Identical Structural Changes Induced in Glycogen Phosphorylase by Two Nonexclusive Allosteric Inhibitors†

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ABSTRACT: Recent kinetic studies on the regulation of glycogen phosphorylase by the synergistic inhibitors caffeine and glucose, in conjunction with X-ray crystallographic data, have led to the proposal of a molecular mechanism for this process in which the two effectors bind at separate sites. These effects have now been studied in solution at two different structural levels by other techniques. Ultracentrifugation studies of the quaternary structure of phosphorylase *a* showed the formation of dimers from tetramers on addition of these ligands independently and cooperative promotion of dimer formation when added together. The effect of the ligands on tertiary structure was investigated by studying the protection against iodoacetamide inactivation of enzyme activity afforded by these ligands. Phosphorus-31 (^{31}P) nuclear magnetic resonance studies of the catalytically essential pyridoxal phosphate residue of adenosine 5′-phosphate (AMP) activated phosphorylase *b* indicated a conversion from the active to the inactive form, possibly involving a protonation of the coenzyme phosphate, on addition of either of these ligands. This suggests a similar active-site environment in the two cases, identical with that observed in the absence of the activator, AMP. The effect of these ligands on the ^{31}P resonance of the analogous adenosine 5′-*O*-thiophosphate activator was also similar. Despite separate and distant binding sites for the two inhibitors, the effects on the gross structure of the enzyme at the quaternary and tertiary levels and their effect on a catalytically essential group are both identical and cooperative.

It is well-known that glucose acts as an allosteric inhibitor of both glycogen phosphorylase *a* and *b* (EC 2.4.1.1) (Wang et al., 1965; Wang & Black, 1968; Helmreich et al., 1967; Graves & Wang, 1972). The nature of this inhibition was clarified on the X-ray crystallographic identification of the binding site of glucose as the active site of the enzyme (Sygusch et al., 1977). Further crystallographic studies (Kasvinsky et al., 1978a) identified a negative effector site which binds purine nucleosides and bases in a crevice on the exterior of the molecule, 10 Å from the active site. This site is presumably the one originally suggested by calorimetric studies as a secondary binding site for AMP (Ho & Wang, 1973). It was later shown to bind adenine or adenosine more tightly (Morange et al., 1976). A combination of kinetic and crystallographic studies demonstrated that compounds binding at this site show competitive inhibition with respect to glucose 1-phosphate at the active site, yet by definition the inhibition is allosteric (Kasvinsky et al., 1978a,b). Studies with caffeine, a strong inhibitor, also showed a synergistic relationship with the inhibition by glucose binding at the active site. A similar kinetic effect could therefore be observed on binding of dissimilar ligands at distinct sites, as well as cooperative interactions between them. Similar results have been obtained with phosphorylase *b*.[1]

It therefore seemed of interest to investigate by solution techniques the nature of the molecular changes induced in the molecule by these ligands at both the tertiary and quaternary levels. Comparison of results obtained in this way with structural information obtained by X-ray crystallographic techniques should yield a greater insight into the molecular basis for this control mechanism. The present paper describes such a study using several different physical and chemical techniques.

An ultracentrifugal analysis was employed to investigate changes in quaternary structure. Some previous work of this kind has been carried out by using other ligands (Wang et al., 1965; Metzger et al., 1967). The differential inactivation of the enzyme upon iodoacetamide modification of sulfhydryl groups in the presence and absence of these ligands was used as an indicator of enzymic conformation. This approach has been used previously with phosphorylase to study changes induced by substrates and activators (Madsen et al., 1973, 1976). Information on the active site was obtained by observation of the phosphorus-31 nuclear magnetic resonance signal of the catalytically essential pyridoxal phosphate residue. Previous studies (Feldmann & Hull, 1977) have demonstrated the sensitivity of this signal to activation of the enzyme but not to its inhibition. Supporting evidence from other kinetic and X-ray crystallographic studies is also presented.[2]

Materials and Methods

Caffeine, iodoacetamide, and buffer chemicals were obtained from Sigma Chemical Co., except for DTT[3] which was obtained from Bio-Rad Laboratories. AMPS was obtained from Boehringer-Mannheim, glucose was from Fisher Chemical Co., and D_2O was from Bio-Rad Laboratories. A Radiometer PHM 62 pH meter was used for all pH measurements, and those measurements made in D_2O buffer are uncorrected.

Rabbit muscle phosphorylase *b* was prepared by the method of Fischer & Krebs (1962) using mercaptoethanol instead of

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[1] Kasvinsky et al. (unpublished experiments).

[2] After this paper was submitted, a report appeared by Hoerl et al. (1979) which showed that glucose has effects on the ^{31}P NMR spectra of pyridoxal 5′-deoxymethylenephosphonate reconstituted phosphorylase *a* which are similar to those we describe herein for phosphorylase *b*.

[3] Abbreviations used: DTT, dithiothreitol; AMPS, adenosine 5′-*O*-thiophosphate; NMR, nuclear magnetic resonance; Mops, 3-(*N*-morpholino)propanesulfonic acid; PLP, pyridoxal phosphate; Bes, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; glucose-1-P, α-D-glucopyranosyl 1-phosphate.

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cysteine and recrystallized at least 3 times before use. Phosphorylase *a* was prepared from phosphorylase *b* with phosphorylase kinase (EC 2.7.1.38) (Krebs et al., 1964). Protein concentration was determined from absorbance measurements at 280 nm, using the absorbance index, $E_{1cm}^{1\%}$, of 13.2 (Buc & Buc, 1968). Rabbit liver glycogen (type III) purchased from Sigma Chemical Co. was purified on a Dowex 1-Cl column and assayed by the method of Dishe (Ashwell, 1957). The concentration of glycogen is expressed as the molar equivalent of its glucose residues. Iodoacetamide was recrystallized according to Battel et al. (1968).

Ultracentrifugal experiments were performed on a Spinco Model E analytical ultracentrifuge at a rotor speed of 56 000 rpm and a temperature of 20 ± 1 °C. Sedimentation coefficients determined from Schlieren patterns were corrected for the viscosity and density of the buffer to water at 20 °C. Sedimentation experiments were carried out at pH 7.0 in 10 mM Bes, 1 mM EDTA, and 2 mM DTT. Protein concentration was 5 mg/mL.

Studies of the effects of caffeine and glucose on the rate of iodoacetamide inactivation of phosphorylase *a* were carried out by incubating enzyme (5 mg/mL) in 10 mM Bes buffer containing 0.15 mM mercaptoethanol and 10 mM EDTA (pH 6.8), 30 °C, with 10 mM recrystallized iodoacetamide in the presence or absence of caffeine or glucose. Samples were withdrawn at timed intervals, diluted with 20 mM glycerophosphate, 1.0 mM EDTA, and 20 mM mercaptoethanol (pH 6.8), and assayed. Initial reaction rates were determined by the Fiske–Subbarow phosphate analysis in the direction of saccharide synthesis, as described by Engers et al. (1970). Reaction mixtures were 0.5 mL and contained 2 mM sodium β-glycerophosphate (pH 6.8), 0.15 mM EDTA, 1 mM DTT, 28 mM glycogen, 75 mM glucose-1-P, and 2–4 μg of phosphorylase *a*.

^{31}P NMR spectra were recorded at 109.29 MHz on a Bruker HX270 superconducting spectrometer operating in the Fourier transform mode with quadrature phase detection at 28 °C. Exponential line broadening used prior to Fourier transformation was generally 20 Hz, and all line width data have been corrected for this. A spectral width of ±5000 Hz was generally employed, with a 50–70° pulse angle (15–20 μs) and a repetition time of 2.0 s.

Sample size was 1.5 mL in a 10-mm tube, with enzyme concentration around 1 mM in monomers. The buffer used in most of the NMR experiments was 50 mM triethanolamine hydrochloride, 100 mM KCl, 1 mM DTT, and 1 mM EDTA (pH 6.8 meter reading) made up in D_2O. Mops buffer, used in one NMR experiment, contained 100 mM 3-(*N*-morpholino)propanesulfonic acid, 50 mM mercaptoethanol, and 2 mM EDTA (pH 6.8) made up in D_2O. The D_2O present in the buffer was used for field/frequency lock, and a 1.0-mm tube containing 85% phosphoric acid was inserted for chemical shift referencing.

AMP was removed from phosphorylase *b* used in NMR experiments by two extended dialyses against 500 volumes of the triethanolamine buffer made up with H_2O containing 50 mM glucose, followed by dialysis against 50 volumes of this same buffer containing 50 mM glucose and charcoal (1 g) in suspension. Further dialysis against H_2O NMR buffer and finally against smaller volumes of D_2O NMR buffer removed glucose and introduced the D_2O. Enzyme prepared in this way gave no observable signal for AMP in the NMR spectrum. Solutions of effectors dissolved in D_2O NMR buffer at pH 6.8 were added directly to the NMR tube as required.

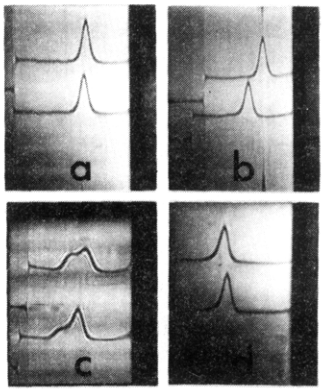

FIGURE 1: Effect of inhibitory ligands on the sedimentation properties of phosphorylase *a*. (a) (top) 1 mM AMP, 13.3 S; (bottom) control, 13.5 S; (b) (top) 0.05 mM caffeine, 12.7 S; (bottom) 5 mM glucose, 12.6 S; (c) (top) 10 mM glucose + 0.05 mM caffeine, 8.9 and 13.1 S; (bottom) 5 mM glucose + 0.05 mM caffeine, 8.9 and 12.5 S; (d) (top) 2 mM caffeine, 8.7 S; (bottom) 50 mM glucose, 8.5 S. Pictures were taken 24 min after attaining full speed. Conditions were as explained under Materials and Methods.

A BASIC program written for a NOVA 1220 computer was used to perform a complete line shape analysis of the binding of AMPS. This program calculates the complete line shape according to the modified Bloch equations [see, for example, Pople et al. (1959)], assuming a two-site exchange situation. The observed line shape is influenced by the natural line width, as measured in the absence of exchange, and a line broadening contribution from exchange of this species between two sites, i.e., free and bound AMPS. Introduction of estimated values for the natural line widths $\Delta\nu^\circ$ and chemical shifts δ of the free and bound AMPS plus estimated values for the off-rate k_{-1} and dissociation constant K_D for AMPS binding allowed simulation of a theoretical spectrum which could be fitted to the observed spectrum by adjustment of the parameters entered. The use of this approach requires the assumption of direct proportionality between peak area and concentration of the respective species. The fast exchange condition for the spin–lattice relaxation rates, $\tau/T_{1B} \ll 1$, where τ is the exchange lifetime and T_{1B} is the spin–lattice relaxation time for bound AMPS (Hull et al., 1976), must therefore apply. This is satisfied in these cases since the maximum value of τ observed is 0.01 s and T_1 values are of the order of 1 s.

Results

Ultracentrifugation Studies. Figure 1 shows the ultracentrifugation patterns observed for phosphorylase *a* in the presence and absence of various ligands. As has been shown previously (Wang et al., 1965), under these conditions phosphorylase *a* sediments as a tetramer of sedimentation constant 13 S in the absence of effectors or in the presence of AMP (Figure 1a). Addition of glucose or caffeine to the enzyme promotes dimer formation, sedimentation constant 8.7 S. This is seen in Figure 1b, where 5 mM glucose and 0.05 mM caffeine have been added independently to the enzyme. The concentrations of ligand utilized are not sufficient to saturate the enzyme, and this is reflected in the conversion of only a small percentage to the dimeric form. Addition of the two ligands together at these concentrations results in cooperative formation of dimer, as is clearly shown in Figure 1c. Here a greater percentage of conversion to dimer is observed than would be expected on a simple additive basis.

Glucose alone at high concentrations (50 mM) can cause complete conversion to dimers, as also can caffeine at a concentration of 2 mM (Figure 1d). A similar result with caffeine has been described elsewhere (Bot et al., 1977).

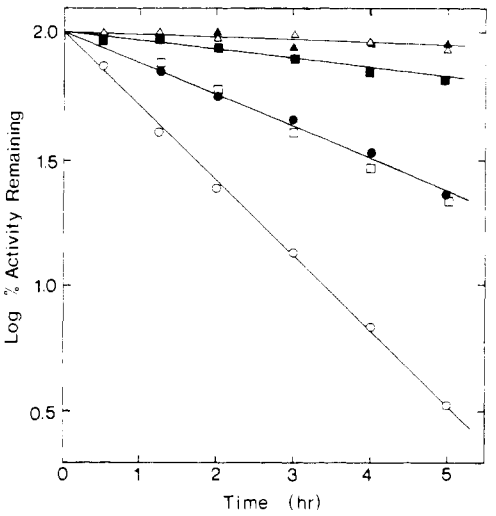

FIGURE 2: Effect of inhibitory ligands on the iodoacetamide inactivation of phosphorylase *a*; apparent second-order inactivation rate constants are shown in parentheses. (○) Control of iodoacetamide alone (1.16 M^{-1} min^{-1}); (□) 0.2 mM caffeine (0.46 M^{-1} min^{-1}); (●) 5 mM glucose (0.48 M^{-1} min^{-1}); (■) 0.2 mM caffeine + 5 mM glucose (0.15 M^{-1} min^{-1}); (▲) 50 mM glucose (0.07 M^{-1} min^{-1}); (△) 2 mM caffeine (0.07 M^{-1} min^{-1}). Conditions were as described under Materials and Methods.

Chemical Modifications. The results of the iodoacetamide inactivation of phosphorylase *a* in the presence of various ligands are shown in Figure 2. Both glucose and caffeine protect against this inactivation and at the concentrations employed offer a similar degree of protection. When present together, however, the degree of protection offered is higher than would be expected in the absence of synergism. Cooperativity between these two sites is therefore exhibited. This is seen more clearly in the rates of inactivation given in the legend to Figure 2. Addition of glucose or caffeine alone at saturating concentrations affords a similar degree of protection in either case.

^{31}P NMR Studies. The ^{31}P NMR spectra for phosphorylase *b* in the presence of various ligands are shown in Figure 3. The spectrum for enzyme alone (Figure 3a) shows a single resonance of line width 80 Hz and chemical shift of 0.6 ppm (relative to H_3PO_4). This is the resonance from the phosphate group of the covalently bound active-site PLP residue. The same chemical shift and a slightly narrower line width [70 Hz, using a lower spectrometer frequency (72.8 vs. 109.29 MHz) and in the presence of ^1H decoupling] have been observed by other workers (Feldmann & Hull, 1977) and designated by them as form I. The small peak to the left of the major resonance at 2.0 ppm has been assigned to inorganic phosphate.

The nucleotide-activated form of phosphorylase *b* was investigated in the presence of adenosine 5′-*O*-thiophosphate (AMPS). This AMP analogue is used (Feldmann & Hull, 1977) because it resonates in a remote part of the spectrum without obscuring the signal for PLP. On addition of AMPS to the sample (Figure 3b), the major resonance for the PLP shifts downfield to a new position at 3.8 ppm with a line width of ~160 Hz, designated as form III, leaving a small percentage of the form I resonance. The binding of AMPS to the enzyme is in the NMR slow-exchange limit under these conditions since two distinct resonances are observed corresponding to the free and bound cofactor. The signal for bound AMPS, δ = 40.8 ppm, has an observed line width of ~100 Hz, whereas that for free AMPS, δ = 43.9 ppm, has an observed line width of ~40 Hz. The line shapes observed for the signals corresponding to the AMPS activator in all experiments were subjected to a complete line shape analysis (see Materials and Methods) to determine values for the off-rate constant (k_{-1}) and the dissociation constant for AMPS and the line widths of the free and bound AMPS in the absence of exchange. These rate and equilibrium constants are given in Table I, and the natural line width, i.e., the line width in the absence of exchange, calculated for bound AMPS was 80 Hz. This is equal to the line width of 80 Hz observed for form I of the PLP resonance. The nucleotide off-rate constant appears to be highly dependent on the particular buffer utilized. This has been observed kinetically (Helmreich et al., 1967) and is now seen in these NMR experiments. Redetermination of the spectrum for enzyme plus AMPS in Mops buffer (Figure 3c) shows that somewhat more intermediate exchange conditions

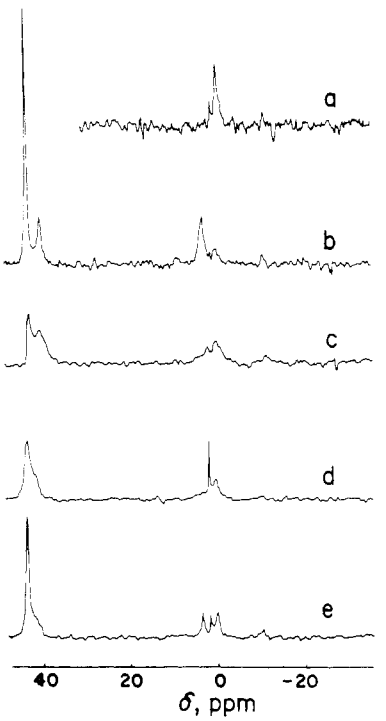

FIGURE 3: ^{31}P NMR spectra of phosphorylase *b* in the presence of various ligands. (a) Phosphorylase *b*, 0.46 mM monomers, 27 687 acquisitions; (b) phosphorylase *b*, 0.85 mM monomers plus 2.43 mM AMPS, 25 233 acquisitions; (c) phosphorylase *b*, 1.03 mM monomers plus 1.83 mM AMPS in Mops buffer, 30 671 acquisitions; (d) phosphorylase *b*, 0.88 mM monomers plus 1.78 mM AMPS plus 50.33 mM glucose, 41 335 acquisitions; (e) phosphorylase *b*, 0.70 mM monomers plus 2.11 mM AMPS plus 0.96 mM caffeine, 33 862 acquisitions. Conditions were as described under Materials and Methods.

Table I: Off Rates and Dissociation Constants for AMPS Binding Calculated from NMR Data[a]

sample	off-rate k_{-1} (s^{-1})	dissocn constant K_D (mM)
phosphorylase *b* + AMPS	200	0.05
phosphorylase *b* + AMPS in Mops buffer	350	0.06
phosphorylase *b* + AMPS + glucose (50 mM)	800	0.3
phosphorylase *b* + AMPS + caffeine (1 mM)	800	0.6
phosphorylase *b* + AMPS (1.26 mM) + glucose (50 mM) + caffeine (0.92 mM)	1000	0.3
phosphorylase *b* + AMPS (1.16 mM) + glucose (50 mM) + arsenate (34 mM)	1000	0.5

[a] Concentrations of enzyme and AMPS were as described in Figure 3.

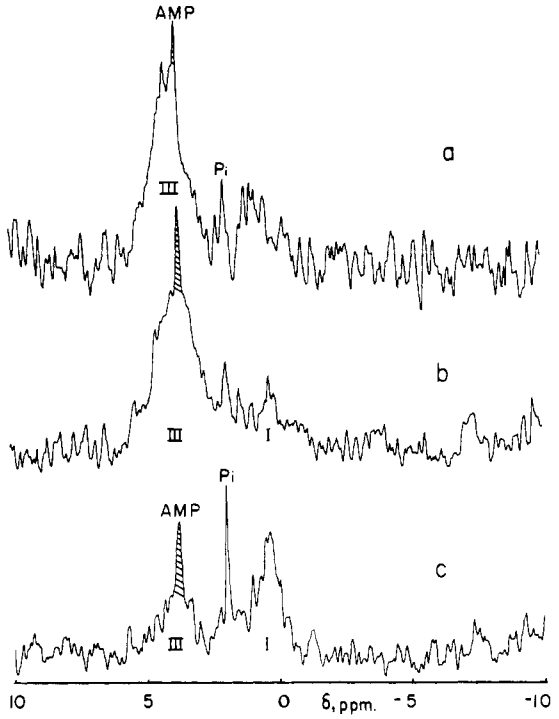

FIGURE 4: Expansion (+10 to −10 ppm) of the PLP region of the ^{31}P NMR spectrum of phosphorylase *b*, using exponential line broadening of 10 Hz. (a) Some conditions as in Figure 3b, after exhaustive dialysis; (b) phosphorylase *b*, 1.07 mM monomers plus 1.34 mM AMPS, 28 362 acquisitions, before exhaustive dialysis; (c) same conditions as in Figure 3e. The cross-hatched portion of each spectrum represents the unliganded AMP.

now apply to the AMPS region of the spectrum (see Table I) and that the predominant PLP resonance is form I.

On addition of glucose (50 mM) to the nucleotide-activated enzyme in the triethanolamine hydrochloride buffer (Figure 3d), two effects are observed. Firstly, the PLP resonates primarily as form I, leaving a little of form III, and, secondly, the signals for free and bound AMPS collapse into a single exchange-averaged signal. The latter effect indicates that the off-rate constant for the activator has increased (see Table I) and that intermediate exchange conditions now apply to this region of the spectrum. The sharp resonance observed at 2.0 ppm is due to contaminating inorganic phosphate which is released by the enzyme upon simultaneous addition of AMPS and glucose. A kinetic determination of the binding constant for AMP in this buffer in the presence of 12 mM glucose-1-P and 1% glycogen gave a value of $K_D(AMP) = 2.3 \times 10^{-5}$ M; addition of 50 mM glucose to the assay mix increased this binding constant to 2.6×10^{-4} M. Therefore, a 10-fold increase in K_D is observed.

In the presence of 1 mM caffeine (Figure 3e), a very similar spectrum is observed. The resonances for free and bound AMPS have collapsed into an exchange-averaged signal, and form I of the PLP is predominant. Some form III is still present due to nonsaturating concentrations of caffeine, and a small amount of AMP is seen on top of the form III peak. This is seen more clearly in Figure 4c, which is an expansion of the PLP region of the same spectrum using a smaller exponential line broadening of 10 Hz. The free AMP is distinguished by its narrow relative line width, as is the free inorganic phosphate seen at a chemical shift of 2.0 ppm. This contaminating AMP was occasionally observed in the first experiments and was subsequently consistently removed by the exhaustive dialysis treatment described under Materials and Methods. Figure 4b shows the PLP region of a typical spectrum of AMPS-activated phosphorylase *b* prepared by the less rigorous dialysis procedure. Here the concentration of contaminating AMP represents ~5% of the enzyme concentration. After the exhaustive dialysis described, spectra such as Figure 4a were obtained, showing only 1% contaminating AMP.

Therefore, both ligands have apparently induced the same changes in the enzyme, namely, a weakening of the binding of the nucleotide activator and conversion of the active form III of the PLP resonance to the inactive form I. Addition of these two ligands together at these near-saturating concentrations caused no significant further change in the spectrum (not shown), and this is reflected in the values shown in Table I.

The K_i measured for phosphorylase *b* in the presence of 0.3 mM AMP was similar to that previously measured for phosphorylase *a* in the absence of AMP, namely, 0.1 mM caffeine (Kasvinsky et al., 1978a). Therefore, it was not feasible to demonstrate a cooperative interaction of glucose with caffeine because of the high concentration of enzyme used, around 1 mM. At equimolar caffeine, more than 70% of ligand and enzyme are combined; a glucose-enhanced binding would therefore be difficult to observe. Neither was it possible to reverse the glucose inhibition on addition of 34 mM arsenate, a substrate for the enzyme. This is clearly seen in Table I. Addition of glucose or caffeine alone to the non-nucleotide-activated enzyme had no effect on the PLP resonance, as expected, giving an identical spectrum with that of the unliganded enzyme (spectrum not shown).

Discussion

The recent discovery of a new series of high-affinity inhibitors of phosphorylase action and the X-ray crystallographic identification of a distinct binding site noncoincident with existing effector sites have opened exciting new possibilities for a further refinement of the control mechanisms exhibited by phosphorylase (Kasvinsky et al., 1978a,b). The results described herein, together with previous data, help to delineate a possible mechanism for this control process by investigation of structural changes induced by these ligands and the allosteric effector glucose. The observation of noncoincident binding of two competitive inhibitors also serves to highlight the dangers of overinterpretation of such kinetic data.

The results obtained by ultracentrifugal analysis of the enzyme in the presence of glucose or caffeine indicate that they promote identical changes in the quaternary structure of the enzyme. Previous ultracentrifugal work (Wang et al., 1965; Metzger et al., 1967) had shown that phosphorylase *a* exists as a tetramer of sedimentation constant 13 S in the absence of ligands but that maltoheptose and glucose both promote dissociation to dimers of sedimentation constant 8.9 S. Addition of AMP or glucose-1-P to the enzyme appeared to stabilize the tetrameric form of the enzyme (Wang et al., 1965; Helmreich et al., 1967). Phosphorylase *b* exists as a dimer in the absence of ligands but associates to a tetramer on binding AMP (Wang et al., 1970; Kastenschmidt et al., 1968), and this association could be enhanced by divalent metal ions, anions, and substrates (Graves & Wang, 1972). Glucose, however, prevents this nucleotide-induced association. Interestingly, concentrations of AMP up to 1 mM were found to promote tetramer formation, but higher concentrations caused dissociation to dimers (Wang et al., 1970). This was interpreted in terms of a second site for AMP binding and can now be rationalized on the basis of binding a second AMP molecule at the nucleoside site (Kasvinsky et al., 1978b).

The results presented here are in agreement with these

findings and further demonstrate that caffeine induces dissociation of phosphorylase *a* to dimers in the same manner as glucose. At saturating concentrations of either 50 mM glucose or 2 mM caffeine a complete conversion to dimers is observed (Figure 1). At low ligand concentration, however, each ligand alone is incapable of shifting the dimer–tetramer equilibrium toward dimer. Only in the presence of both ligands is significant dimer formation observed. The extent of dimer formation observed for the concentrations of glucose and caffeine utilized is less than would have been expected on the basis of kinetic experiments since 5 mM glucose (K_D = 3 mM) would have been expected to cause at least 50% dimerization, yet only a small amount (less than 5%) of dimer is observed. It should be recalled, however, that the quaternary interactions between dimers of phosphorylase *a* are highly dependent upon protein concentration and temperature (Wang & Graves, 1964), with tetramer favored at higher concentrations and lower temperatures. Thus, it is not surprising that the ultracentrifugation experiments, which contain 1000 times the protein concentration used in kinetic studies and are performed at lower temperatures, would contain less dimers in the presence of similar ligand concentrations. In fact, calculations based on published dissociation constants (Metzger et al., 1967; Huang & Graves, 1970) predict this low amount of dimer formation under these conditions in the presence of 5 mM glucose.

The chemical reactivity of the sulfhydryl groups in phosphorylase has been used previously (Madsen et al., 1976) as an indicator of the conformational states induced in the enzyme by various ligands. By choosing conditions under which the inactivation reaction proceeds at a measurable rate and then measuring the inactivation rate in the presence of various ligands, it was possible to draw some conclusions about the conformation changes induced by these ligands on the basis of protection or activation afforded. In this way it was shown that the iodoacetamide or *p*-hydroxymercuribenzoic acid inactivation of phosphorylase *b* was protected against by AMP, yet enhanced by glucose-1-P (Madsen et al., 1973). It was therefore concluded that the enzymic conformations induced by these two ligands were not identical. Similar studies showed that inactivation of phosphorylase *a* by iodoacetamide was the result of modification of cysteine residues 108 and 142. Strong protection against this inactivation was exhibited by glucose (Madsen et al., 1973; Wang & Black, 1968) and all substrates and activators tested.

In the presence of either glucose or caffeine at concentrations roughly equivalent to their K_i values, a fairly large degree of protection was observed (Figure 2), as much as would be expected from their kinetically determined binding constants. This is in contrast to the case observed in the ultracentrifugation studies, where higher apparent dissociation constants would appear to prevail. This is presumably a direct consequence of the lower temperatures used in the ultracentrifugation studies. At saturating concentrations of either of these inhibitors a similar degree of protection is observed. This is strong evidence for the postulate that similar conformations are induced by the two ligands. When the two ligands are added together, cooperative protection against inhibition is observed. This is best seen in the inactivation rates (Figure 2), where it is apparent that the two ligands together lower the rate dramatically. Similar protection against inactivation has also been observed in a study of the effects of substrates or AMP on the iodoacetamide inactivation of phosphorylase *a*.

It has been proposed previously (Helmreich et al., 1967) that glucose stabilizes an inactive T form of the enzyme and that substrates stabilize the active R conformation. Since alkylation of cysteine residue 142 is known to be a major contributing factor to iodoacetamide inactivation, protection against inactivation presumably involves protection of this residue. Access to cysteine-142 is via the central cavity of the dimer and may require breathing of the molecule to permit entry of reagents into this cavity (Fletterick et al., 1979). Any ligands which stabilize either the inhibited T conformation on the one hand (glucose and caffeine) or the active R conformation on the other hand (AMP and glucose-1-P) would be expected to decrease the flexing of the dimer which is necessary for access to cysteine-142.

In a recent ^{31}P NMR study (Feldman & Hull, 1977), it was proposed that the phosphate moiety of the pyridoxal phosphate cofactor covalently bound at the active site of phosphorylase could exist in three different forms, forms I, II, and III, in the presence of various effectors. In the absence of effectors, only form I, line width 70 Hz, was observed. On addition of the allosteric effector AMPS, a partial conversion to form II, line width 30 Hz, was noted, while addition of arsenate to this sample then caused almost complete conversion to form III, line width 70 Hz. Addition of arsenate also caused a tightening of the binding of the effector AMPS. In the absence of arsenate, the free AMPS is in intermediate exchange with bound AMPS and a near coalescence of the two signals was observed. Addition of arsenate sufficiently tightens the effector binding so that slow-exchange conditions now apply, and the two separate signals for free and bound AMPS are observed. The change in the PLP resonance from inactive form I to active form III upon allosteric activation was interpreted in terms of a deprotonation of the phosphate group.

The results of our experiments are in good agreement with these findings. Addition of AMPS in the absence of arsenate, however, gave a much more complete conversion to form III, and this was also reflected in tighter AMPS binding (K_D = 0.05 mM), resulting in slow-exchange conditions. This experiment was performed in a different buffer system from that used by Feldmann & Hull (1977). Redetermination of this spectrum in their Mops buffer showed a predominance of form I of the PLP resonance and an increased off-rate constant for AMPS (k_{-1}), in agreement with their findings. This difference in behavior in different buffers is unsurprising, as it has been noted previously (Helmreich et al., 1967) in kinetic studies of the binding of activators. A possible explanation could be nonproductive competitive binding of the *N*-morpholinopropanesulfonic acid in the nucleotide binding site or even binding of this same compound in the nucleoside site, producing effects similar to those observed with caffeine.

The species previously reported as form II of the PLP resonance was occasionally observed in these studies. We, however, have assigned this as free AMP rather than as a form of the PLP resonance. This assignment is based on the fact that the chemical shift is identical with that of AMP at this pH and on the basis of its ionization characteristics. The similarity between the pH dependence of the chemical shift of form II determined previously (Feldmann & Hull, 1977) and the pH dependence of the chemical shift of AMP (Jaffe & Cohn, 1978) is strong evidence for their identity. The small line width observed for this species (30 Hz) is also incompatible with that expected for a phosphate group covalently bound to a protein of this size. In our hands this resonance could be removed by the exhaustive dialysis procedures described under Materials and Methods.

Addition of glucose or caffeine to the nucleotide-activated enzyme caused two major changes in the spectrum: firstly,

a reversion of the PLP resonance to predominantly form I, and, secondly, a loosening of the binding of AMPS, resulting in near coalescence of the free and bound AMPS resonances. Inspection of the AMPS region of the spectrum in the two cases illustrates the similarity in effects induced by these two ligands, and this is further reinforced on inspection of the data generated through the complete line shape analysis. An increase in the off rate (k_{-1}) of AMPS binding from 200 to 800 s^{-1} is observed in each case, along with an increase in the dissociation constant. This latter parameter can only be approximated rather crudely but is in broad agreement with published values for this dissociation constant. A 10-fold increase in the dissociation constant for AMP in this buffer system on addition of 50 mM glucose was observed by kinetic measurements as described earlier, in agreement with the NMR measurements. The reversion of the PLP resonance from form III to form I, possibly a protonation, on addition of glucose or caffeine indicates that the two ligands are inducing similar conformation changes in the molecule, producing similar environments for the PLP residue in either case. Similar changes in the ^{31}P NMR spectrum were described recently (Hoerl et al., 1979) on addition of glucose to pyridoxal 5'-deoxymethylenephosphonate reconstituted phosphorylase *a*, complementing this work with phosphorylase *b*. Failure of the addition of high concentrations of caffeine to glucose-saturated enzyme to cause any further weakening of nucleotide binding or any further change in the PLP resonance is indicative of the similarity of conformations adopted in each case.

The addition of arsenate, a competitive inhibitor of phosphate binding and itself a substrate, to the glucose-inhibited enzyme could not reverse the spectral changes brought about by the binding of glucose (spectrum not shown). This is seen clearly in the line shape analysis data and is consistent with previous kinetic data (Helmreich et al., 1967) which had shown the inability of phosphate to reverse glucose inhibition of the enzyme.

Inhibition of enzymic activity by glucose or caffeine has therefore been shown to be accompanied by a conformation change leading to weakening of the nucleotide binding. This implies that these two inhibitors induce a conformational state similar to that adopted in the absence of AMP, and the NMR and ultracentrifuge experiments provide strong evidence for this. A structural rationalization for this mechanism has been proposed (Kasvinsky et al., 1978a,b), based on the 3-Å resolution X-ray structure of muscle phosphorylase *a*, as outlined below. One side of the binding pocket for caffeine, 10 Å away from the active site on the exterior of the monomer, is formed in part from a loop of 56 amino acids (residues 242–297) which shifts position during allosteric transitions. In order for glucose-1-P to bind to the enzyme, this loop has to move 5 Å away from its position in the presence of glucose alone. Binding of nucleosides or derivatives such as caffeine prevents the movement of this loop since they lock it into its glucose-inhibited position. This would account directly for the competitive inhibition of glucose-1-P binding and the similarity of the conformations induced in either case. The synergism exhibited between the two ligands can be explained on the basis of each ligand helping to cement the conformation induced by the other. This loop also extends as a chain which projects up into a tower of polypeptide interacting with the other protein subunit and then returns to the region of the AMP binding site. Movement of this chain is seen on binding glucose-1-P. This type of direct interaction between the monomeric subunit interface, the nucleoside binding site, and the AMP binding site could well account for the observed changes in quaternary organization and AMP binding constant on binding of these effectors. In conclusion, crystallographic studies strongly suggest that glucose and caffeine stabilize the same conformation, since either singly or together these ligands bind to the tetragonal crystals without inducing any conformational changes while together they stabilize the crystal structure against radiation damage (Sprang & Fletterick, 1979). The results in this paper suggest strongly that the same relationship obtains for the enzyme in solution.

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Phospholipase D from Savoy Cabbage: Purification and Preliminary Kinetic Characterization†

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ABSTRACT: Phospholipase D has been purified 680-fold from an acetone powder of savoy cabbage in an overall yield of 30%. The purification involves solubilization of the acetone powder in a Ca^{2+}-containing buffer and subsequent ammonium sulfate fractionation. Gel filtration on Sephadex G-200 and hydrophobic affinity chromatography using a γ-aminopropane–agarose gel complete the purification. The two chromatographic steps were conducted in buffers containing 50% ethylene glycol, which was necessary in order to maintain stability of the enzyme. Purity was established on the basis of gel electrophoresis and ultracentrifugation. A preliminary kinetic characterization of the enzyme was carried out by using lecithins with short-chain fatty acids below the critical micelle concentration. A complex series of results were obtained which demonstrated the following. (1) The enzyme is quite sensitive to ionic strength, being inhibited at high ionic strength. (2) The pH optimum depends on the concentration of Ca^{2+} used in the assay. At 0.5 mM Ca^{2+} the pH optimum is 7.25, but it is 6.0 at 50 mM Ca^{2+}. (3) The effect of substrate concentration at a given pH and ionic strength did not show simple hyperbolic kinetics but rather regions of parabolic and hyperbolic kinetics.

Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) catalyzes the hydrolysis of the ester linkage between the phosphatidic acid and alcohol moieties of phospholipids; additionally, this enzyme may catalyze a transferase reaction by which the phosphatidic acid moiety of the phospholipid substrate is transferred to an acceptor alcohol. This transphosphatidylation may be considered the general reaction, with hydrolysis repesenting a specific case in which the acceptor alcohol is water.

A partial purification of the cabbage enzyme was reported by Davidson & Long (1958) and Dawson & Hemington (1967). Yang et al. (1967) extended the original procedure of Davidson & Long to achieve a 110-fold purification with 20% recovery of activity.

Dawson & Hemington (1967) have reported several characteristics of the hydrolytic activity of cabbage phospholipase D by using egg lecithin dispersions. Ca^{2+} was found to be essential for hydrolysis with an optimum concentration of ~40 mM. The reaction had a sharp pH optimum at pH 5.4. Hydrolysis was stimulated by diethyl ether and by anionic amphipathic substances. Quarles & Dawson (1969), also utilizing egg lecithin as substrate, found that the pH optimum of the reaction was shifted with the addition of anionic amphipathic activators. Long et al. (1967) found that the action of phospholipase D upon lysolecithin required the presence of Ca^{2+}, with an optimum around 25 mM. A broad pH optimum at about pH 5.8 was observed.

The first observations of transferase activity by phospholipase D were reported by Yang et al. (1967) and Dawson & Hemington (1967). A variety of water-soluble primary alcohols were active as acceptors for the phosphatidyl group transfer. When both acceptor alcohol and water were present, the transphosphatidylation was usually the preferred reaction [a recent review has appeared by Heller (1978)].

Materials and Methods

Materials. Phospholipase D (grade B), lyophilized, salt-free, and free of choline-destroying activity, was purchased from Calbiochem (San Diego, CA). It was isolated, according to the manufacturer, from cabbage by the heat coagulation and acetone precipitation procedures of Yang et al. (1969). Different lots gave essentially identical results. Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], buffer grade, was from Pierce Chemicals (Rockford, IL). Ethylene glycol, analytical grade, was from Mallinckrodt (St. Louis, MO). 1,3-Diaminopropane was from Aldrich Chemical Co. (Milwaukee, WI). Sephadex G-200 was from Pharmacia Fine Chemicals (Piscataway, NJ). Agarose Bio-Gel A-5m was from Bio-Rad Laboratories (Richmond, CA). Acrylamide and *N*,*N*′-methylenebis(acrylamide) were recrystallized from chloroform and acetone, respectively. Dialysis tubing was prepared by boiling in a 50% ethanol solution and then a 1 mM EDTA solution followed by rinsing in distilled water. Short-chain lecithins were prepared as described by Wells (1972) and Yabusaki (1975). 1,2-Dihexanoylphosphatidic acid was prepared from *sn*-3-glycerobromohydrin (Bird & Chadha, 1966) and dibenzyl phosphate by the method of Hessel et al. (1954). It was isolated as the barium salt and gave analyses of P, 6.19%, and Ba, 26.7% (theoretical: P, 6.15%; Ba, 27.3%), $[\alpha]^{25}_{546} = +9.3°$ (*c* 8.5, chloroform; as free acid). Distilled water was further cleansed by passage through an organic filter and an inorganic ion-exchange resin from Continental Deionized Water Service (Tucson, AZ). This highly purified water

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