Active Form of Pyridoxal Phosphate in Glycogen Phosphorylase. Phosphorus-31 Nuclear Magnetic Resonance Investigation†

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ABSTRACT: The substrate analogue α-D-glucopyranosyl cyclic 1,2-phosphate has been confirmed to be a good competitive inhibitor of glycogen phosphorylases *a* and *b* isolated from rabbit muscle. Effects on tertiary and quaternary structure of the enzyme have been shown to be similar to those induced by the substrate glucose 1-phosphate and different from those of the coincidently binding inhibitor glucose. This information was obtained from study of the ultracentrifugation patterns of the enzyme–inhibitor complex and by determination of its effect on the binding constant for the activator AMP. ^{31}P NMR investigation of the binding of this inhibitor to the enzyme has demonstrated that it both tightens the binding of the nucleotide activator and shifts the resonance of the phosphate group of the pyridoxal phosphate residue to a broad signal around 0 ppm. This situation is further reinforced in the presence of the second substrate, maltopentaose, giving a fully potentiated, but inactive, enzyme–substrate complex. This has not been studied previously by ^{31}P NMR. The active form of the pyridoxal phosphate (PLP), in the presence of all substrates or their analogues, is not therefore a mobile dianionic phosphate as has been previously proposed. It may represent a tightly bound and constrained dianionic phosphate or possibly a protonated phosphate in intermediate exchange. The implications of this finding are discussed.

Despite many years of investigation, the role of pyridoxal phosphate (PLP)[1] in facilitating the glycogen phosphorylase catalyzed transfer of glucose units from glucose-1-P to glycogen is still unclear. Removal of this cofactor leads to complete loss of activity (Cori & Illingworth, 1957), suggesting that it is a catalytically essential group, and studies of various kinds, but particularly those involving the reconstitution of apoenzyme with PLP analogues, have provided evidence that the coenzyme phosphate is the functional group most likely involved in catalysis. (Kastenschmidt et al., 1968; Shaltiel et al., 1969; Pfeuffer et al., 1972; Feldmann et al., 1972, 1974; Vidgoff et al., 1974; Feldmann & Helmreich, 1976; Parrish et al., 1977; Shimomura & Fukui, 1978; Hoerl et al., 1979).

A predominantly hydrophobic environment for the PLP has been implied by several studies [e.g., Johnson et al. (1970)], including work on model compounds (Shaltiel & Cortijo, 1970; Kupfer et al., 1977). This has been borne out by the crystallographic work, in addition to proving the presence of the cofactor in the active site (Sygusch et al., 1977). It should be noted, however, that ionic forces may be involved in the recognition and binding of the substituted pyridine ring (Fletterick & Madsen, 1980). Other studies (Parrish et al., 1977; Shimomura & Fukui, 1978) have implied that the phosphate group of the PLP is placed very close to a second phosphate binding pocket, possibly that of the substrate, and crystallographic studies have again shown this to be the case (Sygusch et al., 1977). No other portion of the pyridoxal phosphate is close enough to interact with the substrate. The phosphate of the PLP was shown to be situated some 7 Å, phosphorus to phosphorus, away from the presumed position of the phosphate of the substrate glucose-1-P in the glucose-inhibited form of the enzyme. This presumed position of the phosphate has been confirmed, within experimental error by low-resolution crystallographic studies of the binding of glucose-1-P (Sygusch et al., 1977). Some catalytic role for the phosphate therefore appears likely. However, it was clearly shown (Illingworth et al., 1958) that the phosphate of the PLP does not exchange with substrate phosphate during reaction. Consequently, various roles for the phosphate have been forwarded, but none as yet indicated.

A more recent approach to this problem has been the ^{31}P NMR study of the enzyme. This has allowed a direct observation of any phosphate groups bound to or associated with the enzyme (Feldmann & Helmreich, 1976; Feldmann & Hull, 1977; Hoerl et al., 1979; Withers et al., 1979). Changes in the local environment and ionization state of phosphate groups may be reflected in changes in the chemical shift and/or line width of the ^{31}P NMR signal. Both the serine-14 phosphate of phosphorylase and bound AMP give a signal in the same region as PLP. It is usually necessary, therefore, to use thiophosphate analogues, which have different chemical shifts, in order to clearly observe the PLP signal, as was pioneered by Feldmann & Hull (1977). Their work has shown that the PLP can exist in two different forms depending on its activation state. These were designated as form I and form III. Form I is the species observed in phosphorylase *b* in the absence of activators (Feldmann & Hull, 1977) or in the presence of the inhibitors glucose or caffeine (Withers et al., 1979) and presumed to be the inactive form. Form III, however, was observed in phosphorylase *a* or in nucleotide-activated phosphorylase *b* (Feldmann & Hull, 1977) and was thus presumed to be the active form of the cofactor. From the relative chemical shifts of the two forms it was proposed that this activation process involves a deprotonation of the phosphate such that form III was dianionic and form I monoanionic. Similar results have been observed more recently with potato phosphorylase (Klein & Helmreich, 1979).

Much other evidence has been accumulated to show that further conformational rearrangements take place upon addition of either of the substrates, and especially in the presence of both, but such a substrate-activated ternary enzyme complex has not previously been studied by ^{31}P NMR. It is, however, impracticable at present to study the enzyme in the presence

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[1] Abbreviations used: AMP, adenosine 5′-phosphate; AMPS, adenosine 5′-*O*-thiomonophosphate; ATPS, adenosine 5′-*O*-(3-thio)triphosphate; NMR, nuclear magnetic resonance; DTT, dithiothreitol; BSA, bovine serum albumin; glucose-1-P, α-D-glucose 1-phosphate; PLP, pyridoxal phosphate; ppm, parts per million; cyclic phosphate, α-D-glucose cyclic 1,2-phosphate; EDTA, ethylenediaminetetraacetic acid.

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of all its substrates and activators, because of the rapid turnover of substrate to product with the concentration of enzyme required for NMR. In addition, the signals for inorganic phosphate and glucose-1-P would obscure that from PLP. A substrate analogue which produces all the conformational changes associated with the true substrate but does not actually undergo enzymic decomposition was therefore required. Glucosyl fluoride was investigated as a potential analogue, but was found to promote the inactive T state of the enzyme. The compound selected was α-D-glucopyranosyl cyclic 1,2-phosphate. This compound has been previously found to be a good inhibitor of both the potato enzyme (Kokesh et al., 1977) and rabbit muscle glycogen phosphorylase (Hu & Gold, 1978). We confirmed it to be a good inhibitor of both phosphorylase *a* and phosphorylase *b*, and, also, of great importance for this study, its phosphate group resonates some 7–10 ppm downfield from enzyme-bound pyridoxal phosphate, allowing the study of the enzyme–inhibitor complex. This low chemical shift is presumably a consequence of ring strain distorting the O–P–O bond angle. Similar chemical shifts have been observed for other five-membered rings (Gorenstein, 1975). The pyranose ring of this compound, however, has been shown to exist in an unstrained $^4C_1$ conformation (O'Connor et al., 1979). The use of this nondegradable analogue thus also allowed the addition of the second oligosaccharide substrate to produce a fully activated enzyme system. It had been shown previously for this enzyme, which follows a rapid equilibrium random bi-bi mechanism, that no half reactions could be observed in the presence of a single substrate (Cohn & Cori, 1948). Neither was it possible to observe scrambling of substrate phosphate oxygen atoms in the absence of primer. Such scrambling has been observed for potato phosphorylase in the presence of cyclodextrins (Kokesh & Kakuda, 1977). It is thus essential to produce the ternary complex to observe any significant physical or chemical change representing the active state of the substrates. Investigation of such a ternary complex by ^{31}P NMR is described in this paper, and possible roles for the PLP in the catalytic mechanism of the enzyme are discussed in this light.

Material and Methods

All buffer chemicals and substrates were obtained from Sigma Chemical Co., except for DTT[1] which was obtained from Bio-Rad Laboratories. AMPS and ATPS were obtained from Boehringer-Mannheim, and D_2O was from Bio-Rad Laboratories. A Radiometer PHM62 pH meter was used for all pH measurements, and those measurements made in D_2O buffer are uncorrected. Maltopentaose was a kind gift of Dr. T. Fukui. Maltoheptaose was prepared as previously described (Kasvinsky & Madsen, 1976). Glucose cyclic 1,2-phosphate was synthesized according to the method of Zmudzka & Shugar (1964) modified by a procedure described to us by Dr. H. Buc. This procedure omitted the acidic ion-exchange step which was found to cause decomposition of the product and employed a DE52 ion-exchange column at pH 8 to remove contaminants from the cyclic phosphate. In this way it was possible to obtain the pure cyclic phosphate free of all phosphate impurities, as determined by ^{31}P NMR. This procedure is described in detail in Dreyfus et al. (1980).

Rabbit muscle phosphorylase *b* was prepared by the method of Fischer & Krebs (1962), using DTT instead of 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). Thiophosphorylase *a* was similarly prepared by using ATPS in place of ATP (Gratecos & Fischer, 1974). Protein concentration was determined from absorbance measurements at 280 nm by 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.

^{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 either 28 or 37 °C. A spectral width of 5000 Hz was generally employed, with a 50–70° pulse angle (15–20 μs) and a repetition time of 2 s. Exponential line broadening used prior to Fourier transformation was generally 20 Hz, and all line width data have been corrected for this.

Sample size was 1.5 mL in a 10-mm tube, with enzyme concentrations between 0.4 and 1 mM calculated for the phosphorylase monomer molecular weight of 97 412. The buffer used in all NMR experiments was 50 mM triethanolamine hydrochloride, 100 mM KCl, 1 mM EDTA, and 1 mM DTT (pH 6.8 meter reading) made up in D_2O previously treated with Chelex to remove any paramagnetic impurities. The D_2O present in the buffer was used for field/frequency lock, and a 1-mm tube containing 85% phosphoric acid was inserted for chemical shift referencing. Computer-assisted line shape analysis of the binding of AMPS was performed as described previously (Withers et al., 1979).

AMP was removed from phosphorylases *a* and *b* as described earlier (Withers et al., 1979), and, when necessary, enzyme was concentrated by use of a Millipore immersible concentrator. Solutions of effectors dissolved in D_2O NMR buffer at pH 6.8 were added directly to the NMR tube as required.

The ultracentrifugation experiments were performed on a Spinco Model E analytical ultracentrifuge at a rotor speed of 56 000 rpm and a temperature of 20 ± 0.1 °C. Sedimentation coefficients determined from Schlieren patterns were corrected for the viscosity and density of the buffer to water at 20 °C. Protein concentration was 5 mg/mL. The buffer used was the same triethanolamine buffer as used for the NMR experiments. 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 0.2 or 0.5 mL, and reactions were performed at 30 °C, pH 6.8, in the same buffer as used for NMR. The inhibition constant for cyclic phosphate with phosphorylase *b* was determined as above with 1 mM AMP, 1.6–24 mM glucose-1-P, 1% glycogen, and 7 μg of enzyme per reaction mix. Phosphorylase *a* was assayed similarly either in the presence of 1 mM AMP or in its absence by using 1–3 μg of enzyme and including 0.1 mg of BSA/reaction. The dissociation constant for AMP in the presence of cyclic phosphate was determined similarly by using a range of AMP concentrations from 1.7×10^{-5} to 1.01×10^{-3} M and 2.5 mM glucose-1-P.

Paper chromatography was performed in the descending mode with Whatman No. 1 paper. Solvent systems used were 1-butanol–pyridine–water (6:4:3) and ethyl acetate–pyridine–water (8:2:2). Papers were visualized with either the silver nitrate dip or an aniline hydrogen phthalate spray (Block et al., 1958).

Results

Determination of Inhibition Constants for Cyclic Phosphate. Both phosphorylase *a* and phosphorylase *b* have been shown to follow rapid equilibrium random order reaction kinetics.

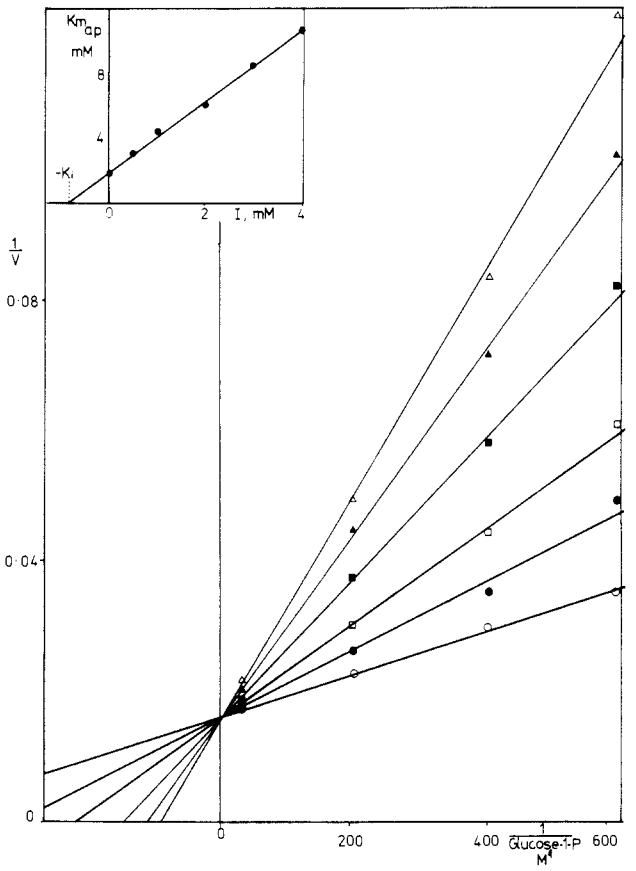

FIGURE 1: Double-reciprocal plot of cyclic phosphate inhibition of phosphorylase *b* with constant (1 mM) AMP. Concentrations of cyclic phosphate (millimolar) are (○) 0, (●) 0.5, (□) 1, (■) 2, (▲) 3, and (△) 4. Inset represents the replot of the apparent K_m values vs. inhibitor concentration.

The dissociation constant for a competitive inhibitor may thus be obtained by replots from a Lineweaver–Burk analysis as demonstrated in Segel (1975), representing dissociation from the enzyme–glycogen complex or the enzyme–glycogen–AMP complex where the activator is present in saturating concentrations. The determination of the effects of cyclic phosphate on the kinetic constants of both phosphorylase *a* in the presence and absence of AMP and phosphorylase *b* in the presence of AMP showed the cyclic phosphate to be a good inhibitor of the enzyme and directly competitive with glucose-1-P binding in each case. The Lineweaver–Burk plot for phosphorylase *b* in the presence of different concentrations of cyclic phosphate is shown in Figure 1. The slopes of these lines were used to determine the apparent K_m for glucose-1-P in the presence of each concentration of inhibitor. The inset shows the replot of K_m(app) vs. inhibitor concentration used to determine the constant, K_i from the intersection on the abscissa. Similar plots were obtained with phosphorylase *a* and thiophosphorylase *a*. The inhibition constants determined in this way are given in Table I. The values of the dissociation constants for the cyclic phosphate are all equivalent to, or smaller than, the corresponding K_m values for glucose-1-P. The tighter binding of cyclic phosphate to nucleotide-free enzyme may indicate that the cyclic phosphate is more effective at promoting the R state of the enzyme than is glucose-1-P. Since glucose-1-P binding is known to improve the binding of AMP to phosphorylase, the effect of the cyclic phosphate inhibitor on the binding of the nucleotide activator was studied. Reaction rates were measured at varying concentrations of AMP and cyclic phosphate by using a fixed concentration (2.5 mM) of glucose-1-P. Data obtained in this way were analyzed according

Table I: Kinetic Binding Constants for Glucose 1-Phosphate and Glucose Cyclic 1,2-Phosphate

enzyme system	K_m, glucose-1P (mM)	K_i, cyclic phosphate (mM)
phosphorylase *b* + 1 mM AMP	2.00	0.90
phosphorylase *a*	2.26	0.28
phosphorylase *a* + 1 mM AMP	0.20	0.25
thiophosphorylase *a*	2.86	0.37
thiophosphorylase *a* + 1 mM AMP	0.25	0.27

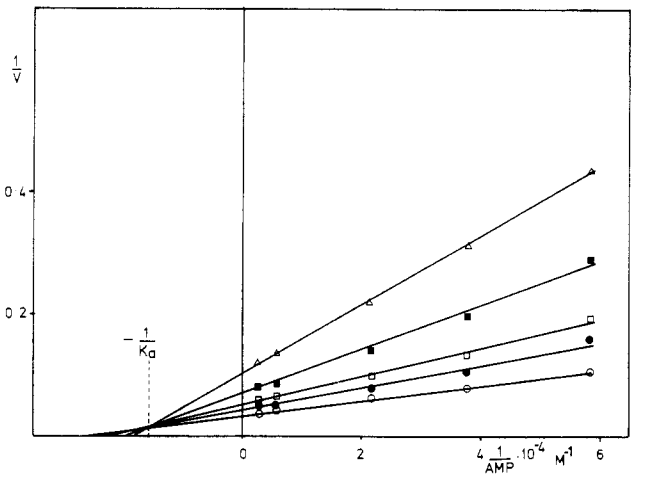

FIGURE 2: Double-reciprocal plot of cyclic phosphate inhibition of phosphorylase *b* with constant (2.5 mM) glucose-1-P and varying AMP. Concentrations of cyclic phosphate (millimolar) are (○) 0, (●) 1, (□) 2, (■) 3, and (△) 4.

to Segel (1975) for an inhibitor competing with one substrate in a random bireactant system. These results are given in Lineweaver–Burk format in Figure 2. The value of the binding constant for AMP in the presence of cyclic phosphate is obtained from the value of 1/[AMP] at the intersection of the plots. The dissociation constant, K_d, for AMP binding to the enzyme–inhibitor complex (5×10^{-5} M) was thus shown to be slightly greater than that for the enzyme–substrate complex (3.5×10^{-5} M), as measured in a control experiment (data not shown), but much smaller than that for the binding of AMP to free enzyme (3×10^{-4} M) (Mott & Bieber, 1970).

Ultracentrifugation Studies. Figure 3 shows the ultracentrifugation patterns observed for AMP-activated phosphorylase *a* in the presence and absence of cyclic phosphate. As has been shown previously, (Wang et al., 1965) under these conditions, in the absence of effectors or in the presence of AMP, phosphorylase *a* sediments as a tetramer of sedimentation constant 13 S. Addition of saturating concentrations of cyclic phosphate to the enzyme causes no change in the sedimentation pattern of the enzyme; thus this inhibitor does not destabilize the tetrameric form of the enzyme. This same situation obtains with the substrate glucose-1-P (Wang et al., 1965; Helmreich et al., 1967). Inhibitors such as glucose or caffeine, however, have been shown to promote dimerization of the enzyme at equivalent saturating concentrations (Wang et al., 1965; Withers et al., 1979).

^{31}P NMR Studies. (1) Effects of Ligands on Binding of Nucleotide to Phosphorylase b. In the absence of any other effectors, AMPS is known to bind sufficiently tightly to phosphorylase *b* to be within the slow exchange limit on the NMR time scale. This is seen in Figure 4a, the ^{31}P NMR spectrum of phosphorylase *b* plus AMPS. Here, two distinct resonances are seen due to free and bound nucleotide. The

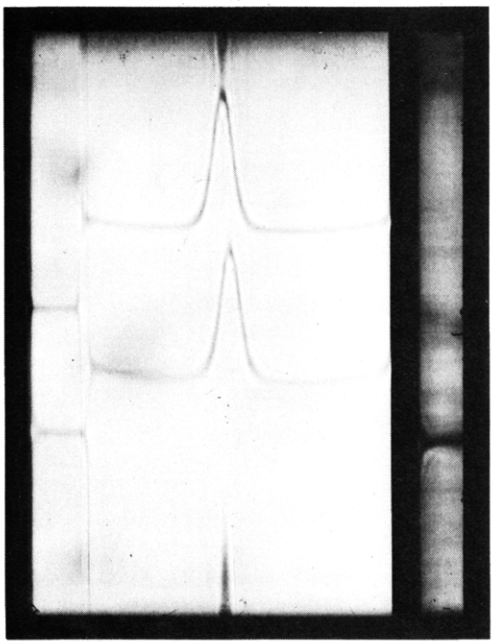

FIGURE 3: Effect of AMP and cyclic phosphate on sedimentation properties of phosphorylase *a*. (Top) AMP (1 mM) plus cyclic phosphate (2 mM) (13.4 S); (bottom) AMP (1 mM) (13.4 S). Pictures were taken 24 min after attaining full speed. Conditions were as explained under Materials and Methods.

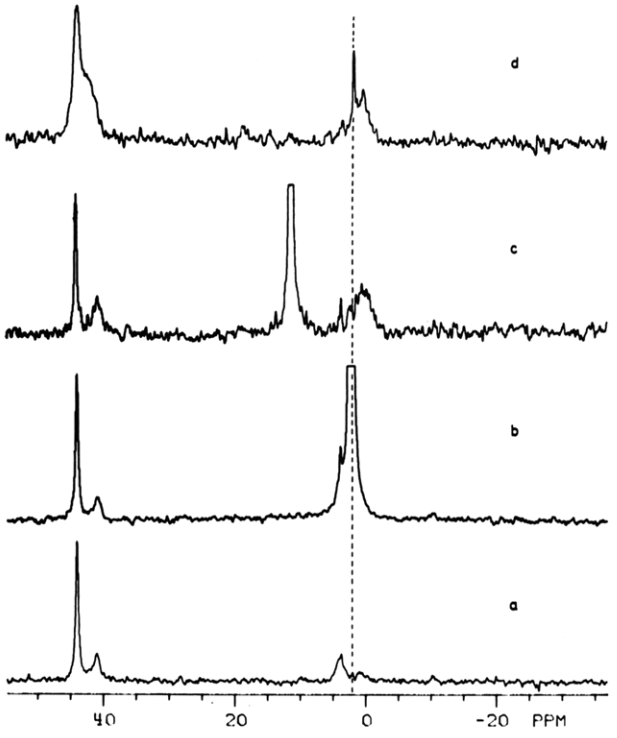

FIGURE 4: ^{31}P NMR spectra of phosphorylase *b* in the presence of various ligands. (a) Phosphorylase *b* (0.85 mM monomers) plus 2.43 mM AMPS, 25 233 acquisitions; (b) phosphorylase *b* (0.93 mM monomers) plus 2.29 mM AMPS and 11.4 mM glucose-1-P, 18 000 acquisitions; (c) phosphorylase *b* (1.01 mM monomers) plus 2.34 mM AMPS and 5.33 mM cyclic phosphate, 5000 acquisitions; (d) phosphorylase *b* (0.85 mM monomers) plus 1.83 mM AMPS and 5.07 mM glucosyl fluoride, 28 461 acquisitions. Conditions were as described under Materials and Methods.

signal for bound AMPS, δ 40.8, has an observed line width of 100 Hz, whereas that for free AMPS, δ 43.9, has an observed line width of 40 Hz. Figure 4b shows the spectrum for phosphorylase *b* plus AMPS and glucose-1-P. The signal for free AMPS is now narrower than previously, δ 43.9 and $\Delta\nu_{obsd}$ = 30 Hz, indicating that the nucleotide is now binding more tightly than in the absence of substrate; this is reflected mainly in a decrease in its off rate constant (k_{-1}) (Table II). This is consistent with kinetic data (Madsen, 1964) which demonstrated tightening of nucleotide binding upon binding glucose-1-P. Addition of the cyclic phosphate to nucleotide-activated phosphorylase *b* induced very similar changes in the thiophosphate region of the spectrum (Figure 4c). Here again, the signal from free AMPS is narrow, δ 44.0 and $\Delta\nu_{obsd}$ = 26 Hz. Slight broadening of the signal for bound AMPS in the presence of cyclic phosphate (δ 40.7 and $\Delta\nu_{obsd}$ = 115 Hz) may be related to a further restriction in rotational freedom upon tightening of nucleotide binding.

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

sample	figure	off rate, k_{-1} (s^{-1})	dissociation constant, K_D (μM)
phosphorylase *b* + AMPS	4a	210	200
phosphorylase *b* + AMPS + glucose-1-P	4b	150	150
phosphorylase *b* + AMPS + cyclic phosphate	4c	110	80
phosphorylase *b* + AMPS + glucosyl fluoride	4d	750	800
phosphorylase *b* + AMPS + maltoheptaose	6a	410	800
phosphorylase *b* + AMPS + maltopentoase	6b	750	600
phosphorylase *b* + AMPS + maltopentaose + cyclic phosphate	6c	120	300
thiophosphorylase *a* + AMPS	5a	25	2
thiophosphorylase *a* + AMPS + cyclic phosphate	5c	20	0.5
thiophosphorylase *a* + AMPS + glucose-1-P	5d	30	0.5
thiophosphorylase *a* + AMPS + maltopentaose + cyclic phosphate	6d	20	0.5

[a] Enzyme and ligand concentrations are as described in corresponding figures.

This is in marked contrast to the situation observed with other inhibitors such as glucose and caffeine which were shown to drastically weaken the binding of nucleotide (Withers et al., 1979), producing a single exchange-averaged signal for both free and bound AMPS.

α-D-Glucopyranosyl fluoride has been shown previously (Ariki & Fukui, 1975) to be a good specific inhibitor of phosphorylase *b*, competitive with glucose-1-P. It binds to the enzyme almost 10 times more tightly than glucose (K_i for glucosyl fluoride = 0.43 mM, K_i for glucose = 3 mM), and on this basis it had been proposed that it occupied both the glucose and the phosphate subsites of the active site. We have confirmed these kinetic constants and have also performed an X-ray difference Fourier analysis of the binding of glucosyl fluoride which showed binding in the active site totally coincident with the binding of glucose and no significant structural changes within the protein (R. J. Fletterick et al., unpublished results). Addition of glucosyl fluoride to the activated enzyme (Figure 4d) weakens nucleotide binding, producing a single exchange-averaged signal for the free and bound AMPS. Such an effect on the nucleotide binding constant therefore accords with this compound being a true glucose analogue in its mechanism of inhibition and not a glucose-1-P analogue.

The line shapes observed for the signals corresponding to the AMPS activator in these experiments were subjected to

a complete line shape analysis to determine values for the off-rate constant (k_{-1}) and dissociation constant for AMPS and the line widths of the free and bound AMPS in the absence of exchange as explained previously (Withers et al., 1979). The rate and equilibrium constants obtained in this way by assuming a natural line width (no exchange) for bound AMPS of 80 Hz are given in Table II.

(*2*) *Effects of Ligands on PLP Coenzyme.* Figure 4a also shows the ^{31}P NMR spectrum of the PLP of nucleotide-activated phosphorylase *b*, with the major resonance being form III (δ 3.8 and $\Delta\nu$ = 160 Hz). A small portion of form I (δ 0.6) is still observed in addition to a small amount of contaminating inorganic phosphate. Glucosyl fluoride has the same effects on the ^{31}P NMR spectrum of nucleotide-activated phosphorylase as does glucose, but at lower concentrations since it binds 10 times tighter. It therefore promotes form I of the PLP resonance δ 0.39 as seen in Figure 4d, in addition to weakening the binding of the nucleotide. The sharp signal at δ 1.9 is due to inorganic phosphate. Addition of sufficient glucose-1-P to saturate the enzyme results in a huge signal for glucose-1-P, completely masking the PLP region and rendering it useless for such a study (Figure 4b). However, the glucose cyclic 1,2-phosphate resonates at a position some 7–10 ppm downfield from enzyme-bound PLP (δ 11.3) and will not, therefore, obscure the PLP region. Also, saturation of the enzyme with this ligand can be achieved at a lower concentration than is necessary for glucose-1-P, which leads, in turn, to a smaller signal from the free cyclic phosphate.

The ^{31}P NMR spectrum of nucleotide-activated phosphorylase *b* in the presence of cyclic phosphate is shown in Figure 4c. The major signal observed is that of free cyclic phosphate (δ 11), with no separate signal for bound cyclic phosphate being in evidence. The PLP now resonates in a position equivalent to form I, with a very broad line width (δ 0.3 and $\Delta\nu$ = 250 Hz). The signal observed at δ 3.6 corresponds to free AMP contaminant.

Since the cyclic phosphate inhibitor binds even more tightly to phosphorylase *a* and thiophosphorylase *a* in the presence of AMP, similar studies were carried out on this latter enzyme system to see if cyclic phosphate effects the same change on the PLP resonance. The spectrum of thiophosphorylase *a* plus AMPS is shown in Figure 5a. All spectra of phosphorylase *a* were run at 37 °C to increase the solubility of the enzyme. This spectrum is essentially identical with that published by Feldmann & Hull (1977). Here the PLP is seen to exist completely in form III, δ 3.8 and $\Delta\nu$ = 140 Hz, as would be expected from the results with phosphorylase *b*. The narrow lines for free and bound AMPS (δ 44.0, $\Delta\nu_{obsd}$ = 20 Hz and δ 40.7, $\Delta\nu_{obsd}$ = 75 Hz, respectively) indicate very tight binding of the nucleotide, and the signal observed between these two resonances (δ 42.5 and $\Delta\nu_{obsd}$ = 80 Hz) is due to the thiophosphoseryl residue. Addition of 50 mM glucose to a sample of thiophosphorylase *a*, in the absence of AMPS (Figure 5b), results in partial change of the PLP resonance back from form III to the form I (δ 0.5 and $\Delta\nu$ = 65 Hz) as would be expected from the results with phosphorylase *b* (Withers et al., 1979) and with 5′-deoxypyridoxal 5′-methylenephosphonate reconstituted phosphorylase *a* (Hoerl et al., 1979). In the presence of glucose the thiophosphoseryl residue resonates at lower field with a narrow line width (δ 43.4 and $\Delta\nu$ = 60 Hz). This species has previously been assigned to a less tightly bound form of the thiophosphate (Hoerl et al., 1979). However, its predominance in the presence of inhibitory glucose was not reported previously. Addition of glucose-1-P to phosphorylase *a* in the presence of AMPS allowed observation of the thiophosphoseryl and AMPS resonances but again obscured the PLP region. Here the thiophosphoseryl residue resonated as a single high-field species (δ 42.5) (Figure 5d). Unliganded thiophosphorylase *a* shows these two forms of the thiophosphoserine in intermediate exchange.

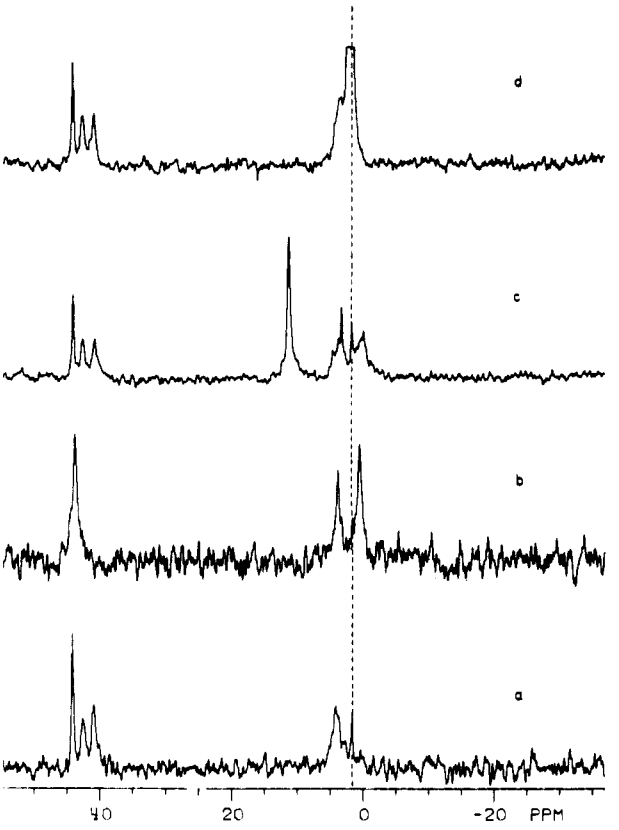

FIGURE 5: ^{31}P NMR spectra of thiophosphorylase *a* in the presence of various ligands. (a) Thiophosphorylase *a* (0.49 mM monomers) plus 0.92 mM AMPS, 21 000 acquisitions; (b) thiophosphorylase *a* (0.45 mM monomers) plus 60 mM glucose, 24 650 acquisitions; (c) thiophosphorylase *a* (0.54 mM monomers) plus 0.88 mM AMPS and 2.76 mM cyclic phosphate, 25 000 acquisitions; (d) thiophosphorylase *a* (0.49 mM monomers) plus 0.92 AMPS and 2.04 mM glucose-1-P, 27 600 acquisitions. Conditions were as described under Materials and Methods.

Figure 5c shows the spectrum obtained for thiophosphorylase *a* plus AMPS and cyclic phosphate. As was the case with phosphorylase *b*, the predominant form of the PLP is now of the broadened form I type (δ 0.3), with some form III left. Sharp signals at 1.9 and 3.6 ppm correspond to contaminating phosphate and AMP, respectively. The thiophosphoseryl residue still occurs as a single high-field peak (δ 42.5), as was observed also in the presence of glucose-1-P. It therefore appears that in phosphorylase *a* also, the cyclic phosphate promotes this same broadened form I of the PLP and an activated R conformation, as evidenced by the position of the thiophosphoseryl residue.

(*3*) *Binding of Oligosaccharide Substrate.* The oligosaccharides maltoheptaose and maltopentaose can be used as substrates for phosphorylase in place of glycogen. These oligosaccharides have several advantages over glycogen phosphorylase for use in these NMR studies. Since they have relatively low molecular weights, the problems of sample viscosity and high enzyme–substrate complex molecular weights encountered with the use of glycogen are minimized. It is also then possible to observe the different effects due to binding to the storage site alone or to both the storage site and the active site. Binding constants for these two sites on phosphorylase *a* in the absence of AMP have been calculated

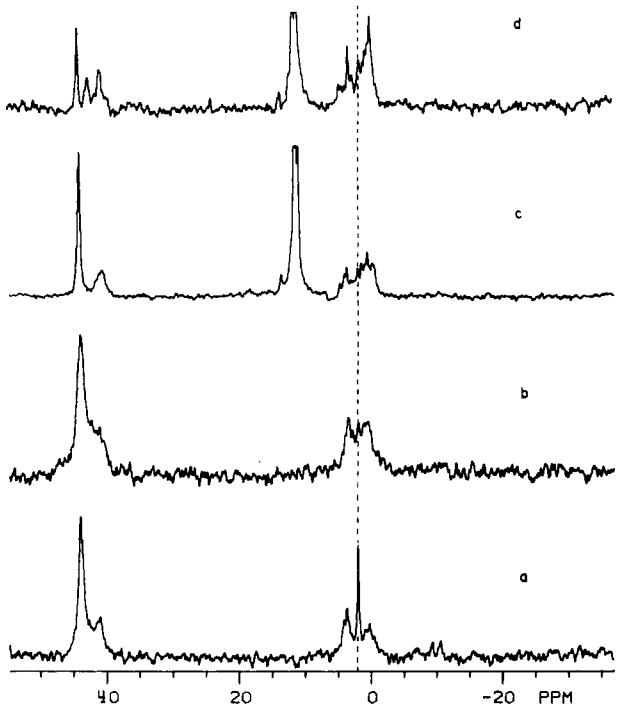

FIGURE 6: ^{31}P NMR spectra of phosphorylase *b* and thiophosphorylase *a* in the presence of oligosaccharide. (a) Phosphorylase *b* (1.06 mM monomers) plus 2.06 mM AMPS and 8.4 mM maltoheptaose, 19 567 acquisitions; (b) phosphorylase *b* (1.20 mM monomers) plus 2.43 mM AMPS and 67 mM maltopentaose, 15 324 acquisitions; (c) phosphorylase *b* (1.01 mM monomers) plus 2.12 mM AMPS, 5.55 mM cyclic phosphate, and 59.5 mM maltopentaose, 27 000 acquisitions; (d) thiophosphorylase *a* (0.54 mM monomers) plus 0.88 mM AMPS, 4.54 mM cyclic phosphate, and 70 mM maltopentaose, 10 000 acquisitions. Conditions were as described under Materials and Methods.

at 1 and 22 mM, respectively (Kasvinsky et al., 1978). These values were not determined in the presence of AMP, but binding of glycogen to the enzyme in the presence of substrate is not greatly affected by AMP addition (Engers et al., 1970).

The spectrum of nucleotide-activated phosphorylase *b* in the presence of 8 mM maltoheptaose is shown in Figure 6a. Under these conditions the storage site will be ~95% occupied, but the active site will be <25% filled. Part of the PLP exists in form I, δ 0.4, with the narrow peak at δ 1.9 being due to inorganic phosphate. Binding of AMPS is weakened by the binding of oligosaccharide. Under conditions where a reasonable amount of oligosaccharide might be expected to be binding at the active site, slightly more conversion to form I is observed. This is shown in Figure 6b, which is the spectrum of nucleotide-activated phosphorylase *b* plus 60 mM maltopentaose. Here the storage site will be fully saturated and the active site at least 75% occupied. Binding of AMPS is also slightly further weakened as evidenced by a further collapse of the signals for free and bound AMPS toward a single signal. The possibility of this effect being due to contaminating glucose, either in the original maltopentaose sample or produced in the tube by contaminating α-amylase, was eliminated by paper chromatographic examination of the deproteinized sample. No glucose could be detected.

(*4*) *Ternary Complex.* The spectrum of the fully potentiated conformation of the enzymic complex of phosphorylase *b* plus AMPS, maltopentaose, and glucose cyclic 1,2-phosphate is shown in Figure 6c. No reaction whatsoever has occurred in this case, since there is no indication of any significant disappearance of cyclic phosphate when spectra were inspected at 3-h time intervals, and the small amounts of contaminating phosphates produced were also shown to appear in nonenzymic controls. The binding of AMPS does not seem to be affected significantly by this further addition of oligosaccharide, (relative to Figure 4c), and it is therefore significantly tighter than in the presence of oligosaccharide alone (Figure 6b). The cyclic phosphate therefore reverses the original effect of maltopentaose on the binding of AMPS, to give an off-rate constant for AMPS very similar to that of the activated enzyme plus cyclic phosphate and a dissociation constant considerably lower than that observed in the presence of oligosaccharide alone. The PLP resonates in a position corresponding to a broadened form I (δ 0.3 and $\Delta\nu$ = 250 Hz). This is consistent with the tendency of both ligands individually to promote form I. A similar situation obtains with the ternary complex of thiophosphorylase *a* in Figure 6d. Here the thiophosphoserine and AMPS signals do not alter significantly upon subsequent binding of oligosaccharide, but there is a slightly greater conversion of form III to form I and a narrowing of the signal (δ 0.3 and $\Delta\nu$ = 200 Hz). Contaminants of inorganic phosphate and AMP are in evidence here. It therefore seems that the same effects are observed with the two forms of the enzyme.

Discussion

Previous attempts to investigate the nature of the active form of PLP in glycogen phosphorylase by ^{31}P NMR have generally included studies of the activated enzyme alone or in the presence of one of its substrates. Under such conditions it has been demonstrated that the PLP phosphate exists in a mobile dianionic form and as such may be capable of acting as a base or possibly a nucleophile in the catalytic reaction. Such studies have not, however, included observation of the ternary complex of activated enzyme in the presence of both substrates or their analogues.

By use of the substrate analogue glucose cyclic 1,2-phosphate, we have been able to investigate the activated ternary complex and have found quite different results from those reported previously. This compound was shown to bind in a mode similar to that of glucose-1-P rather than glucose by the following criteria. Linear rather than curved Lineweaver–Burk plots were obtained for its inhibition, and positive allostery in its effect on AMP binding. Stabilization of a tetrameric rather than a dimeric form of phosphorylase *a* was shown by ultracentrifugal analysis. A high-field ^{31}P NMR resonance for the thiophosphoserine of thiophosphorylase *a* was observed in its presence. Crystals of phosphorylase *a* grown in the glucose-inhibited form cracked on exposure to the cyclic phosphate, and direct observation of its binding at the active site by X-ray difference Fourier analysis has been achieved (R. J. Fletterick et al., unpublished results).

Results obtained with α-D-glucopyranosyl fluoride indicate that this compound is acting as a glucose rather than a glucose 1-phosphate analogue as evidenced by its curved Lineweaver–Burk plots and its weakening of AMP binding. The ^{31}P NMR spectrum of the enzyme in the presence of this analogue was identical with that observed with glucose and represents binding of the analogue to the T state of the enzyme. Production of an active ternary complex in the R state, therefore, appears to require correct occupancy of both glucosyl and phosphate subsites simultaneously in order to promote the correct conformational changes. It therefore appears that cyclic phosphate is capable of producing this activated ternary complex in the R state. Moreover, the tighter binding of cyclic phosphate than of glucose-1-P to the nucleotide-free phosphorylase *a* may imply that it is even more effective at producing the R state of the enzyme. This suggests, in turn, that the conformation of the cyclic phosphate is similar to the

conformation of bound glucose-1-P. Similar conclusions were drawn previously on the basis of kinetic evidence alone (Kokesh et al., 1977; Hu & Gold, 1978).

The enzyme has been proposed to contain the phosphate of the PLP in its dianionic form in the active configuration. It would seem likely, however, on the basis of electrostatic interactions, that the introduction of a substrate or its analogue bearing negative charge(s) into the active site would cause the protonation, or something equivalent, of the coenzyme phosphate in order to minimize charge–charge repulsion. A deprotonation of the PLP phosphate upon substrate binding would seem inherently unlikely because of the resulting proximity of two negatively charged species. X-ray crystallographic studies have shown the two phosphates to be ~7 Å apart, as measured from phosphorus atom to phosphorus atom, in phosphorylase *a* (Sygusch et al., 1977). A distance of 6 Å was reported for phosphorylase *b* (Johnson et al., 1980). Some caution must be exercised in interpretation of this active-site geometry for phosphorylase *a* since the enzyme was crystallized in the glucose-inhibited form and some conformation changes are known to occur upon substrate addition. However, the crystal structure of phosphorylase *b* was determined in the presence of the weak activator IMP, and a similar measurement was obtained. It is thus certain that these two phosphate moieties bind very close together and that the binding of substrate must affect, electrostatically, the ionization state of the coenzyme phosphate. Further evidence for the proximity of these two subsites comes from studies of pyridoxal-reconstituted phosphorylase (Parrish et al., 1977). This study suggested that a single molecule of pyrophosphate was capable of simultaneously occupying both the coenzyme and substrate phosphate binding pockets. Studies of the rate of reconstitution of apoenzyme with pyridoxal phosphate analogues (Shimomura & Fukui, 1978) provide further evidence for this hypothesis. The highest rate of reconstitution measured was for the pyridoxal diphosphate analogue, indicating a strong binding site for such a pyrophosphate moiety.

The shift of the ^{31}P NMR resonance of coenzyme phosphate to higher field upon binding of cyclic phosphate to the nucleotide-activated enzyme is therefore reasonable on the basis of electrostatic interactions. Reduction of electrostatic repulsion between the two moieties could most easily be achieved either by protonation of one or both of the phosphates or by tighter coordination to neighboring basic amino acid side chains. Either mechanism would reduce the overall amount of negative charge present. If protonation occurs, the dianionic coenzyme phosphate is the most likely to protonate since protonation of cyclic phosphate would only occur at much lower pH values. Protonation of cyclic phosphate could also lead to its decomposition, and no evidence of any phosphorylase-catalyzed decomposition, which would be manifested by the appearance of new ^{31}P NMR signals, is observed during these NMR experiments. A very small amount of decomposition is occasionally observed, but this can be accounted for by spontaneous decomposition or possibly catalytic decomposition by small quantities of contaminating phosphatases.

Such a lack of decomposition, as observed by ^{31}P NMR even at almost stoichiometric levels of enzyme and inhibitor, is also evidence against the presence of an acid catalytic group on the enzyme which can facilitate C–O bond fission by protonation of the glycosidic oxygen atom. The cyclic phosphate analogue is quite acid labile and would be expected to decompose if an acidic group were brought close in the activated complex. It thus appears that acid catalysis of aglycon departure is unlikely to occur in the catalytic event. Similarly, no hydrolysis of cyclic phosphate by potato phosphorylase was observed previously (Kokesh & Kakuda, 1977).

The shift of the pyridoxal phosphate signal to higher field with a broad resonance (250 Hz) at around 0 ppm is thus probably due either to protonation or to tighter binding with neighboring lysine and arginine residues and possible strain of the phosphate moiety. Changes in the O–P–O bond angle of ~2° can result in chemical shift changes of ~4 ppm (Gorenstein, 1975). It is difficult to differentiate between these two possible interpretations of the data. The broad line width observed (250 Hz) may be explicable on both accounts. In the former case, the broad line width observed could be a result of intermediate to fast exchange of this proton with another enzymic group. Alternatively, if the chemical shift observed is due to tight binding and concomitant distortions, the large line width may well be a result of hindered rotation of the coenzyme phosphate or changes in the chemical shift anisotropy of the bound phosphate which will increase the chemical shift anisotropic contribution to the line width.

A similar high-field shift, but of lesser magnitude, was observed with potato phosphorylase (Klein & Helmreich, 1979) upon addition of arsenate, an analogue of phosphate. It also caused a broadening of the resonance. However, subsequent addition of maltoheptaose led to a small shift back downfield. This latter experiment must, however, be interpreted with great care since arsenolysis, and hence glucose production, will be occurring in this system, and this could confuse the interpretation.

Addition of oligosaccharide to nucleotide-activated enzyme was shown to be accompanied by a partial conversion of the PLP from form III to form I as seen in Figure 6a,b. This may be explained on the assumption that the oligosaccharide may bind at the active site, occupying the terminal glucose binding site, and thus enforce conformation changes similar to those promoted by glucose, as long as nothing occupies the phosphate site. The possibility of this effect being caused by contaminating glucose in the oligosaccharide was eliminated by paper chromatographic examination of the oligosaccharide used and the deproteinized NMR sample. No significant amount of glucose could be detected. Subsequent addition of oligosaccharide to the enzyme cyclic phosphate complex at concentrations which should allow a reasonable occupancy at the active site does not lead to any significant further change in the NMR spectrum of the enzyme. In the case of phosphorylase *a*, a more complete conversion to the high-field resonance is observed, which is presumably the result of allosteric improvement of cyclic phosphate binding. Quite probably some further conformational changes do occur on binding of the oligosaccharide, but these do not affect the immediate environment of the coenzyme phosphate. This may argue against some form of direct interaction between the oligosaccharide and the coenzyme phosphate.

Inspection of the thiophosphate regions of these spectra allows further deductions regarding the conformation changes taking place in this part of the molecule. The thiophosphoseryl resonance of thiophosphorylase *a* in the presence of cyclic phosphate and AMPS occurs at δ 42.5 and $\Delta\nu$ = 80 Hz. This is the same chemical shift as was observed with glucose-1-P but different from that observed in the presence of glucose (δ 43.4 and $\Delta\nu$ = 60 Hz). The narrower line width in the latter case may be interpreted in terms of increased mobility of the residue. In its activated conformation, the thiophosphoryl group may be held in a stable salt bridge, but in its glucose-inhibited form it is unbound and more mobile. Similar observations were made previously (Hoerl et al., 1979) for

succinylated thiophosphorylase *a* and thiophosphorylase *a* at high pH. The greater mobility of the thiophosphoseryl residue in the presence of glucose would account for higher V_{max} values observed for phosphorylase phosphatase acting on phosphorylase *a* in the presence of glucose than observed in its absence (Detwiler et al., 1977). Phosphorylase with a mobile N-terminal polypeptide containing the thiophosphoseryl residue would be a better substrate than the same molecule with the N-terminal region held tightly in by salt bridges between the thiophosphate and various arginine residues.

A molecular basis for the similarity of the effects of the PLP region of the ^{31}P NMR spectrum caused by glucose and glucose cyclic 1,2-phosphate can be forwarded. In the nonactivated enzyme and in the presence of glucose part of a large loop of polypeptide is known to be present within the active site (Madsen et al., 1978). In particular, Asn-284 may make a hydrogen bond with the C-2 oxygen of the glucose while Asp-283 occupies part of the same subsite as would the substrate phosphate if present (Fletterick & Madsen, 1980). Presumably its carboxylate Asp-283 helps to neutralize the high degree of positive charge present at this binding pocket (e.g., Arg-568). Addition of AMP causes this loop to swing out of the active site removing the carboxylate from the phosphate binding pocket, thus allowing the binding of substrate. The NMR observations may therefore be rationalized on this basis. The native or glucose-inhibited enzyme has this site occupied by the aforementioned negative carboxylate ion which is close enough to the pyridoxal phosphate to force the latter to protonate. Activation by AMP is accompanied by removal of this charge, and the coenzyme phosphate is then allowed to deprotonate. Subsequent addition of cyclic phosphate causes reprotonation or something equivalent in response to the presence of the negatively charged phosphate. Thus, tight ionic coordination of the pyridoxal phosphate with the positive charges in the active-site region could account for the observed NMR spectrum, and it may be noted that chemical studies have placed Arg-568 close to the phosphates (Dreyfus et al., 1980) whereas in the glucose-inhibited form it is too far away to interact (Fletterick & Madsen, 1980).

Regardless of the difficulty in deciding between the alternative interpretations of our data at this stage, the form of PLP observed in this activated ternary complex is not a mobile dianionic phosphate as has been proposed previously. The suggested role of the pyridoxal phosphate as a base (Helmreich & Klein, 1980) or as a nucleophile (Johnson et al., 1980) is therefore unlikely on the basis of this evidence. It may exist in a protonated form, but it would not appear that this is exactly the same species as the form I observed in the nonactivated enzyme. As discussed above, the alternative is a tightly bound, constrained dianion.

Any role of the pyridoxal phosphate in a catalytic mechanism must take into account the high possibility of some type of direct phosphate to phosphate interaction as evidenced by the X-ray data, the pyrophosphate inhibition of pyridoxal-reconstituted phosphorylase, and the high rate of reconstitution with pyridoxal pyrophosphate. This mechanism would also require that the coenzyme phosphate be in some other form than a free dianion and may have to allow for the absence of acid catalytic assistance. Such a mechanism is presently being formulated in concert with a detailed X-ray crystallographic analysis of the binding of cyclic phosphate and other substrate analogues (R. J. Fletterick et al., unpublished results).

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Synthesis of 1-Chloro-2-oxohexanol 6-Phosphate, a Covalent Active-Site Reagent for Phosphoglucose Isomerase†

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ABSTRACT: A new covalent active site reagent, 1-chloro-2-oxo-6-hexanol 6-phosphate, has been synthesized from glutaric acid monomethyl ester and characterized by NMR spectroscopy. Inactivation of phosphoglucose isomerase, when incubated with various modifier concentrations, was found to be pseudo first order with respect to enzyme concentration (half-life of inactivation 6 h at pH 7.5 (30 °C) and 2.0 μM active site concentration) but showed saturation kinetics for the dependence on inactivator concentration. This saturation phenomenon demonstrates the occurrence of a reversible enzyme–inhibitor complex (K_{diss} = 14.3 mM) preceding the irreversible inactivation via the chloromethyl oxo groups. Substrate or competitive inhibitors such as 6-phosphogluconate or 5-phosphoarabinonate protect against inactivation of the isomerase by the modifying reagent.

Kinetic studies on the effect of pH and temperature on substrate and inhibitor binding to phosphoglucose isomerase [D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9] yielded a proposal for the mechanism of this enzyme that involves both a histidine and a lysine residue as catalytically functional groups of the enzyme protein (Dyson & Noltmann, 1968; Noltmann, 1972). Efforts have been expended over the past several years to find suitable modifying reagents for these two amino acid residues in order to determine the validity of this proposal. Techniques such as dye-sensitized photooxidation (Chatterjee & Noltmann, 1967), carboxamidomethylation (Schnackerz & Noltmann, 1970), and Schiff base formation with pyridoxal 5′-phosphate followed by borohydride reduction (Schnackerz & Noltmann, 1971) have provided supporting evidence for this mechanism. Whereas our proposal calls for the involvement of the imidazolyl nitrogen of a histidine in the proton transfer between carbons 2 and 1 of the hexosephosphates, Rose's laboratory has advanced the concept that a glutamic carboxyl performs this function (Rose, 1975). For further study of this question and specifically determination of an active-site label that could be used for structural studies, the present investigation was directed toward the synthesis of a halomethyl ketone derivative of the substrate. It was hoped that such a reagent could be covalently bound to the active-site of phosphoglucose isomerase and, after appropriate cleavage of the enzyme molecule into its peptides, serve to identify an amino acid residue involved in the isomerization process, i.e., histidine or glutamic acid or both.

Precedents for the use of halomethyl ketone reagents to label enzymes with similar functions, notably triosephosphate isomerase and aldolase, have been provided by Hartman (1970 a,b; Hartman et al., 1973), Coulson et al. (1970), and Burton & Waley (1966). We are particularly interested in resolving the question of whether a histidine or a glutamic acid residue is involved in the phosphoglucose isomerase reaction. Evidence provided for triosephosphate isomerase at the level of total sequence analysis appears to indicate unequivocally that for this enzyme a glutamic acid is the critical residue. The use of a covalently bound reagent will also be helpful in providing chemical evidence for the hypothesis advanced by Shaw & Muirhead (1976) and Bruch et al. (1976) that the active sites of phosphoglucose isomerase are located at the interface be-

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