Mechanism of Allosteric Activation of Glycogen Phosphorylase Probed by the Reactivity of Essential Arginyl Residues. Physicochemical and Kinetic Studies†

Marc Dreyfus,* Bernard Vandenbunder, and Henri Buc

ABSTRACT: The modification of phosphorylase with the arginine-specific reagent 2,3-butanedione in borate buffers has been investigated. When phosphorylase *b* in the absence of any effectors or phosphorylase *a* in the presence of the allosteric inhibitors caffeine or glucose is incubated with 2,3-butanedione, the nucleotide binding site is rapidly lost, but the enzyme is inactivated only very slowly, indicating no modification of the active site. By contrast, phosphorylase *a* alone or phosphorylases *a* and *b* in the presence of allosteric activators are inactivated rapidly by 2,3-butanedione. A correlation exists between the activation of the enzyme, as measured by its K_m for glucose 1-phosphate under the same conditions, and the rate of 2,3-butanedione inactivation. In all cases, glucose 1-phosphate protects the enzyme efficiently; moreover, the inactivated enzyme has lost its binding site for glucose cyclic 1,2-phosphate, a glucose 1-phosphate analogue. From these observations, it is proposed that phosphorylase has two (or two groups of) crucial arginine residues, presumably involved in the binding of nucleotides at the activator site on one hand and in the binding of phosphorylated substrates at the active site on the other. The latter locus is accessible to chemical modification in only the active conformations of the enzyme, and its increased reactivity must reflect the catalytic-site rearrangement brought about by activation. A variety of physicochemical probes indicates that, after chemical modification in the presence of activators, phosphorylase *b* is irreversibly trapped in an "*a*-like" conformation. It is thus concluded that the phosphorylation of Ser-14 or modification of an active-site arginine residue, two loci situated 40 Å apart, can stabilize similar conformations of this large globular protein. The impact of this finding on our current interpretation of allosteric transitions is briefly discussed.

The activity of glycogen phosphorylase (EC 2.4.1.1), the first enzyme implied in the breakdown of glycogen in muscle, is regulated by an elaborate control mechanism [for reviews see Graves & Wang (1972), Busby & Radda (1976), and Fletterick & Madsen (1980)]. The *b* form of the enzyme can be activated allosterically by binding of nucleoside monophosphates, notably AMP,[1] and can be inhibited by binding of the allosteric inhibitors Glc-6-P, ATP, glucose, or purines. Moreover, under hormonal control, it can be converted into phosphorylase *a* through the phosphorylation of a single serine residue (Ser-14). Phosphorylase *a* barely has a nucleotide requirement for enzymatic activity, although AMP causes a decrease of the K_m for substrates, but can still be allosterically inhibited by glucose and by a variety of fused-ring compounds, notably free purines (Fletterick & Madsen, 1980).

Although these allosteric properties have been extensively studied by a variety of kinetic and physicochemical approaches, still little is known of the underlying conformational changes at a molecular level. Recently, however, new information has been provided by X-ray crystallography. Isomorphous crystals suitable for high-resolution studies have been obtained from phosphorylase *b* in the absence of effectors and from phosphorylase *a* in the presence of the allosteric inhibitor glucose, and the two structures have been solved to a resolution of 3 and 2.5 Å, respectively (Weber et al., 1978; Sprang & Fletterick, 1979). These studies, together with the elucidation of the complete amino acid sequence (Titani et al., 1977), have led to the description of the folding of the whole molecule and to the localization of several binding sites for substrates and effectors. Among the most significant results was the discovery that both activator sites, the Ser-14 residue and the AMP binding site, are very distant from the catalytic site. Moreover, some structural information about the activation process itself has been obtained, at least at low resolution (Madsen et al., 1978). However, in the latter studies, the lattice constraints may restrict the structural changes brought about by activators. Therefore, it will presumably be necessary to solve separately the structure of the enzyme cocrystallized with activators before a reliable description of the allosteric transition can be given (Blake, 1979).

Another approach to the study of enzyme structure, which is complementary to X-ray diffraction work, consists of the chemical identification of the amino acid residues that are essential for enzyme structure and function, i.e., for catalytic activity or ligand binding capacity. In this respect, it seems especially promising to investigate the role of arginine residues in phosphorylase, because it has been recognized recently, since the advent of specific modifying reagents, that arginine is quite generally present in enzyme binding sites for phosphoric esters (Riordan et al., 1977). Since both the allosteric effectors and substrates of phosphorylase belong to this class, essential arginyl residues are expected to be present both at the allosteric and at the active site of the enzyme. Indeed, such a situation has been found to hold in several other allosteric enzymes, including rabbit muscle fructose 1,6-diphosphatase (Riordan et al., 1977), *Escherichia coli* aspartate transcarbamylase (Kantrowitz & Lipscomb, 1976, 1977), and bovine liver glutamate dehydrogenase (David et al., 1976; Pal & Colman, 1976).

† From the Institut Pasteur, Unité de Physicochimie des Macromolécules Biologiques, 25 rue du Docteur Roux, 75724 Paris, Cédex 15, France. *Received February 1, 1980.* This work was supported by grants from the Centre National de la Recherche Scientifique (L.A. 270), the Délégation Générale de la Recherche Scientifique et Technique (Grant MRM 76.7.1191), and the Institut National de la Santé et de la Recherche Médicale (Grant No. 77.84).

[1] Abbreviations used: AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; Glc-1-P, α-D-glucopyranose 1-phosphate; Glc-1,2-P, α-D-glucopyranose cyclic 1,2-phosphate; Glc-6-P, α-D-glucopyranose 6-phosphate; DTE, dithioerythritol; DTT, dithiothreitol; butanedione, 2,3-butanedione; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal 5′-phosphate.

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Recently, Li et al. (1977) described the modification of phosphorylase with the arginine-specific reagent 2,3-butanedione in borate buffer. The reagent was reported to inactivate both the *a* and *b* forms of the enzyme. In both cases, the inactivation process was shown to be first order with respect to butanedione, suggesting that the modification of only one crucial arginine residue caused inactivation, and the influence of the various phosphorylase ligands on the inactivation rate was studied. However, since the only enzyme property recorded during modification was the catalytic activity, it is difficult in each case to decide whether inactivation results from a modification of the active site, of an effector site, or of another locus. The only evidence presented for an "active-site" modification was an X-ray analysis of crystalline phosphorylase *a* after modification with 2,3-butanedione. The interpretation of this experiment relied on an active-site localization which was later shown by the same group to be incorrect (Sygusch et al., 1977). These facts encouraged us to reinvestigate the field. The present study has taken advantage of our observation (Dreyfus et al., 1978), also reported by others (Uhing et al., 1979), that phosphorylase *b* can be activated to a considerable degree even in the complete absence of nucleotides if suitable organic solvents are present in the assay medium; this allowed us to record the enzyme activity and thus to probe the active site integrity, independently of that of the nucleotide binding site.

In the first paper of this series, we shall describe physicochemical results on the modification of phosphorylase with 2,3-butanedione. It will be shown that essential arginine residues are present at both the allosteric and the active sites and that the reactivity of these sites toward butanedione varies widely depending upon the presence of allosteric effectors. Moreover, it will be shown that, under certain conditions, the enzyme can be locked by butanedione treatment in the conformation prevailing during modification, even after removal of allosteric effectors and excess reagents. In the second paper (Vandenbunder et al., 1980), similar experiments using [^{14}C]phenylglyoxal as a modifying reagent will be presented, and the essential active-site and allosteric-site arginine residues will be identified to allow comparison with X-ray diffraction data. A preliminary report of this work has been presented (Vandenbunder et al., 1979).

Materials and Methods

Enzymes and Reagents. The preparation and purification of phosphorylases *a* and *b* and the procedures routinely used to remove bound nucleotides or to measure the enzyme concentration were described in our previous publications (Dreyfus et al., 1978; Morange & Buc, 1979). All reagents used were analytical grade and were not further purified. The glycogen analogue maltoheptaose was prepared by Dr. Olivier Bensaude according to French et al. (1949).

The substrate analogue glucose cyclic 1,2-phosphate was synthesized on a 100-mg scale by cyclization of cold or ^{14}C-labeled glucose 1-phosphate, according to Zmudzka & Shugar (1964), except for the following modification. After pyridine and dicyclohexylurea had been removed by ether extraction, the reaction mixture was poured onto a 50-mL column of DEAE-52 equilibrated in 2 mM ammonium bicarbonate. The column was washed with 50 mL of the same buffer and then eluted with a linear gradient of ammonium bicarbonate concentration (2–100 mM; 2 × 100 mL) and finally with 50 mL of 100 mM ammonium bicarbonate. Glucose cyclic 1,2-phosphate was eluted near the middle of the gradient and was either used as a concentrated solution in ammonium bicarbonate or lyophilized. The product, which strongly inhibits phosphorylase, is very sensitive to hydrolysis under mild acid conditions. For this reason all stages of the preparation and handling of the compound were controlled by ascending paper chromatography in 2-propanol–ammonia–water (7:1:2). Chromatograms (20 cm) were cut into 1 cm long pieces and counted for radioactivity or assayed for inorganic phosphate after extraction by boiling in 10^{-1} N HCl for 2 min. Occasionally, glucose cyclic 1,2-phosphate ($R_f \simeq 0.4$) was contaminated with an impurity having an R_f of 0.95. However, equilibrium dialysis experiments, followed by chromatographic analysis of the composition of the "free ligand" and "enzyme plus ligand" compartments, showed that this impurity does not bind to phosphorylase.

Enzyme activity was measured at 24 °C in the direction of glycogen synthesis. Standard assay media contained 0.1% oyster glycogen, 0.5 mg/mL bovine serum albumin, 1–20 μg/mL phosphorylase, and either 1 mM AMP or 10% v/v *tert*-butyl alcohol in 50 mM glycylglycine buffer, pH 6.8.[2] The reaction was initiated by adding 10 mM glucose 1-phosphate. It has previously been shown that under these conditions native phosphorylase is nearly saturated with substrates (Dreyfus et al., 1978). Since the phosphorylase *b* activity measured in the presence of alcohol is very sensitive to traces of AMP, suitable precautions were taken to free all reagents from possible contamination by nucleotides (Dreyfus et al., 1978).

Modification of Phosphorylase. Unless otherwise specified, modification of phosphorylase (1–20 mg/mL) was carried out at 30 °C in 20 mM sodium tetraborate buffer, pH 7.5, containing 1 mM EDTA, and, when appropriate, specific ligands. The reaction was initiated by adding a portion of a concentrated solution of a freshly prepared 2,3-butanedione solution in the same buffer, adjusted to pH 7.5. At suitable time intervals, samples were removed and assayed for activity in standard assay media. The dilution was sufficient to ensure that any effector present in the modification medium had only a negligible effect on the activity measurements.

The partially labeled enzymes were isolated from the reaction mixture by the addition of an equal volume of saturated ammonium sulfate at 0 °C. When modification was performed in the presence of alcohol, an equal volume of buffer was added to the enzyme solution to reduce the alcohol content before ammonium sulfate addition. The precipitated enzyme was collected, resuspended in borate buffer, eventually treated with Norite A, and finally purified by gel filtration in borate buffer.

Binding of Effectors and Substrates to Native and Modified Enzyme. Although the ultracentrifugation method (Howlett et al., 1978) was used in some preliminary binding experiments, precise binding isotherms on the native or modified enzyme were obtained by equilibrium dialysis. Since it is known that the modification of arginine residues by 2,3-butanedione is easily reversed after removal of excess reagent but can be stabilized by borate (Riordan et al., 1977), all dialysis experiments were performed in 20 or 50 mM sodium tetraborate buffers. Even under these conditions, some loss of label occurred, as judged from the increase of the activity after the

[2] The specific activity of phosphorylase *b* in the presence of *tert*-butyl alcohol decreases at low enzyme concentration, especially if the organic solvent content is high. This previously unrecognized effect accounts for our observation (Dreyfus et al., 1978) that high concentrations of alcohol inhibit the enzyme reversibly. In the present report, the concentration of the enzyme was kept above 5 μg/mL when assayed in the presence of 10% *tert*-butyl alcohol: this is sufficient in all cases to allow the specific activity to reach a plateau. Under our standard assay conditions, this plateau is ~60% of the AMP-induced activity.

lengthy dialysis process (15–20 h). In all experiments reported here, this increase remained within 10% of the activity of the native enzyme.

Since borate is known to complex polyhydroxylic compounds, we investigated the possible influence of the buffer on the interaction of native phosphorylase with its ligands. The affinity of the enzyme for AMP or glucose cyclic 1,2-phosphate was found to be only slightly lowered in 20 mM sodium tetraborate, pH 7.5, with respect to 50 mM glycylglycine buffer, pH 6.8. On the other hand, at pH 7.5 and in the absence of AMP, the activity of phosphorylase *a* was completely inhibited by high borate concentrations ($K_{1/2}$ = 22 mM when the Glc-1-P and glycogen concentrations were 10 mM and 1%, respectively). This inhibitory effect, which was largely reversed by AMP, is not specific for borate since other large, easily polarizable anions are known to inhibit the enzyme (Engers & Madsen, 1968; Sealock & Graves, 1967).

Resolution of the Enzyme. The rate of resolution of the native and modified enzyme was compared as follows: the resolution reaction was initiated by mixing, at 0 °C, 1 volume of 0.4 M imidazole and 100 mM cysteine hydrochloride buffer brought to pH 6.2 (20 °C) with citric acid with 1 volume of a 10 mg/mL enzyme solution in 20 mM sodium tetraborate buffer, pH 7.5. At timed intervals, aliquots (400 μL) were removed and mixed with 600 μL of saturated ammonium sulfate. The precipitated enzyme was rapidly collected, washed with 60% ammonium sulfate, collected again, and resuspended in borate buffer. The concentration of the partially resolved enzyme was measured by using $E_{1\%}^{1cm}$ = 13.2 at 280 nm, and an aliquot was treated with perchloric acid (final 0.3 M) to precipitate the protein (Baranowski et al., 1957). The concentration of residual PLP was then measured at 295 nm after centrifugation. As a control, a 1:1 stoichiometry of PLP to phosphorylase was always obtained at t = 0, and the time course of the resolution of the native enzyme measured by this procedure paralleled that of the loss of activity (Figure 4).

Other Physical Measurements. Fluorescence spectra, sedimentation coefficients, and fast kinetic data on the nucleotide–phosphorylase interaction were obtained as previously described (Vandenbunder et al., 1976, 1978). Unless otherwise stated, all these experiments were run in 20 or 50 mM sodium tetraborate buffer, pH 7.5, containing 1 mM EDTA and 1 mM DTE (or DTT).

Results

Reaction of Phosphorylase b with the Arginine-Specific Reagent 2,3-Butanedione. Figure 1a shows the time course of the inactivation of phosphorylase *b* by the arginine-directed reagent 2,3-butanedione in 50 mM sodium tetraborate buffer, pH 7.5. At suitable time intervals, aliquots were removed from the reaction mixture and assayed for activity in standard media containing either AMP or alcohol. It may be seen that the AMP-induced activity was rapidly lost, while the alcohol-induced one was not. This suggests that the primary effect of butanedione is to modify the AMP site, without affecting the active site. This was confirmed by physicochemical studies on the partially modified enzyme after elimination of the excess reagent. Loss of the AMP binding site was evident from equilibrium dialysis measurements (Figure 1b), but no other gross changes were noticed in the enzyme's properties (Table I). Thus, the kinetic response of the modified enzyme vs. glucose 1-phosphate is very similar to that of the native enzyme, provided the activity is measured in the presence of alcohol. Similarly, the fluorescence quantum yield of the coenzyme, pyridoxal 5′-phosphate, as well as the kinetics of resolution of the enzyme in imidazole–citrate buffer, was barely

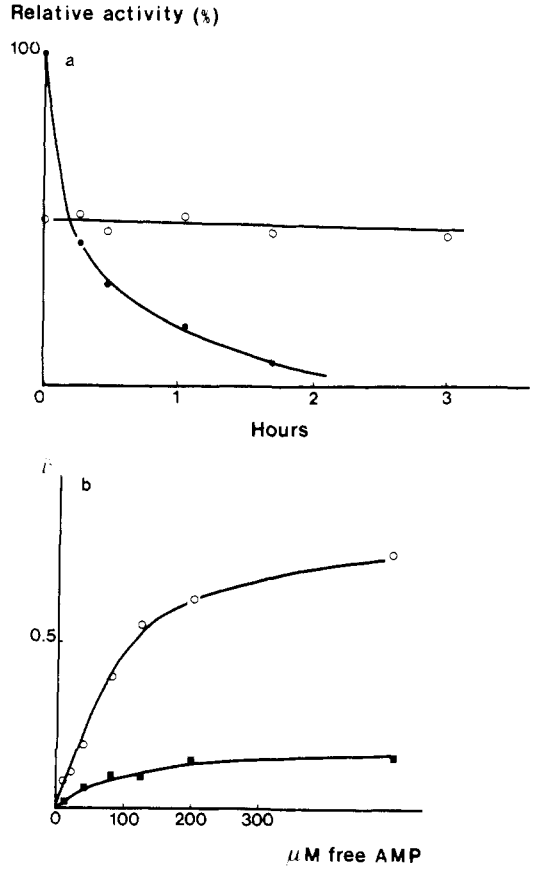

FIGURE 1: (a) Inactivation of glycogen phosphorylase *b* by 2,3-butanedione. Phosphorylase *b* (20 mg/mL) was incubated with 5 mM butanedione in 50 mM sodium tetraborate, 1 mM DTE, and 1 mM EDTA, pH 7.5, at 30 °C. At suitable time intervals, aliquots were assayed for activity in standard media containing 1 mM AMP (●) or 10% *tert*-butyl alcohol (○). (b) Binding of AMP on phosphorylase *b*. Equilibrium dialysis experiments were performed in 20 mM sodium tetraborate, 1 mM EDTA, and 1 mM DTE, pH 7.5 at 25 °C. $\bar{r}$ represents the concentration ratio [ligand bound]/[total phosphorylase]. (○) Native enzyme; (■) enzyme extensively modified by butanedione. The modified enzyme retained 12 and 90% of the activity of the native enzyme in standard assay media containing 1 mM AMP and 10% *tert*-butyl alcohol, respectively.

Table I: Influence of Modification by 2,3-Butanedione on Some Physicochemical Properties of Phosphorylase *b*

properties	native enzyme	modified enzyme	act. retention of modified enzyme (%)
high-affinity AMP binding sites/monomer of phosphorylase *b* (25 °C)	1.0	0.22	12
half-reaction time for resolution (min)	20	25	42
sedimentation coeff in borate buffer (20 °C) (S)	8.6	8.4	24
kinetic response with respect to Glc-1-P [K_m ($\bar{n}_{Hill}$)][b] (mM)	2 (1.6)	2 (1.6)	24

[a] Percent activity (with respect to the control) retained when assayed in the presence of 1 mM AMP. [b] The kinetic response with respect to Glc-1-P was studied in an assay medium containing 0.5% oyster glycogen, 0.5 mg/mL BSA, 0.1 mM EDTA, and 10% *tert*-butyl alcohol in 50 mM glycylglycine buffer, pH 6.8. Glc-1-P concentration was varied from 0.7 to 20 mM.

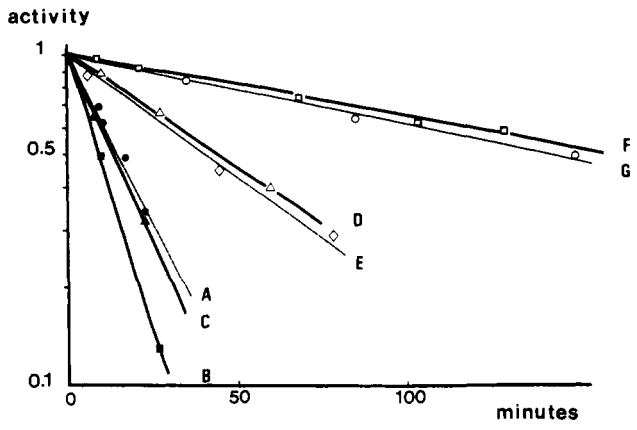

FIGURE 2: Time course of the inactivation of phosphorylase *a* (1 mg/mL) by butanedione (10 mM) in the presence of different ligands. (A) No ligand added to the modification medium; (B) 300 μM AMP; (C) 300 μM AMP plus 2% oyster glycogen; (D) 300 μM AMP plus 10 mM glucose 1-phosphate; (E) 300 μM AMP plus 10 mM Glc-1-P plus 36 mM P_i; (F) same as (E) plus 2% oyster glycogen; (G) same as (E) plus 60 mM maltoheptaose. The inactivation buffer was 20 mM sodium tetraborate, 1 mM DTE, and 1 mM EDTA, pH 7.5, at 30 °C. Activities were measured at 24 °C in a standard assay medium containing AMP (Dreyfus et al., 1978).

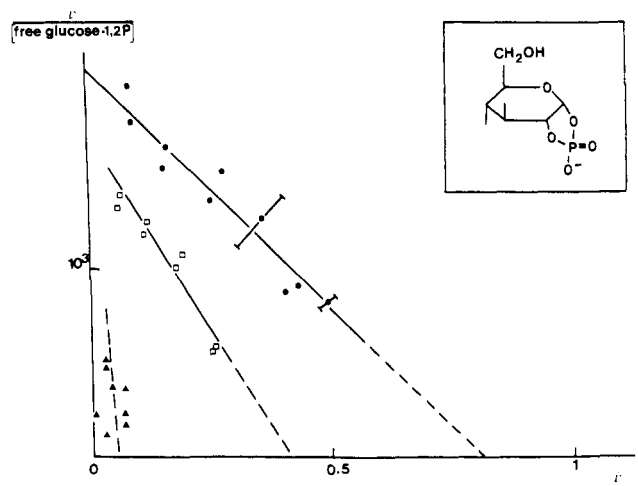

FIGURE 3: Scatchard representation of the binding of the substrate analogue glucose cyclic 1,2-phosphate to phosphorylase *a* (20–25 mg/mL) at 30 °C in 50 mM sodium tetraborate, 1 mM DTE, and 1 mM EDTA, pH 7.5. $\bar{\nu}$ was as in Figure 1b. (●) Native enzyme; (▲) enzyme sample isolated after modification by butanedione in the presence of 100 μM AMP (15% residual activity); (□) sample modified under the same conditions, except that glucose (50 mM) was present instead of AMP (55% residual activity).

modified. Moreover, no change in the aggregation state of the enzyme was apparent in ultracentrifugation studies. Thus, it is clear that the "inactivation" of phosphorylase *b* by 2,3-butanedione reported by Li et al. (1977) simply results from a loss of its nucleotide binding capacity and that neither the structure of the enzyme nor its intrinsic catalytic properties have been appreciably modified.

Reaction of Phosphorylase a with 2,3-Butanedione. In view of the above findings, it was first anticipated that phosphorylase *a*, which does not require AMP for catalytic activity, would not be affected by butanedione treatment. On the contrary, phosphorylase *a* was found to be rapidly inactivated when incubated with butanedione in the absence of other ligands. Equilibrium dialysis studies on the partially inactivated enzyme showed clearly that this activity loss was much more rapid than the modification of the AMP binding site and was therefore unrelated to it: thus, an enzyme sample showing only 28% of the initial activity (as measured in the absence of AMP) retained at least 80% of the AMP binding sites. Moreover, the inactivated enzyme did not recover any activity when AMP or alcohols were added to the assay medium. In order to interpret this inactivation process, we investigated how far the various phosphorylase ligands protect the enzyme when added to the modification buffer (Figure 2). AMP, which is known to increase the affinity of the enzyme for its substrates, also increases the inactivation rate. On the other hand, when the substrate glucose 1-phosphate was present in addition to AMP, the inactivation was slowed by a factor of 5, the concentration required for half-maximal protection ($\sim$1.5 mM) being close to the K_m reported for this substrate (Helmreich et al., 1967; Dreyfus et al., 1978). Inorganic phosphate also protected the enzyme, although less efficiently: the inactivation rate was reduced by a factor of $\sim$2 when 40 mM P_i was present. Glycogen had but a small protective effect by itself but acted synergistically with other substrates so that a virtually complete protection (>95%) was eventually obtained when all substrates were present together. Similar effects were observed with the glycogen analogue amyloheptaose (Figure 2).

The protection pattern offered by various ligands suggests that the inactivation of phosphorylase *a* is not due to the destruction of the nucleotide binding site but rather to the modification of an essential arginine residue possibly involved in the binding of phosphorylated substrates at the active site. In order to probe the integrity of this substrate site after modification, we undertook equilibrium binding studies with a structural analogue of glucose 1-phosphate, glucose cyclic 1,2-phosphate (Zmudzka & Shugar, 1964). Glucose 1-phosphate itself could not be used for binding studies because of its poor affinity for the enzyme (K_m in the millimolar range) and because of its slow polymerization by phosphorylase, even in the absence of primer (Illingworth et al., 1961). On the other hand, glucose cyclic 1,2-phosphate has been shown by Hu & Gold (1978) to be a potent competitor with glucose 1-phosphate on phosphorylase *a* ($K_I \simeq$ 300 and 500 μM in the absence and presence of AMP, respectively), and on phosphorylase *b* in the presence of AMP.

In our hands, the analogue was found to bind to phosphorylase *a* (but not to the inactive phosphorylase *b*) even in borate buffers. Binding was only slightly decreased by AMP but was completely abolished by an excess of glucose 1-phosphate. In the absence of AMP, it proved possible to obtain a correct binding isotherm despite the high dissociation constant ($K_D \simeq$ 500 μM) (Figure 3). The deviation from a stoichiometry of one molecule bound per monomer arises presumably from some inactivation of the enzyme during the lengthy dialysis process (1 day at 30 °C). After extensive modification with butanedione in the presence of AMP, this binding site was abolished (Figure 3), while it was largely protected when glucose 1-phosphate was present in addition to AMP (not shown). In fact, in all cases investigated, a close correlation between the residual activity after modification and the number of remaining Glc-1,2-P binding sites seems to exist (Figure 3).

Modification of Glycogen Phosphorylases a and b in the Presence of Allosteric Effectors. The above results suggest that the inactivation of phosphorylase *a* results from the modification of one (or several) arginine residue(s) involved in the binding of the substrates. This residue is not reactive in phosphorylase *b*, and this might reflect the different conformations of the catalytic site in the inactive and active conformations of the enzyme. To test this hypothesis further,

Table II: Effect of Allosteric Inhibitors on the Inactivation Rate of Phosphorylase *a* by 2,3-Butanedione

ligand added	half-inactn time[a] at saturation of ligand (min)	concn required for half-max protectn[b] (mM)
none	12	
caffeine	100	0.1
glucose	100	4

[a] The enzyme was incubated with 10 mM butanedione and, where indicated, specific ligands, in 20 mM sodium tetraborate and 1 mM EDTA, pH 7.5, at 30 °C and assayed for activity after dilution in a medium containing 0.5 mg/mL BSA, 10 mM Glc-1-P, and 0.1% oyster glycogen in 50 mM glycylglycine buffer, pH 6.8, at 24 °C. [b] Concentrations were varied from 0 to 70 mM (glucose) and 0 to 6 mM (caffeine).

we added various allosteric activators and inhibitors to the modification medium and tested their influence on the inactivation rate of phosphorylases *a* and *b*.

As stated above, the allosteric activator AMP increases the rate of the inactivation of phosphorylase *a* by butanedione (Figure 2). Conversely, the allosteric inhibitors glucose and caffeine very efficiently protect the enzyme (Table II). The concentrations required for half-maximal protection were 4 and 0.1 mM, respectively, for glucose and caffeine, in good agreement with the inhibition constants reported for these compounds (Fletterick & Madsen, 1980). Equilibrium dialysis studies showed that, when the enzyme was modified in the presence of glucose, the loss of AMP binding sites occurred as fast as, or faster than, the loss of enzymatic activity: an enzyme sample modified in the presence of 100 mM glucose and showing 66% of the native activity (in the absence of AMP) retained only 59% of the AMP binding sites. This situation contrasted with that observed with unliganded enzyme. Nevertheless, the slow inactivation observed at saturating glucose concentrations still correlated with the loss of the Glc-1,2-P binding site (Figure 3).

The effect of allosteric activators on the phosphorylase *b*–butanedione reaction is summarized in Table III. To avoid complications arising from partial destruction of the nucleotide site, we measured all the activities reported here in the presence of 10% *tert*-butyl alcohol. It is seen that, when activators are included in the modification buffer, the phosphorylase *b* activity can be abolished by butanedione. Moreover, there is a close parallelism between the inactivation rate and the affinity of the enzyme for its substrate, glucose 1-phosphate, measured under the same conditions, at saturating glycogen concentration. In particular, when both AMP (1 mM) and alcohol (10% *tert*-butyl alcohol) are present in the modification medium, the enzyme is inactivated very rapidly, like phosphorylase *a* (Table III); we have shown previously that, under these conditions, phosphorylase *b* is a Michaelian enzyme with high V_{max} and low K_m for substrates, like the *a* form (Dreyfus et al., 1978). The protection offered by the various ligands was also very similar: glucose 1-phosphate decreased the inactivation rate by a factor of 4–5, the concentration needed for half-maximal protection being ~5 mM. Inorganic phosphate was somewhat less effective in protecting the enzyme, and glycogen had no effect alone but, together with the other substrates, it offered virtually complete protection (>95%).

Phosphorylase b Can Be Irreversibly Trapped in an a-like Conformation after Modification with 2,3-Butanedione. After extensive modification with 2,3-butanedione in the presence of activators [usually 1 mM AMP and 10% (or 5%) *tert*-butyl alcohol], phosphorylase *b* is trapped in a conformation having many properties in common with native phosphorylase *a* (Table IV). Thus, at 20 °C and at 60000 rpm, the modified protein (3 mg/mL) sedimented as a poorly resolved dimer–tetramer mixture, while under the same conditions the native enzyme was essentially dimeric. The PLP fluorescence showed the low quantum yield characteristic of the *a* enzyme, and the resolution in imidazole–citrate–cysteine buffer was very much slower than that of the native enzyme (Figure 4), except for an initial drop of PLP content, possibly due to the resolution of the unmodified fraction of the enzyme. Phosphorylase *a* also is known to be resistant to resolution under the mild conditions employed here (Shaltiel et al., 1969).

An even more striking result was observed when the nucleotide binding properties of the modified enzyme were investigated (Figures 5 and 6). Not only was the AMP site fully protected from butanedione modification, as expected since

Table III: Effect of Allosteric Activators on the Inactivation Rate of Phosphorylase by 2,3-Butanedione[a]

enzyme form	ligand added	half-inactn time[a] (min)	K_m ($\bar{n}_{Hill}$) for Glc-1-P at saturating glycogen[b] (mM)
phosphorylase *b*	none	~450[c]	
	IMP (4 mM)	~270[c]	40 (2.0)
	tert-butyl alcohol (10%)	80[d]	2 (1.6)
	AMP (1 mM)	60	2 (1.4)
	AMP (1 mM) plus *tert*-butyl alcohol (5%)	22[d]	ND[e]
	AMP (1 mM) plus *tert*-butyl alcohol (10%)	10[d]	1.1 (1.0)
phosphorylase *a*	none	18	1.0 (1.0)
	0.5 mM AMP	12	0.8 (1.0)

[a] Conditions of modification and assay were the same as those in Table II, except that the assay medium contained 10% *tert*-butyl alcohol (phosphorylase *b*) or 1 mM AMP (phosphorylase *a*). [b] Kinetic response of the enzyme vs. Glc-1-P, in the presence of the activators listed in column 2, at 24 °C in 50 mM glycylglycine buffer, pH 6.8. [c] Extrapolated. [d] Controls showed no loss of activity when butanedione was omitted. [e] ND, not determined.

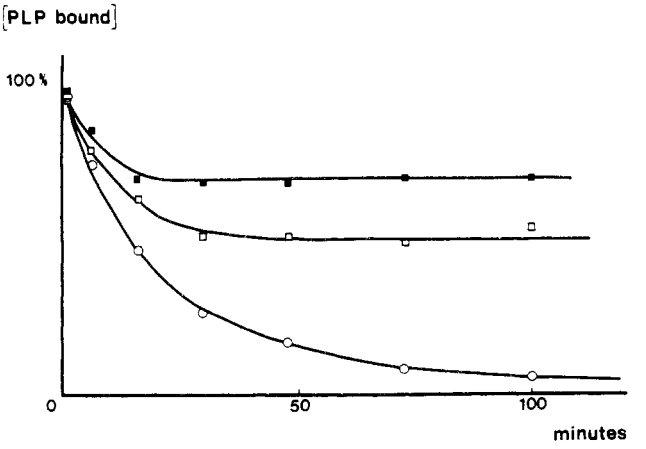

FIGURE 4: Time course of the resolution of phosphorylase *b* in 0.2 M imidazole citrate and 50 mM cysteine (pH 6.2 at 20 °C) at 0 °C. (○) Native enzyme; (■) enzyme isolated after modification by butanedione in the presence of AMP (1 mM) and *tert*-butyl alcohol (10%) (11% residual activity in a standard AMP medium); (□) idem, except that Glc-1-P (10 mM) was also present in the modification medium (42% residual activity).

Table IV: Comparison between Some Physicochemical Properties of Phosphorylase *a*, Phosphorylase *b*, and Modified Phosphorylase *b*[a]

	phosphorylase *b* modified in the presence of			
	1 mM AMP plus 10% *tert*-butyl alcohol	1 mM AMP plus 10 mM Glc-1-P	native phosphorylase *a*	native phosphorylase *b*
sedimentation coeff (3 mg/mL; 20 °C) (S)	8.7 (19) 12.5	NT[f]	13.6	8.7
PLP fluorescence quantum yield (30 °C; native phosphorylase *b* = 100) (%)	75 (16)	87 (65)	70	100
resolution of enzyme (% PLP released after 100 min)	30 (11)	50 (42)	NR[e]	95
K_D for AMP binding (high-affinity site; 20 °C) (μM)	18 (16)	40 (56)	5	75
residence time of ϵAMP (ms)	10 (11)	biphasic relaxation process[d] (42)	20	0.9
−AMP/+AMP act. ratio[b]	0.05 (16)	NT[f]	0.8	0.003
kinetic response with respect to Glc-1-P[c] (mM)				
K_m	0.9 (13)	1.5 (60)	0.8	2.0
n_{Hill}	1.0		1.0	1.4

[a] Procedures for the physicochemical measurements, as well as for the preparation and isolation of the modified enzymes, are described under Materials and Methods. In each case, the enzyme samples used in the first and second columns were prepared under exactly the same conditions, except for the presence or absence of Glc-1-P during modification. The figures in parentheses indicate the residual activity of the modified enzymes (in percent with respect to native) in standard media containing 1 mM AMP. [b] The assay medium contained 0.1% oyster glycogen, 0.5 mg/mL BSA, 10 mM Glc-1-P in 50 mM glycylglycine buffer, pH 6.8 (24 °C), and ±1 mM AMP. [c] AMP assay medium as in footnote *b*. Glc-1-P concentration was varied from 0.3 to 10 mM. [d] Visual inspection of the ϵAMP modified enzyme binding curves showed a biphasic process, with time constants in the 1- and 10-ms time range. This presumably reflects binding on the unmodified and modified enzyme subpopulations, respectively. [e] In our hands, phosphorylase *b* plus 1 mM AMP was also resistant to resolution under these conditions. NR, no resolution. [f] NT, not tested.

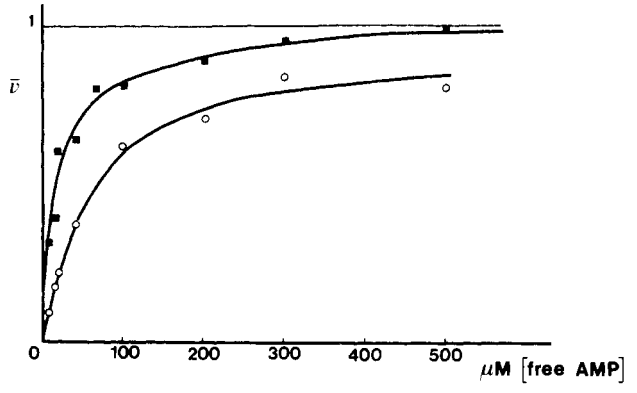

FIGURE 5: Fixation of AMP on phosphorylase *b* at 20 °C. (○) Native enzyme; (■) enzyme sample isolated after modification by 10 mM butanedione in the presence of 1 mM AMP and 10% *tert*-butyl alcohol at 30 °C (16% residual activity). $\bar{\nu}$ was as in Figure 1b. The buffer was 20 mM sodium tetraborate, 1 mM EDTA, and 1 mM DTE, pH 7.5, for both the modification and the equilibrium dialysis experiments.

AMP was present in the modification medium, but also the affinity of the modified enzyme for AMP was greatly *increased* (Figure 5). The increased affinity for nucleotides was also evident when we studied the fixation of the fluorescent AMP analogue, ϵAMP, on the native and modified enzyme, using a temperature-jump relaxation technique (Vandenbunder et al., 1978). We have shown previously that the fluorescent analogue has a much higher affinity for the *a* than for the *b* enzyme and that this affinity increase is due to a longer residence time of the bound nucleotide on the *a* form. As seen in Figure 6, the equilibrium dissociation constant for the enzyme–ϵAMP complex, obtained from fast kinetic measurements, is decreased by a factor of ~10 after extensive modification with butanedione. This increased affinity is essentially due to a much longer residence time of the nucleotide: indeed, this residence time now approaches the one observed for the *a* enzyme (Table IV).

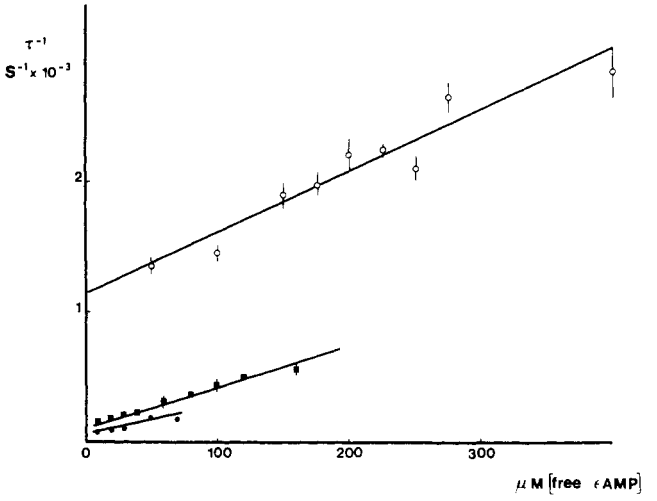

FIGURE 6: Plot showing the inverse of the relaxation time (τ^{-1}) characteristic of phosphorylase *b*–ϵAMP interaction vs. concentration of ϵAMP. (○) Native phosphorylase *b*; (●) native phosphorylase *a*; (■) phosphorylase *b* isolated after extensive modification by butanedione in the presence of 10% *tert*-butyl alcohol and 1 mM AMP (11% residual activity). Relaxation data were obtained with a temperature-jump spectrofluorometer (Vandenbunder et al., 1978) in 20 mM sodium tetraborate, 1 mM DTE, and 1 mM EDTA, pH 7.5, at 11 °C (final temperature). Provided the concentration of ϵAMP is much larger than that of phosphorylase (as in the experiments showed here), the inverse of the relaxation time for the simple bimolecular process [enzyme] + [ϵAMP] $\rightleftharpoons$ (k_{on}, k_{off}) [complex] is $\tau^{-1} = k_{on}$-[ϵAMP] + k_{off}. Therefore, the plot of τ^{-1} vs. [ϵAMP] should be linear, as observed, and the slope and intercept are k_{on} and k_{off}, respectively.

The kinetic properties of phosphorylase *b* after partial inactivation by butanedione were investigated next. As shown in Table IV, the modified enzyme resembles native phosphorylase *b* in being far less active in the absence of AMP than in its presence. On the other hand, at saturating AMP levels, the K_m for glucose 1-phosphate was much lower than that of the unmodified enzyme and similar to that of phosphorylase

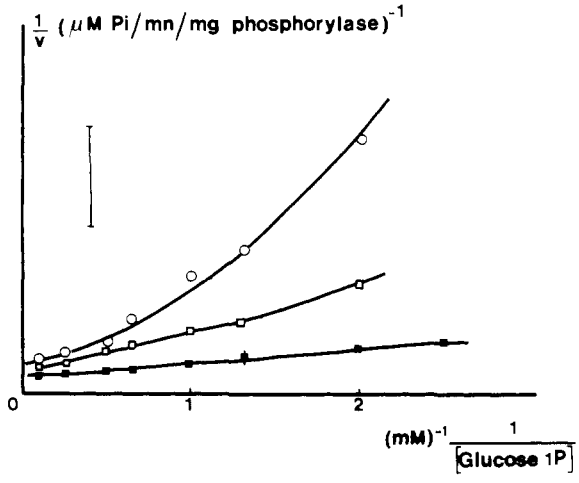

FIGURE 7: Double-reciprocal plots showing the kinetic response of phosphorylase *b* vs. Glc-1-P concentration in 50 mM glycylglycine buffer, pH 6.8, containing 1 mM AMP and 0.25% oyster glycogen, at 24 °C. (○) Native enzyme; (■) enzyme sample isolated after modification by 10 mM butanedione in the presence of 1 mM AMP and 10% *tert*-butyl alcohol; (□) idem, except that Glc-1-P (10 mM) was also present during modification. The vertical bar represents 0.1, 0.2, or 1.0 (μM P_i/min^{-1} mg^{-1})$^{-1}$ for curves (○), (□), and (■), respectively.

a (Figure 7 and Table IV). This lowered K_m eventually overcompensates for the decreased V_{max}; thus, when the activity was measured at low (0.5 mM) Glc-1-P concentration, a slight activity *increase* was observed at the beginning of the modification reaction.

Finally, we compared the properties of the enzyme modified in the presence or absence of glucose 1-phosphate, in addition to activators. In all cases, the changes in the physicochemical properties of the enzyme were less marked when the modification was performed in the presence of the substrate (cf. Figures 4 and 7 and Table IV). This strongly suggests that these changes, like the activity loss, are primarily due to the modification of the same arginine residue(s) located at the active site.

Discussion

We have provided evidence that phosphorylase *a* can be inactivated by 2,3-butanedione, through the modification of one (or one group of) essential arginine residue(s). The protection pattern offered by substrates, together with the finding that the inactivation of the enzyme parallels the loss of binding sites for the substrate analogue, glucose cyclic 1,2-phosphate, suggests that this residue is located at the active site. In a following paper, it will be shown that glucose 1-phosphate does in fact protect only one arginine residue with concomitant protection of the enzymatic activity; moreover, this residue will be chemically identified as Arg-568, which is indeed located in the vicinity of the active site. At this point we shall anticipate the result and assume that the rate of inactivation of the enzyme (or the rate of disappearance of the glucose cyclic 1,2-phosphate binding site) represents the rate of modification of this residue.

In the absence of effectors, phosphorylase *b* is not significantly inactivated by butanedione, and, therefore, this crucial residue is not reactive in the inactive conformation of the enzyme ("T" conformation in Monod's nomenclature). The apparent "inactivation" of phosphorylase *b* reported by Li et al. (1977) simply results from a loss of the AMP binding site, and the modified enzyme is still fully active when assayed in the presence of organic solvents. However, when allosteric activators are included in the modification medium, inactivation does occur. The effect is gradual: the more efficient the activator, as judged by the Michaelis constant for Glc-1-P and the associated Hill coefficient, under the same conditions, the higher the inactivation rate (Table III). When fully activated, phosphorylase *b* can be inactivated by butanedione as rapidly as phosphorylase *a*. Conversely, phosphorylase *a* can be efficiently protected from butanedione inactivation by the addition of the allosteric inhibitors caffeine and glucose. The concentration of these compounds required for half-maximal protection agrees with their inhibition constants. Since both compounds are known to inhibit the enzyme through stabilization of the same inactive (T) conformation (Withers et al., 1979), it is likely that the observed protection is also a consequence of this conformational shift. Thus, we conclude that the crucial arginine residue is unreactive in the T conformations of both forms of the enzyme but becomes highly reactive in their R forms. Clearly, it can be used as a local probe to follow the molecular rearrangement of the active site brought about by the activation or inhibition of the enzyme.

These simple conclusions were not reached by Li et al. (1977) in their first study of the phosphorylase–butanedione interaction. These authors reported mainly measurements of enzymatic activity during modification, and the ligand-binding properties of the modified enzyme were not investigated. Moreover, since the phosphorylase *b* activity was measured in the presence of AMP, it was impossible to decide, simply from observation of the loss of activity during modification, whether the active site, the AMP site, or both were modified. These features largely obscured the interpretation of the results obtained with phosphorylase *b*. On the other hand, phosphorylase *a* data were more readily interpretable, and the observed inactivation was attributed to a modification of the active site, as also suggested here. However, one of the major arguments supporting the conclusion of Li and co-workers was an X-ray analysis of the phosphorylase *a* crystals after butanedione treatment, which showed a modification of the "active site". Since the same group (Sygusch et al., 1977) later demonstrated that this site was actually the allosteric site and that the active site was located in a remote region of the molecule where no butanedione modification was apparent, this observation seems a posteriori to contradict solution studies. We believe that this discrepancy arises from the fact that the phosphorylase *a* crystals used in X-ray work are grown in the presence of glucose and thus contain an inhibited conformation of the enzyme. We have shown here that, in the presence of glucose, the active-site modification is comparatively slow. Indeed, Li and co-workers observed that their microcrystals were inactivated 20 times slower than the solution enzyme and that the most prominent effect of butanedione is the modification of the allosteric site, as was indeed observed here.

After extensive modification by butanedione in the presence of activators, followed by the removal of activators and excess reagents, phosphorylase *b* is irreversibly trapped in an *a*-like conformation. To reach this conclusion, we used several probes which should be primarily sensitive either to the conformation of the active site (the quantum yield of PLP fluorescence and the ease of removal of the coenzyme in deforming buffers) or to the conformation of remote regions of the molecule (the affinity for nucleotides and the tendency to tetramerize). When Glc-1-P is present and partially protects the enzyme from inactivation, the observed physicochemical changes are far less pronounced. This suggests that the shift toward the *a*-like character arises from the modification of the crucial

arginine residue. Conceivably, this residue, once modified, cannot be fitted back into the "inactive" conformation of the active site, and this is sufficient to lock the entire enzyme structure into the conformation prevailing during modification, even after the removal of activators.

Much evidence suggests that similar conformational changes can be induced in phosphorylase *b* either by phosphorylation of Ser-14 or by noncovalent binding of AMP, especially at low temperature, or in the presence of additional effectors such as anions high in the Hofmeister series, including P_i, or certain organic solvents (alcohols, etc.). This view is supported by studies of the conformation of the AMP site (Shimomura & Fukui, 1976; Vandenbunder et al., 1978; Dreyfus et al., 1978), on the environment of the PLP, probed by its ionization state (Feldmann & Hull, 1977) or ease of removal from the holoenzyme (Hedrick et al., 1969; Shaltiel et al., 1969), and on the aggregation state of the enzyme (Kastenschmidt et al., 1968; Sealock & Graves, 1967; Dreyfus et al., 1978). We have shown here that the modification of an arginine residue located at the active site, a locus distant from both known activator sites, can also induce similar conformational changes. We do not imply that phosphorylase adopts in each case strictly the same R conformation: for instance, the affinity of the butanedione-treated enzyme toward AMP, although greatly increased with respect to native phosphorylase *b*, is still lower than that of phosphorylase *a*. Despite these quantitative differences, there is no doubt that different local perturbations can induce qualitatively similar structural changes in remote regions of this large globular protein. This, of course, agrees with a basic postulate of the concerted model for allosteric transitions (Monod et al., 1965).

The residual activity of the partially inactivated phosphorylase *b*, measured in the presence of saturating AMP, is characterized by a low *a*-like K_m for glucose 1-phosphate. On the other hand, the modified enzyme differs from phosphorylase *a* in having a low activity in the complete absence of nucleotides. If we assume that the residual activity arises from hybrid dimers in which only one monomer has been modified at the active site, our results indicate that the modified subunit cannot force its unmodified partner to adopt its own "activated" conformation in the absence of AMP. A similar effect was noted by Battersby & Radda (1979), who observed that a subunit activated with [*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine, a covalent activator which binds to the AMP site (Anderson & Graves, 1973), could not induce AMP-independent activity in an unmodified subunit. On the other hand, in the presence of AMP, the K_m for Glc-1-P is markedly lowered with respect to native phosphorylase *b*; thus, under these circumstances, the modified monomer helps its unmodified counterpart to reach the most active conformation.

What is the structural role of the crucial arginine residue in the R conformation? Conceivably, it is involved in the binding of phosphorylated substrates at the active site, since the substrate analogue Glc-1,2-P does not bind to the modified enzyme. It must be stressed, however, that Glc-1-P is not able to fully protect this residue against butanedione modification, since some enzyme inactivation, loss of Glc-1,2-P binding site, or irreversible shift toward the *a*-like conformation are observed even in the presence of saturating Glc-1-P concentration. Full protection requires that glycogen (or its short chain analogue maltoheptaose) be present in addition to phosphorylated substrates. Several other cases are known in which full protection of an active-site arginine residue requires the simultaneous presence of all substrates [see, i.e., Kantrowitz & Lipscomb (1976), Pal & Colman (1976), and Borders et al. (1978)]. In this respect, yeast hexokinase PII (Borders et al., 1978) is especially similar to phosphorylase in that both the uncharged substrate glucose and the negatively charged MgATP are necessary to obtain significant protection, although the addition of substrates is random. In phosphorylase, the additional protection offered by glycogen may arise from a direct effect of the polysaccharide at the active site. Alternatively, it may be simply due to the binding of glycogen to its storage site, which is known to induce conformational changes in the active-site region (Kasvinsky et al., 1978a). Further studies on the protection offered by short-chain glycogen analogues are required to clarify this point.

At present, our most convincing argument for a participation of the crucial residue in the binding of phosphorylated substrates in the R state is still indirect. It lies in the remarkable parallelism which exists between the modification reaction and the binding of Glc-1-P at the active site in a way allowing catalytic activity. Thus, allosteric effectors affect the inactivation rate and the K_m for Glc-1-P in a similar way; conversely, the chemical modification of the active site and the binding of Glc-1-P both increase the affinity of phosphorylase *b* for nucleotides [see Kastenschmidt et al. (1968)]. This parallelism is most readily accounted for if the crucial residue is assumed to be involved in the binding of substrates in the R form. In this conformation, it would be freely accessible for butanedione modification as well as for substrate binding. Conversely, in the T state, it would be buried or shielded in some way: this would make it unreactive toward butanedione and inaccessible for proper binding of substrates, thus accounting for the lack of catalytic activity of this conformation. Deshielding of this residue would then be an essential feature of the activation process. Definite evidence for or against this simple interpretation will require a detailed X-ray analysis of the active-site region for both the inactive and active conformations of the enzyme.

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