised to determine the origin of the unusual changes in behavior observed upon dilution.

The variations in the kinetic parameters may be interpreted in many ways. First, they could be attributed to the presence of inhibitors whose effect could be diminished by dilution (Brandt et al., 1974, 1975; Huang and Glinsmann, 1975, 1976). At least two protein inhibitors have been described in the complex. However, they cannot account for all the data. Second, phosphorylase *b*, which acts as a competitive inhibitor of the phosphatase, is present in the complex at a high concentration (20 mg/mL). On the basis of its K_i given in Table III, this could account for a 3-fold increase in K_m in the absence of AMP or 8-fold when phosphorylase *b* is saturated with the nucleotide, whereas a 15-fold increase in K_m on dilution of the complex was reported (Haschke et al., 1972). On the other hand, the effect of dilution could be attributed to changes in the intrinsic properties of the phosphatase itself since dilution of purified enzyme preparations resulted in the same increase in activity and decrease in K_m observed with the glycogen-protein complex. One possibility is that the enzyme, which has a tendency to aggregate (Gratecos et al., 1974, 1977), undergoes a concentration-dependent polymerization yielding species with lower V_{max} and higher K_m. Some experimental evidence arguing in favor of this hypothesis has been reported in the preceding manuscript (Gratecos et al., 1977). Alternatively, the phosphatase could react with some of the other enzymes involved in the control of glycogen metabolism, particularly with their phosphorylated forms. Because of their high concentration in the sarcoplasm relative to the phosphatase, phosphorylase kinase and glycogen synthase could be prime candidates.

Finally, the differences in kinetic parameters might arise from difficulties inherent to measuring enzyme activities in the glycogen-particulate preparation. By adding labeled phosphorylase *a* to undiluted aliquots of the reaction mixture, the substrate/enzyme ratio departs considerably from ideal Michaelis-Menten assumptions. In the particulate suspension, the molar ratio of phosphorylase *a* (15 mg/mL, 150 μM in enzyme subunits) to phosphatase (0.15 mg/mL, 5 μM based on the purification described in Gratecos et al., 1977) is less than 30 and this value falls to 6 when one considers only the concentration of exogenously added ^{32}P-labeled phosphorylase *a* (3 mg/mL). This unfavorable ratio must surely contribute, at least in part, to the low percentage of activity expressed in concentrated solutions so that the Michaelis-Menten parameters must be interpreted with caution. The above restrictions become even more critical when one compares the values obtained by direct assay of phosphatase activity with estimates based on the rate at which endogenous phosphorylase *a* returns to the *b* form during "flash activation": the former are 7 times lower. Addition of purified phosphatase that brings about an increase in activity toward endogenous phosphorylase *a* results in no increase in activity when release of ^{32}P from added [^{32}P]phosphorylase *a* is measured. Nonetheless, and in spite of these limitations, estimation of phosphorylase *a* turnover during "flash activation" confirmed that phosphorylase phosphatase undergoes a reversible inhibition.

Taken together, these results can account for the changes in the kinetic parameters observed upon dilution of the complex, with little need to invoke specific interactions between the phosphatase and other elements of the complex. By contrast, the lack of sensitivity of the phosphatase to AMP in the particles must be due to some specific interaction involving either the phosphatase or phosphorylase *a* since the purified phosphatase, even at high concentrations, is still inhibited by AMP.

Some molecular species of phosphorylase *a* have been shown to be more susceptible to the phosphatase than others, raising the possibility of an interesting and unusual form of control of enzyme activity, i.e., allosteric regulation of the substrate (Nolan et al., 1964; Martensen et al., 1974; Martensen and Graves, 1974; Busby and Radda, 1976). Glucose activates the reaction by modifying the conformation of the substrate, causing an increase in V_{max} with little change in K_m. Glucose is also known to induce a tetramer to dimer transition of phosphorylase *a* (Wang and Graves, 1964) and this alteration in the state of aggregation of the substrate might also be implicated since sedimentation velocity measurements have confirmed that dissociation does occur under our assay conditions.

All nucleotides tested are inhibitory, in agreement with previous data by Bot and Dosa (1971) and others. Presumably, they act by modifying the substrate but this has been proven only for AMP. The different effects of these inhibitors are perhaps not surprising since IMP and other nucleotides have been shown to activate phosphorylase *b* by a mechanism different from that for AMP (Black and Wang, 1970; Buc et al., 1976; Vandenbunder et al., 1976). Results reported here indicate that under our experimental conditions, AMP inhibits the phosphatase by lowering V_{max} by 95% with no change in K_m; this is in contrast to the increase in K_m with little change in V_{max} observed at a very high AMP/phosphorylase *a* ratio (Martensen et al., 1974). The striking effects of AMP are especially interesting since a condition of high AMP level in the muscle would coincide with conditions of increased need for glycolysis, in which case a low activity of the phosphotase would seem appropriate. AMP could inhibit the phosphatase both by formation of a phosphorylase *a* species that is a very poor substrate and by conversion of phosphorylase *b* to its active conformation, making it a better phosphatase inhibitor. Unfortunately, there is little information on the conformation of phosphorylase in vivo, and, therefore, on the manner in which it can be modified by AMP and other metabolites. Haschke et al. (1972), investigating the AMP binding to phosphorylase in the protein-glycogen complex, concluded that the K_m for this nucleotide is the same as for the crystalline enzyme but that the elements of the complex hinder the nucleotide-induced conformational changes. This could explain the lack of sensitivity of the phosphatase to AMP in the complex, but it would seem to exclude a mechanism by which AMP

would control phosphatase activity in the particulate preparation. Likewise, transient inhibition of the phosphatase during "flash activation" by formation of an IMP-phosphorylase *a* or ATP-phosphorylase *a* complex was previously ruled out (Haschke et al., 1970). Essentially the same inhibition pattern of the phosphatase was observed when a ^{32}P-labeled phosphopeptide derived from phosphorylase *a* was used as substrate in the presence of glycogen particles. Dephosphorylation of the phosphopeptide is totally unaffected by adenine nucleotides. Nevertheless, allosteric modification of phosphorylase remains an intriguing possibility for the control of phosphorylase phosphatase activity in vivo.

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