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INHIBITION OF α-GLUCAN PHOSPHORYLASE BY BISULFITE

COMPETITION AT THE PHOSPHATE BINDING SITE

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SUMMARY

1. $NaHSO_3$ was found to inhibit potato and rabbit muscle phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) at pH 6.0. The inhibition is completely reversible and specific for this salt. Sulfate, azide and cyanide had no effect, but bicarbonate at pH 8 showed weak inhibition.

2. The inhibition by bisulfite was competitive for glucose-1-*P*, P_i and arsenate. The K_i values of bisulfite were determined and found to be smaller than the K_m values. The interaction of the anion at the phosphate binding site occurred in an equimolecular ratio. The inhibition was more pronounced at acidic pH than at basic pH.

3. Bisulfite caused a retardation of the resolution of pyridoxal-5′-*P* from phosphorylase *b*, but not of the reductive fixation. No change in fluorescence and absorption due to the bound cofactor occurred in the reversible inhibition. The addition of hydroquinone had no effect on the rate of inhibition.

4. It is suggested from these results that the inhibition is caused by competition of bisulfite and phosphate at the phosphate binding site of phosphorylase.

INTRODUCTION

α-Glucan phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) catalyzes the reversible phosphorolysis of glycogen and starch. The enzyme has a wide variety of regulatory mechanisms according to the source from which it is isolated. Rabbit muscle phosphorylase exists in two forms, active and inactive, which are interconvertible through enzymic phosphorylation and dephosphorylation reactions. These interconversions are associated with a change in the quarternary structure of the enzyme. The inactive enzyme has another means for activation; it is activated by an allosteric effector, AMP. Phosphorylase from the potato, on the contrary, exists in an active form and is not affected by AMP. All the phosphorylases

isolated hitherto from various sources contain bound pyridoxal-5′-*P*, which is essential for the enzyme activity. These features are well documented by several recent reviews[1–5].

Although much is known about the interaction of these multiple sites, little information is as yet available on the active site. Several investigations on the chemical modification of phosphorylase have been reported[6–18], but they give little information about the active site. Tu *et al.*[19] and Gold *et al.*[20] have recently described that 1,5-gluconolactone competes for the glucosyl transfer site of phosphorylase and that the transition state involves formation of an enzyme–glucosyl complex in which the glucosyl residue is in the half-chair conformation. The present report deals with the competitive inhibition of potato and rabbit muscle phosphorylase by bisulfite. The results are discussed in connection with a structural relationship between bisulfite and phosphate.

MATERIALS AND METHODS

Glucose-1-*P*, glucose-1,6-P_2, AMP, ATP, NADP, hexokinase (EC 2.7.1.1), phosphoglucomutase (EC 2.7.5.1) and glucose-6-*P* dehydrogenase (EC 1.1.1.49) were purchased from Sigma. Soluble starch, Na_2SO_3, $NaHSO_3$, pyridoxal-5′-*P*, *n*-hexylamine and $NaHCO_3$ were obtained from Wako. Shellfish glycogen and Na_3AsO_4 were the products of Nakarai. Amylose (D.P. 30) was a gift from Hayashibara. ^2H_2O was obtained from Schwartz.

Potato phosphorylase was purified according to the method previously reported[21]. Crystalline rabbit muscle phosphorylase *b* was prepared according to the method of Fischer and Krebs[22], and the third crystals were freed from AMP by passage through a charcoal–cellulose column before use. Phosphorylase activity in the direction of polysaccharide synthesis was assayed as described in the previous paper[17]. In the direction of polysaccharide degradation, the rate of phosphorolysis and arsenolysis were determined as follows. The reaction mixture contained 2% amylose, the enzyme and Na_3PO_4 or Na_3AsO_4 in an appropriate buffer, pH 6.0. The total volume was 1 ml. After incubation for 10 min at 30 °C the reaction was terminated by immersing the tube in a boiling water bath for 70 s. The reaction mixture was then cooled rapidly in an ice bath. Bisulfite, which inhibits the glucose-6-*P* dehydrogenase system, was oxidized to sulfate by H_2O_2 in the cold. For the determination of glucose-1-*P* and free glucose, 10 μl each of 6% and 30% H_2O_2 were used, respectively. Amylose precipitated in the cold was removed by centrifugation. Glucose-1-*P* or free glucose in an aliquot of the supernatant fluid was determined with a phosphoglucomutase–glucose-6-*P* dehydrogenase system or a hexokinase–glucose-6-*P* dehydrogenase system, respectively. Concentration of phosphorylase was determined spectrophotometrically using absorbance indices, $A^{1\%}_{278\,nm}$, of 11.5 for the potato enzyme and 11.7 for the muscle enzyme. Resolution of pyridoxal-5′-*P* from muscle phosphorylase *b* was carried out according to Shaltiel *et al.*[23], and reductive fixation according to Strausbauch *et al.* (Variant 1) (ref. 24). Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer MPF-2A. Absorption measurements were carried out with a Hitachi recording spectrophotometer EPS-3T. Nuclear magnetic resonance (NMR) studies were carried out with a Hitachi–Perkin–Elmer high resonance NMR spectrophotometer R-20.

RESULTS

In the course of the study on the effect of various inorganic salts on phosphorylase, it has been found that $NaHSO_3$ specifically inhibits both potato and rabbit muscle phosphorylase. The inhibition is completely reversible; all the original activity could be recovered after either dilution or dialysis against water.

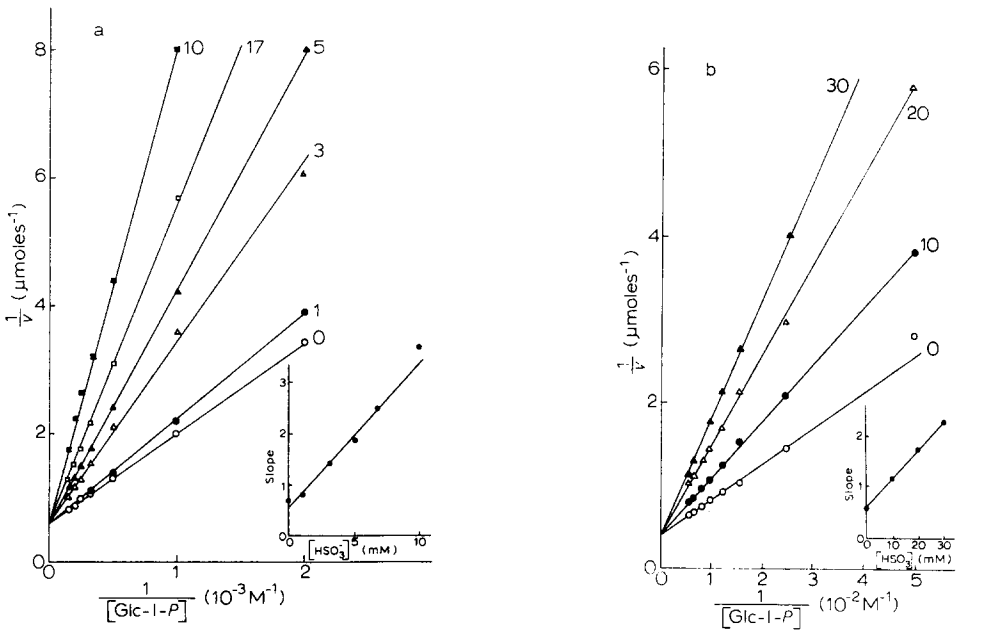


Fig. 1. Double reciprocal plot of initial velocity against glucose 1-*P* concentration in the presence of various concentrations of bisulfite. (a) Potato phosphorylase; at pH 6.0 and 30 °C in 20 mM glycerophosphate buffer (1 ml). 2% soluble starch, 5.7 μg of enzyme and glucose-1-*P*. Bisulfite concentration as indicated in mM. Insert: plot of the slope against bisulfite concentration. (b) Muscle phosphorylase *b*; at pH 6.0 and 30 °C in 20 mM glycerophosphate buffer (1 ml). 1% glycogen, 15 mM cysteine, 1 mM AMP, 7 μg of enzyme and glucose-1-*P*. Bisulfite concentration as indicated in mM. Insert: plot of the slope against bisulfite concentration.

Fig. 1 illustrates double reciprocal plots with respect to glucose-1-*P* for potato phosphorylase and muscle phosphorylase *b*, in the presence of various concentrations of $NaHSO_3$. The plots are linear and show that bisulfite is a competitive inhibitor for glucose-1-*P*. The inserts of Fig. 1 illustrate plots of slopes *vs* inhibitor concentration. The linear relationships obtained for both enzymes implies that one mole of bisulfite interacts with a glucose-1-*P* binding site of phosphorylase. The K_i of bisulfite was calculated from these results to be 1.7 mM for potato phosphorylase and 7 mM for muscle phosphorylase *b*. The values are quite low compared to the K_m values for glucose-1-*P* of the potato and muscle enzymes, 2.5 and 11 mM, respectively. A constant ratio of K_i/K_m for both enzymes suggests that the inhibition occurred at the common active site, regardless of the difference in their regulatory mechanism, and that a possible structural relationship exists between the inhibitor and the substrate.

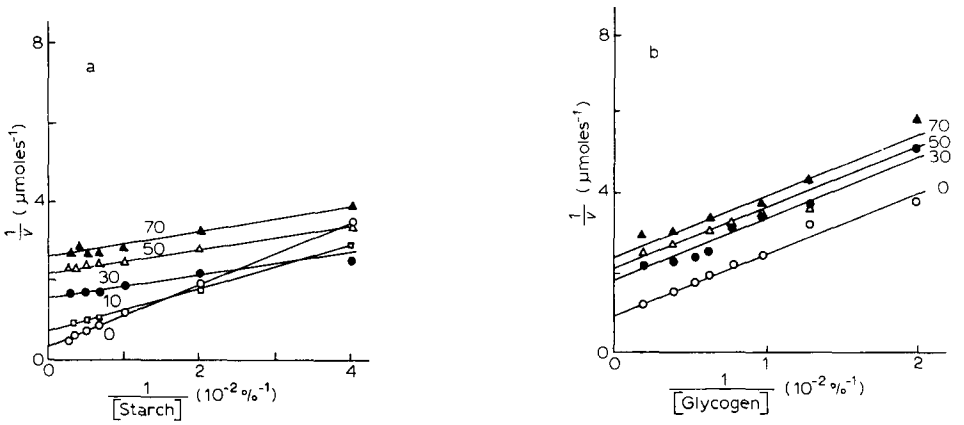
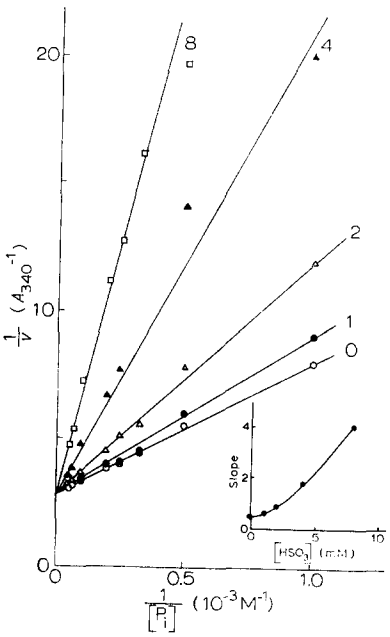


Fig. 2. Double reciprocal plot of initial velocity against polysaccharide concentration in the presence of various concentrations of bisulfite. (a) Potato phosphorylase; at pH 6.0 and 30 °C in 20 mM glycerophosphate buffer (1 ml). 10 mM glucose-1-*P*, 11.4 μg of enzyme and soluble starch. Bisulfite concentration as indicated in mM. (b) Muscle phosphorylase *b*; at pH 6.0 and 30 °C in 20 mM glycerophosphate buffer (1 ml). 16 mM glucose-1-*P*, 15 mM cysteine, 1 mM AMP, 7 μg of enzyme and glycogen. Bisulfite concentration as indicated in mM.

Fig. 2 illustrates double reciprocal plots with respect to polysaccharide for potato phosphorylase and muscle phosphorylase *b*, in the presence of various concentrations of $NaHSO_3$. Soluble starch and glycogen were known to be good primers for the potato and the muscle enzyme, respectively, and used in these experiments as polysaccharide. The plots are linear in both cases. However, in contrast to the results with respect to glucose-1-*P*, the maximal velocities are retarded by the presence of bisulfite. This indicates that in the direction of polysaccharide synthesis, bisulfite is an uncompetitive inhibitor with respect to the polysaccharide. The non-parallel relationship observed in the case of potato phosphorylase can not be explained at present.

The effect of $NaHSO_3$ on the activity of phosphorylase was also determined in the direction of polysaccharide degradation. Fig. 3 shows double reciprocal plots with respect to P_i for potato phosphorylase in the presence of various concentrations of sodium bisulfite. The plots are linear and the values of maximal velocity are the same both with and without bisulfite. This indicates that bisulfite acts as a competitive inhibitor with respect to P_i in polysaccharide degradation, as in the case of glucose-1-*P* in polysaccharide synthesis. These results are quite understandable when one makes the supposition that the binding sites of P_i and of the phosphate moiety of glucose-1-*P* are the same in phosphorylase. This supposition is most conceivable in the reversible reaction catalyzed by the enzyme. However, the plot of slopes *vs* bisulfite concentrations (the insert of Fig. 3) is not straight, in contrast to the result in the case of glucose-1-*P*. The reason for this complex result is obscure.

It is well known that P_i can be replaced by arsenate in the degradation of polysaccharide by phosphorylase. The effect of bisulfite was examined in the arsenolysis of starch by potato phosphorylase. As Fig. 4 shows, bisulfite acted as a potent competitive inhibitor with respect to arsenate in phosphorylase action. A K_i value of 0.02 mM was obtained which is one order of magnitude smaller than the K_m for arsenate, 2.5 mM. In the case of arsenolysis, the slope is directly proportional to the



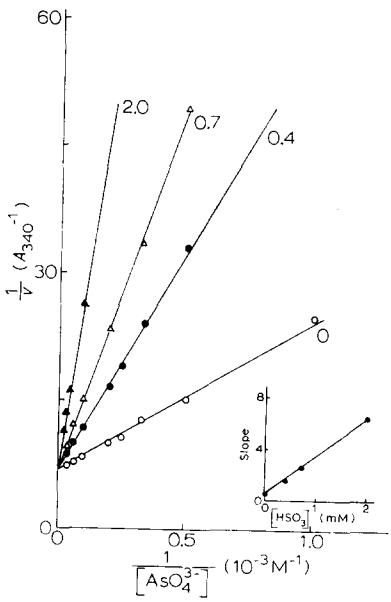


Fig. 3. Double reciprocal plot of initial velocity of potato phosphorylase reaction against orthophosphate concentration in the presence of various concentrations of bisulfite; at pH 6.0 and 30 °C in 50 mM citrate buffer (1 ml). 2% amylose, 9.3 μg of enzyme and Na_3PO_4. Bisulfite concentration as indicated in mM. A 100-μl aliquot was taken for assay of glucose-1-*P*. An $A_{340\ nm}$ increase per ml of the original reaction mixture was used for initial velocity. Insert: plot of the slope against bisulfite concentration.

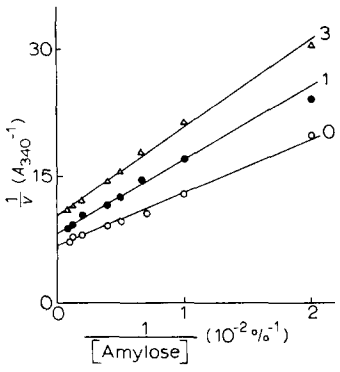
Fig. 4. Double reciprocal plot of initial velocity of potato phosphorylase reaction against arsenate concentration in the presence of various concentrations of bisulfite; at pH 6.0 and 30 °C in 20 mM glycerophosphate buffer (1 ml). 2% amylose, 10 μg of enzyme and Na_3AsO_4. Bisulfite concentration as indicated in mM. A 300-μl aliquot was taken for assay of glucose. An $A_{340\ nm}$ increase per ml of the original reaction mixture was used for initial velocity. Insert: plot of the slope against bisulfite concentration.

concentration of bisulfite, indicating that one mole of HSO_3^- interacts at a phosphate (arsenate) binding site of the enzyme, as the insert of Fig. 4 shows.

The effect of bisulfite was studied at various concentrations of polysaccharide in the arsenolysis by potato phosphorylase. Fig. 5 illustrates the kinetic data. Both K_m and V vary at various concentrations of bisulfite. This infers that bisulfite is a noncompetitive inhibitor with respect to amylose in arsenolysis. It is concluded from all of the above results that bisulfite interacts directly at the phosphate binding site of phosphorylase. Bisulfite may not act as an active substrate for the enzyme, since no evidence for the liberation of either glucose or its ester from amylose in the presence of bisulfite and phosphorylase has been obtained.

Fig. 6 shows the activity of potato phosphorylase at various pH values in the presence and absence of bisulfite. Both curves have a maxima around pH 6. The rate of inhibition is not greatly influenced by pH, but is more pronounced at acidic pH than at basic pH.

The inhibitory action of bisulfite on phosphorylase is highly specific. The inorganic salts which exhibited no effect on the enzyme at 50 mM and pH 6.0 include



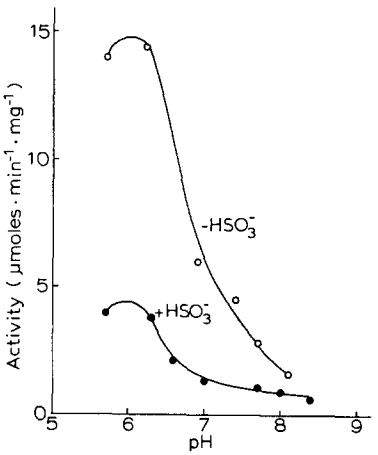


Fig. 5. Double reciprocal plot of initial velocity of potato phosphorylase reaction against amylose concentration in the presence of various concentrations of bisulfite; at pH 6.0 and 30 °C in 20 mM glycerophosphate buffer (1 ml). 100 mM Na_3AsO_4, 10 μg of enzyme and amylose. Bisulfite concentration as indicated in mM. A 300-μl aliquot was taken for assay of glucose. An $A_{340\ nm}$ increase per ml of the original reaction mixture was used for initial velocity.

Fig. 6. Effect of pH on the potato phosphorylase reaction in the presence and absence of bisulfite; in 40 mM glycerophosphate buffer, 1% soluble starch, 10 mM glucose-1-*P* and 12 μg of enzyme in the presence and absence of 20 mM bisulfite at 30 °C. pH was adjusted with NaOH or HCl to the values indicated.

Na_2SO_4, NaN_3, NaCN and others. The only exception we encountered was $NaHCO_3$ at pH 8.0. This salt inhibited the starch synthetic reaction by potatop hosphorylase, as Table I shows. The action of bicarbonate, however, was much weaker than that of bisulfite.

TABLE I

INHIBITORY EFFECT OF $NaHCO_3$ ON POTATO PHOSPHORYLASE*

Concentration (mM)	*Activity remained (%)*
0	100
50	56
100	38
200	34

* The reaction was carried out at pH 8.0 and 30 °C in 50 mM Tris–HCl buffer, 1% soluble starch, 10 mM glucose-1-*P*, 70 μg of the enzyme and various concentration of sodium bicarbonate in a total volume of 1 ml.

Tu *et al.*[19] have shown that 1,5-gluconolactone, which competes for the glucosyl transfer site of phosphorylase, affects the properties of the pyridoxal-5'-*P* binding site. An experiment was carried out to see whether bisulfite shows a similar interaction with the pyridoxal-5'-*P*. Fig. 7 illustrates the effect of bisulfite on the resolution of rabbit muscle phosphorylase *b*. The rate of resolution was determined by the decrease in enzyme activity under the conditions as described by Shaltiel *et al.*[23]. It is clear that the addition of 50 mM bisulfite caused a marked retardation of the

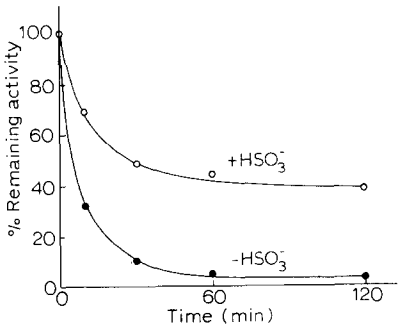


Fig. 7. Effect of bisulfite on the resolution of pyridoxal-5'-G from muscle phosphorylase *b*; 5.05 mg of enzyme in 2.5 ml. 50 mM of bisulfite was added concomitantly with the reagent for resolution at 0 °C and incubated at the same temperature. A 5-μl aliquot was removed for assay of remaining activity, after the periods of time indicated.

resolution of phosphorylase *b*. About 40% of the activity still remained in the presence of bisulfite after a period of time in which all the activity was apparently lost in the absence of this anion. The addition of 2% $HClO_4$ caused pyridoxal-5'-*P* to be almost completely removed from the enzyme which had been treated with bisulfite. A possibility that a part of the bound cofactor was fixed by reduction and was thus resistant against resolution is thus ruled out. Bisulfite, however, did not block the reductive fixation of pyridoxal-5'-*P* in muscle phosphorylase *b* with $NaBH_4$.

Bisulfite functions as a reducing agent and is known to cleave disulfide linkage in protein. However, potato and muscle phosphorylase has been regarded as containing no disulfide linkage, since the numbers of sulfhydryl groups which were reactive with sulfhydryl reagents in the presence of denaturating agents coincide well with those of half-cystine residues determined by amino acid analysis[13,18]. Thus, the possibility that the inhibition observed was due to the cleavage of disulfide bond was excluded.

Another possibility is the addition of bisulfite on the pyridine ring or aldimine linkage of pyridoxal-5'-*P* group bound on phosphorylase; this group is at present thought to participate in the function of the enzyme. However, this addition may not be the case, as judged by the following observations. The inhibition is specific for bisulfite. Cyanide, which is also a good nucleophile, was ineffective for phosphorylase activity. No change in either absorption or fluorescence due to bound pyridoxal-5'-*P* was observed upon the addition of 50 mM bisulfite at pH 6.0. The change of fluorescence was observed only after a prolonged period of time, as stated in the subsequent section. The inhibitory effect of bisulfite was also seen in the muscle phosphorylase *b* which had been treated with $NaBH_4$, which reduces the aldimine linkage. Moreover, titration of potato phosphorylase by alkali in the presence of 50 mM bisulfite caused the same absorption change of pyridoxal-5'-*P* as given in the case of no bisulfite addition.

The Schiff base of pyridoxal-5'-*P* and *n*-hexylamine in ^2H_2O was analyzed by NMR with or without 50 mM bisulfite at pH 6.1 and room temperature. Upon the addition of bisulfite, the signal of 4'-H completely disappeared but the ones of 2-CH_3, 5-CH_2 and 6-H remained unaltered. The results are explained by assuming that the

pyridoxal-5'-*P* forms an addition compound with bisulfite at the 4'-position, but no change occurs on its ring structure.

Since it had been recently shown that hydroxy radicals were produced in a dilute bisulfite solution in the presence of O_2 (ref. 25), experiments were carried out to determine whether the inhibition was caused by radical reaction. The addition of 100 mM hydroquinone as radical scavenger had no effect on the rate of the inhibition by bisulfite.

DISCUSSION

The inhibitory effect of bisulfite on phosphorylase is highly specific for this salt and competitive with respect to phosphate interacting in an equimolecular ratio at the active site. These results might be interpreted by a structural similarity between bisulfite and phosphate, which are, as well as arsenate, salts of weak acids containing an O atom connected by a double bond and two or three dissociable OH groups, and their pK_2 values are around neutrality. Bicarbonate, which exhibited a weak inhibition at pH 8, has a similar structure but a much higher pK_2 value. The structure illustrated schematically in Fig. 8 seems to be a requirement for binding

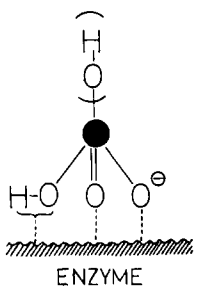


Fig. 8. Schematic representation of an anion bound at the phosphate binding site of phosphorylase.

at the phosphate binding site of phosphorylase. Of such anions, the ones with the third OH group (phosphate and arsenate) act as substrate accepting the glucosyl residue of polysaccharide, and the anions with only two OH groups (bisulfite and bicarbonate) can bind at the active site but not behave as substrate.

Bisulfite was known to be a competitive inhibitor for sulfatase[26], and arsenate inhibited phosphatase[27–29]. Although detailed studies on these inhibitions are not available at present, they seem to support the above discussion on the inhibition of phosphorylase by bisulfite. Many other enzymes are known to be inhibited or activated by various anions[30–32]. In those cases, however, the range of effective anions was broad, irrespective of their structure.

In a previous paper[17], we reported that phosphorylase was inactivated on the reaction with glyoxal. In this reaction, the inactivation was retarded when glyoxal–bisulfite complex was used in place of free glyoxal. The bisulfite might bind at the active site and protect it against the modification, as do the substrates.

Bisulfite affected the properties of the pyridoxal-5'-*P* binding site, as in the case of 1,5-gluconolactone[19] which is a competitive inhibitor with respect to the

glucosyl residue of glucose-1-*P*. The pyridoxal-5'-*P* is essential for the enzyme activity, but its exact role in phosphorylase has yet to be established. The action of bisulfite might be explained either by direct interaction at the pyridoxal-5'-*P* site or by a conformational change of protein due to the binding of bisulfite at a separate active site.

It is difficult to ascertain whether the inhibitory action of bisulfite is biologically significant or not, although the salt is widely distributed in various biological materials as an intermediary compound of sulfur metabolism. The prolonged contact of phosphorylase with bisulfite for several days resulted in the gradual change of fluorescence with the concomitant loss in enzyme activity (Kamogawa, A and Fukui, T., unpublished). The activity thus lost could no longer be restored after dialysis against water. These changes are distinguishable from the inhibitory effect of bisulfite described above, and could be caused by its action as a nucleophile.

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