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Function of Pyridoxal 5′-Phosphate in Glycogen Phosphorylase: A Model Study Using 6-Fluoro-5′-deoxypyridoxal- and 5′-Deoxypyridoxal-Reconstituted Enzymes†

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ABSTRACT: A new vitamin B_6 analogue, 6-fluoro-5′-deoxypyridoxal (6-FDPL), was synthesized and characterized. This analogue, as well as 6-fluoropyridoxal (6-FPAL), 6-fluoropyridoxal phosphate (6-FPLP), and 6-fluoropyridoxine, showed positive heteronuclear ^1H–^{19}F nuclear Overhauser effects between the 5′-protons and the 6-fluorine. Apophosphorylase reconstituted with 6-FDPL showed 1% of the activity of the native enzyme in the presence of phosphite. The kinetic pattern, apparent pH optimum of activity, and the activity–temperature dependency of the 6-FDPL–enzyme were virtually identical with those of phosphorylase reconstituted with the parent compound, 6-FPAL [Chang, Y. C., & Graves, D. J. (1985) *J. Biol. Chem. 260*, 2709–2714], except the K_m of phosphite toward the 6-FDPL–enzyme was 9 times higher than that with the 6-FPAL–enzyme and the 6-FDPL–enzyme showed a lower V_{max} value. Phosphorylase reconstituted with 5′-deoxypyridoxal (DPL) also showed activity in the presence of phosphite. The kinetics and the temperature–activity dependency of this reconstituted enzyme were investigated. ^{19}F nuclear magnetic resonance studies showed that the binding of glucose 1-phosphate to a 6-FDPL–enzyme–adenosine 5′-phosphate (AMP) complex shifted the ^{19}F signal 0.6 ppm upfield, whereas a 2.1 ppm change was observed when the 6-FPAL–enzyme–AMP formed a complex with glucose 1-phosphate [Chang, Y. C., Scott, R. D., & Graves, D. J. (1986) *Biochemistry 25*, 1932–1939]. Analysis of the activation parameters, activation enthalpy and activation entropy, of the reaction of glycogen degradation catalyzed by phosphorylase containing pyridoxal phosphate, 6-FDPL, pyridoxal, or DPL showed that modifications of the coenzyme molecule affected only the activation entropy, not the activation enthalpy. Results of this study indicate that the protein structure surrounding the coenzyme molecule, as well as the coenzyme configuration, is altered upon the binding of ligands. The 5′-OH group of the protein-bound coenzyme is a necessary factor for the completion of these conformational changes. A correct transformation of the protein structure, coordinated by the coenzyme molecule, is required for the high efficiency of catalysis.

Kinetic and equilibrium dialysis studies have shown that the activity of glycogen phosphorylase is determined by an equilibrium between at least two conformers, the active "R" and the inactive "T" conformers (Helmreich et al., 1967; Kastenschmidt et al., 1968). This equilibrium is affected by the binding of different ligands to the protein (Graves & Wang, 1972; Klein & Helmreich, 1980; Madsen & Withers, 1984). The reactivity of phosphorylase toward SH-modifying reagents (Avramovic-Zikic et al., 1970) as well as the electron spin resonance (ESR)[1] and fluorescence spectral studies of

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[1] Abbreviations: 6-FPAL, 6-fluoropyridoxal; 6-FPLP, 6-fluoropyridoxal phosphate; DPL, 5′-deoxypyridoxal; 6-FDPL, 6-fluoro-5′-deoxypyridoxal; NMR, nuclear magnetic resonance; AMP, adenosine 5′-phosphate; glucose-1-P, glucose 1-phosphate; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal 5′-phosphate; TEAA, tetraethylammonium acetate; DTE, dithioerythritol; NOE, nuclear Overhauser effect(s); ESR, electron spin resonance; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; Me_4Si, tetramethylsilane; Me_2SO, dimethyl sulfoxide.

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covalently or noncovalently bound probes (Campbell et al., 1972; Brooks et al., 1974) indicates that the protein structure can be transformed into at least four distinct conformers. The interconversion among these conformers is also induced by the binding of ligands. These observations clearly demonstrate the dynamic nature of the protein structure; however, detailed information about the structures of different conformers is not available. X-ray crystallographic studies showed that the substitution of the substrate glucose-1-P in the catalytic site of phosphorylase with glucose, glucose cyclic 1,2-phosphate, heptenitol, or heptulose 2-phosphate (Madsen et al., 1978; Sprang & Fletterick, 1979; Sprang et al., 1982; Withers et al., 1982; Johnson et al., 1980; McLaughlin et al., 1984) induces substantial rearrangement of the protein structure. These observations indeed provide information about the detailed pictures of phosphorylase–ligand complexes, but the high-resolution structure of the enzyme in the fully activated state, carrying out catalysis, has not been obtained. Recently, apophosphorylase reconstituted with 6-fluoropyridoxal (6-FPAL) or 6-fluoropyridoxal 5′-phosphate (6-FPLP) has been used as a model system to study the conformational changes of different protein–ligand complexes in solution (Chang et al., 1985). The results show that the binding of the substrate, glucose-1-P or phosphate, shifts the NMR signal of the protein-bound fluorine nucleus. NMR studies of macromolecule-bound fluorine nuclei show that the neighboring protons, carbonyl groups, or aromatic rings may interact with the target nucleus and influence its chemical shift [see review of Gerig (1978)]. The magnitudes of these effects are usually determined by the distances between interacting nuclei. Therefore, the chemical shift changes of the 6-FPAL or 6-FPLP–phosphorylase signals indicate that, during catalysis, the protein structure around the coenzyme binding site undergoes changes. These structural changes may affect the interactions between the bound fluorine nucleus with nearby residues, either amino acids or the remaining atoms of the coenzyme, and hence influence the resonance frequency.

To identify the factors influencing the chemical shift of the fluorine nucleus bound to 6-FPAL or 6-FPLP–phosphorylase, another vitamin B_6 analogue, 6-FDPL, was synthesized, and phosphorylase reconstituted with this analogus was thoroughly studied. A comparison of the ^{19}F NMR spectroscopic properties of phosphorylases reconstituted with 6-FPAL and 6-FDPL showed that the nature of the substituent at the 5′-position of the coenzyme, a methyl group in 6-FDPL and a hydroxymethyl group in 6-FPAL, is a determining factor influencing the chemical shift of the ^{19}F NMR resonance.

Studies have shown that the structural characteristics of phosphorylase reconstituted with 5′-deoxypyridoxal (DPL) are virtually identical with those of the native enzyme (Kastenschmidt et al., 1968). This enzyme can also be partly activated by the presence of phosphite (R. F. Parrish and D. J. Graves, unpublished results). A more complete study of the kinetics and the temperature–activity dependency of the DPL–enzyme is carried out in this work. The activation parameters of the glycogen phosphorolytic reaction catalyzed by the apophosphorylase reconstituted with PLP, pyridoxal, DPL, or 6-FDPL are evaluated to elucidate the involvement of the coenzyme in the process of catalysis.

Materials and Methods

Rabbit skeletal muscle was obtained from Pel-Freeze Co. The purchase of [U-^{14}C]glucose-1-P was from Amersham. Pyridoxine hydrochloride was purchased from National Biochemical Corp. Pyridoxal, pyridoxal phosphate, and glucose-1-P were obtained from Sigma Chemical Co. Synthesis of 6-fluoropyridoxal and 6-fluoropyridoxal phosphate and the reconstitution of apophosphorylase with these fluorinated analogues were reported earlier (Chang & Graves, 1985). 5′-Deoxypyridoxal (DPL) was kindly supplied by Dr. David E. Metzler of Iowa State University.

Chemical Synthesis. 5′-Deoxypyridoxine was synthesized from pyridoxine hydrochloride by the methods of Ikawa (1968) and Korytnyk and Ikawa (1970). 5′-Deoxypyridoxine was fluorinated at the 6-position by the method of Korytnyk and Kravatava (1973) with modifications. 5′-Deoxypyridoxine (11 g) in 300 mL of water was slowly mixed with 60 mL of HCl (6 N) containing 5 g of sodium nitrite and 4 g of aniline. During this process, the temperature of the solution was kept between 5 and 10 °C, and the pH was kept at 8.0 by adding 5 N NaOH. After the solution was mixed, it was stirred at room temperature for another hour. The precipitate, 5-deoxy-6-phenylazopyridoxine (I), was collected by filtration, washed with cold water, and dried in a vacuum oven at 40 °C. (The yield of this reaction was ~80%.) A suspension containing 4 g of compound I, 350 mL of ethanol, and 0.5 g of Pd/C (10%) was hydrogenated with Parr hydrogenator under 34 psi of hydrogen for 16 h. After hydrogenation, fine powders (Pd/C) were filtered out through a glass-scintered funnel layered with Celite. The filtrate was concentrated to 60 mL with a rotary evaporator and then kept on ice for 30 min. White crystals that formed in the solution were collected by filtration and washed with cold ethanol. The filtrate was further concentrated by a rotary evaporatory to 30 mL and stored on ice, and the white crystals were again filtered out. The white crystals isolated from the solution were 6-amino-5-deoxypyridoxine (II). Compound II thus obtained weighed 2 g, and the yield of this reaction was 50%. Compound II (0.7 g) was dissolved in 16 mL of fluoroboric acid (40%) and cooled to −5 °C in a salt–ice slurry. Sodium nitrite (0.3 g) was slowly added to the solution while the temperature of the solution was kept below −5 °C. The resulting solution was kept stirring for another 2 h at 10 °C. The solution was then extracted 3 times with an equal amount of ether. Ether was removed from the combined extracts by rotary evaporation, and the solid residue was redissolved in 4 mL of solution containing 0.2 M TEAA and 15% methanol at pH 6.0. This mixture was applied on a reverse-phase C_{18} HPLC column and was developed by using a gradient of 15–40% methanol in 0.2 M TEAA at 1 mL/min. The duration of the gradient was 40 min. Fractions with absorbance at 290 nm were pooled and lyophilized. The product, 6-fluoro-5-deoxypyridoxine (III), (0.12 g) was obtained with 17% yield. Compound III (0.2 g) was dissolved in 30 mL of chloroform. To this solution, 2 g of MnO_2 was added, and the mixture was stirred at room temperature. After 18 h, the black powder was removed by filtration with a glass scintered funnel layered with Celite and then washed several times with chloroform. Chloroform in the filtrate was evaporated by rotary evaporation. The residue was redissolved in a solution containing 0.05 M TEAA and 15% methanol at pH 6.0. The mixture was then applied to a C_{18} HPLC column and eluted by using a gradient of 15–80% methanol in 0.05 M TEAA at pH 6.0. The fractions containing 6-fluoro-5-deoxypyridoxal (IV), with absorbance at 360 nm, were pooled and extracted 6 times with ether. The ether was removed from the combined extracts by rotary evaporation and dried in a desiccator. The resulting yellow powder, 6-fluoro-5-deoxypyridoxal (IV), weighed 52 mg, and the yield was 43%. Properties of compounds III and IV are listed in Table I. Purities of compounds III and IV were checked by analytical HPLC, TLC, and ^1H NMR (Table I),

Table I: Properties of 6-Fluoro-5′-deoxypyridoxine and 6-Fluoro-5′-deoxypyridoxal

	^{1}H NMR[a]		UV–vis absorbance (M^{-1} cm^{-1})			
	chemical shift (ppm)	relative peak area	in 0.1 N HCl	in 0.1 N NaOH	TLC[b] R_f	HPLC[c] (%)
6-fluoro-5′-deoxypyridoxine	4.62	2	$\epsilon_{287} = 6.12 \times 10^3$	$\Delta_{312} = 6.50 \times 10^3$	0.82	50
	2.24	3				
	2.11	3				
6-fluoro-5′-deoxypyridoxal	10.39	1	$\epsilon_{363} = 2.68 \times 10^3$	$\epsilon_{405} = 3.91 \times 10^3$	0.93	65
	2.39	3	$\epsilon_{295} = 1.06 \times 10^3$	$\epsilon_{316} = 1.34 \times 10^3$		
	2.34	3	$\epsilon_{254} = 4.47 \times 10^3$	$\epsilon_{260} = 2.41 \times 10^3$		

[a] Samples were dissolved in Me_2SO. Chemical shift values were relative to the signal of the internal standard Me_4Si. [b] Developing solution was 1-butanol–acetic acid–water (4:15 v/v), and spray solution was Gibb's reagent. [c] A reverse-phase C_{18} microsol column and an eluent consisting of a gradient of 15–80% methanol in 0.05 M TEAA were used.

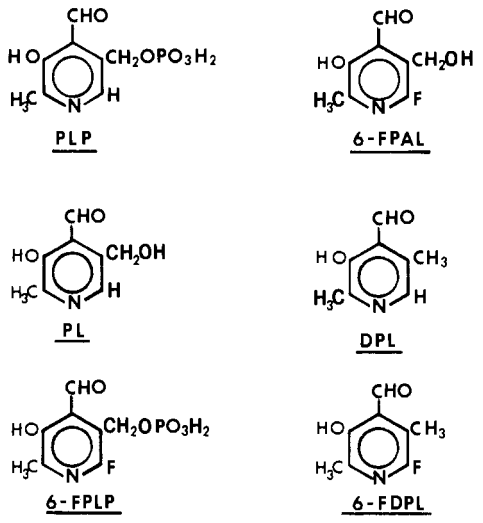

FIGURE 1: Chemical structure of pyridoxal 5′-phosphate (PLP), pyridoxal (PL), 6-fluoropyridoxal 5′-phosphate (6-FPLP), 6-fluoropyridoxal (6-FPAL), 5′-deoxypyridoxal (DPL), and 6-fluoro-5′-deoxypyridoxal (6-FDPL).

and no contaminant was found in any of these tests. Chemical structures of 6-FDPL and other PLP analogues used in this study are shown in Figure 1.

Enzyme. The resolution of PLP from phosphorylase was carried out by the method of Graves et al. (1975). To reduce the residual activity due to the presence of any unresolved enzyme, the resulting apoenzyme was routinely subjected to a second resolution treatment by incubating apoenzyme (1.5 mg/mL) in the deforming buffer (0.1 M cysteine and 0.4 M imidazole–citrate at pH 6.1) on ice for 2.5 h. An equal volume of 100% saturated ammonium sulfate was added to the protein solution at the end of incubation to precipitate the enzyme. Protein was collected by centrifugation, dissolved in a minimal amount of a buffer solution containing 0.05 M cysteine and 0.2 M imidazole–citrate at pH 6.1, and dialyzed against the same buffer overnight. Protein was concentrated by another ammonium sulfate precipitation and dialyzed against a buffer solution containing β-glycerophosphate (0.05 M), DTE (5 mM), and EDTA (2 mM) at pH 6.8. The activity of the enzyme after two consecutive resolution treatments was lower than 0.05% of the native phosphorylase. Phosphorylase reconstituted with 6-FDPL was obtained by incubating apophosphorylase with a 2-fold excess of this fluorinated compound at 30 °C for 30 min. The enzyme solution thus obtained was then dialyzed against the same buffer solution overnight. The UV–vis spectrum of the resulting enzyme showed an absorption band at 339 nm. The stoichiometry of the coenzyme bound to protein was determined by the method of Baranowski et al. (1957). One mole of phosphorylase was found to contain 0.96 mol of 6-FDPL. Phosphorylase reconstituted with DPL was obtained by incubating apophosphorylase with a 1.2-fold excess of DPL at 30 °C for 1 h. The resulting enzyme was used without further treatment. Rates of incorporation of [^{14}C]glucosyl residues into glycogen were determined by utilizing the filter paper assay of Thomas et al. (1968).

NMR Spectroscopy. ^{19}F NMR spectra were routinely obtained at 282.4 MHz on a Bruker WM 300 spectrometer. A spectral width of 20000 Hz was employed with 28-μs (a 60° pulse) pulse width and a repetition time of 1 s. A cylindrical microcell (Wilmad) containing a 1-mL sample solution was fitted in a 10-mm NMR tube containing D_2O (50%) and trifluoroacetate (0.05 mM, pH 6.8). The D_2O outside the microcell was used for field-frequency lock, and the trifluoroacetate signal was used for chemical shift referencing. The ^{1}H NMR spectrum was obtained at 300 MHz on a NT300 NMR spectrometer. A spectral width of 3000 Hz was used with a 7-μs (a 60° pulse) pulse width and a repetition time of 3 s. A capillary (1 mm) containing 1% Me_4Si in $CDCl_3$ was fitted in the center of the NMR tube (5 mm). D_2O in the sample solution was used for a lock, and the signal of Me_4Si in the capillary was used as a chemical shift reference. Preceding NOE experiments, a ^{1}H NMR spectrum of the sample was obtained with the decoupling channel of the ^{19}F probe to determine the frequencies of the proton resonances of the sample. For NOE experiments, a selective radio-frequency pulse was applied for 10 s, followed by the observation pulse and the next preirradiation if more than one transition was required to get accurate measurement of the intensity. The selective irradiation was applied over a wide spectral range, covering the proton resonances of the coenzymes. The changes in intensity were measured from changes in the integrated area.

RESULTS

The pK_a values of the functional groups of 6-FDPL in 20% D_2O were determined by ^{19}F NMR spectroscopy. The ^{19}F NMR spectrum of 6-FDPL at pHs higher than 2 shows two signals. On the basis of the electronic absorption bands of 6-FDPL (Table I), the major signal was assigned to the aldehyde form and the minor signal to the hydrated form of the fluorinated compound. The pH dependencies of the chemical shifts of these species are studied (data not shown). The titration curve of the aldehyde form of 6-FDPL shows two transitions. The first transition with a midpoint of 7.7 was assigned to the ionization of the 3-phenolic group and the second transition with an estimated midpoint lower than 0 to the protonation of the pyridine nitrogen. The first transition for the hydrated form (7.5) is similar to that of the aldehyde form. Low concentration of the hydrated form at pH 2 or lower prevented measurement of the second transition. The pK_a values of 6-FDPL are virtually identical with the corresponding values of 6-fluoropyridoxal phosphate or 6-fluoro-

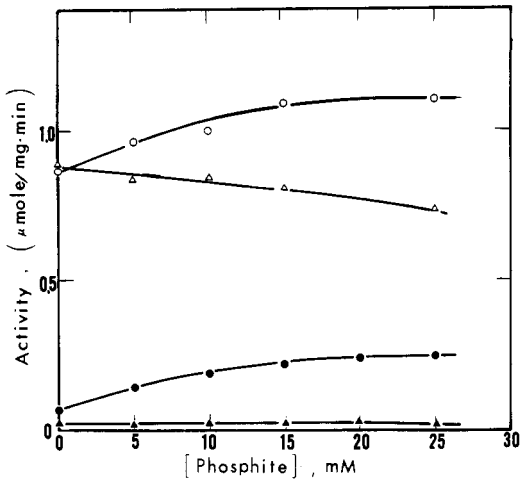

FIGURE 2: Enzymatic activities of apophosphorylase and 6-FDPL–phosphorylase in the presence of varied amounts of phosphite. The activities of apophosphorylase after the first and second resolution treatment are shown as (△) and (▲), respectively. The activities of 6-FDPL–enzymes made of apophosphorylases after the first and the second resolution treatment are respectively shown as (○) and (●). The reaction mixture contains 50 mM glucose-1-P, 1 mM AMP, 40 mM β-glycerophosphate, 2 mM EDTA, 5 mM DTE, 1% glycogen, 0.2 mg/mL apoenzyme or reconstituted enzyme, and varied concentrations of phosphite as indicated in the figure.

pyridoxal (Chang et al., 1985). This result shows that the substitution of the 5′-phosphate group of 6-FDLP or the 5′-OH of 6-FPAL by a hydrogen does not affect the pK_a values of other functional groups of the same molecule. On the basis of a NOE study of *o*-fluorotoluene, Bell and Saunders (1970) found that there are significant dipole–dipole interactions between the fluorine nucleus and the protons of the neighboring methyl group. We also investigated the possibility that there are dipole–dipole interactions between the 6-fluorine with the 5′-protons of 6-FDPL, 6-FPLP, 6-FPAL, or 6-fluoropyridoxine. The change in the intensity of the fluorine signals of these fluorinated compounds was examined after they were exposed to preirradiation at selected radio frequencies, covering the range of resonating frequencies of the protons of these compounds. The maximal NOE of these compounds were found at frequencies where the 5′-protons of these compounds resonate, around 5 ppm for 6-FPLP, 6-FPAL, and 6-fluoropyridoxine, and at 2.3 ppm for 6-FDPL. It is also found that the NOE of 6-FPLP, 6-FDPL, and 6-fluoropyridoxine are around 11%, whereas that of 6-FPAL is only 5%. Because the NOE is primarily due to the dipole–dipole interactions between spins and its magnitude is determined by the distance between interacting spins, the significantly different NOE of these compounds provide direct evidence that the average distance between the 5′-protons and 6-fluorine in 6-FPLP, 6-FDPL, or 6-fluoropyridoxine is smaller than that in 6-FPAL. This observation is consistent with the structures of these fluorinated compounds determined by ^{1}H NMR: In aqueous solutions, most 6-FPAL forms a hemiacetal, where the 4-, 5-, 5′-, and 4′-C's and 5′-O form a closed five-member ring; this particular structure is absent in the remaining compounds (Chang et al., 1985). The rotation around the bond connecting the 5 and 5′-C's of the latter compounds can bring the 5′-protons in close proximity to the 6-fluorine, whereas the ring structure of 6-FPAL hinders such a rotation.

The enzymatic activities of apophosphorylase (after the first or second resolution treatment) reconstituted with 6-FDPL were assayed in the presence and absence of phosphite. These results, along with the activity of apophosphorylase assayed under identical conditions, are shown in Figure 2. This figure clearly shows that phosphite activates the reconstituted enzyme and inhibits the residual activity of the apoenzyme. The same figure also shows that the second resolution treatment of phosphorylase reduces the residual activity of the apoenzyme from 1% to 0.05% but does not significantly affect the degree of activation of the 6-FDPL–enzyme induced by phosphite. This observation thus rules out the possibility that the activity induced by the presence of phosphite is somehow related to the residual activity of the apoenzyme. Kinetic studies of the native and pyridoxal-reconstituted phosphorylase have shown that phosphite at a concentration of 15 mM or higher competes with glucose-1-P for its binding site on the protein (Parrish et al., 1977). A similar inhibitory effect of the same phosphate analogue on the enzymatic activity of phosphorylase containing 6-FDPL or DPL was not observed (Figure 2). This may be due to (1) the protein structure surrounding the glucose-1-P binding site in the 6-FDPL– or DPL–enzyme is different from that in the native or the pyridoxal–enzyme and/or (2) the inhibitory effect of phosphite was masked by the more significant activation effect of the same anion in the 6-FDPL– or DPL–enzyme in the concentration range examined.

Table II: Kinetic Parameters and Activation Entropy Changes, $\Delta S^{\ddagger}$, of Phosphorylase Containing PLP or Its Analogues

	kinetic parameters[a]	$\Delta S^{\ddagger}$ (eu)
PLP–phosphorylase	V_{max} = 66.7 IU[b] K_M(glucose-1-P) = 8 mM[b]	0
6-FPLP–phosphorylase	V_{max} = 17.8 IU[c] K_M(glucose-1-P) = 7.4 mM[c]	−2.6
pyridoxal–phosphorylase	V_{max} = 15.4 IU[d] K_a = 0.8 mM[d] K_a' = 0.6 mM[d] K_s = 18.0 mM[d] K_s' = 22.0 mM[d] K_m = [glycogen] = 0.01%[e] V_{max} = 0.8 IU[f] K_m(glucose-1-P) = 21.3 mM[f] K_m(phosphite) = 0.8 mM[f] K_m(glycogen) = 0.005%[f]	−2.9
6-FPAL–phosphorylase	V_{max} = 4.2 IU[d] K_a = 0.8 mM, K_a' = 0.9 mM[c] K_s = 15.4 mM, K_s' = 13.9 mM[c]	−5.5
5′-DPL–phosphorylase	V_{max} = 2.8 IU K_a = 7.3 mM, K_a' = 9.0 mM K_s = 15.6 mM, K_s' = 12.6 mM	−6.3
6-FDPL–phosphorylase	V_{max} = 0.7 IU K_a = 7.3 mM, K_a' = 7.2 mM K_s = 11.9 mM, K_s' = 12.1 mM	−9.1

[a] K_a = dissociation constant of phosphite from an enzyme–phosphite–AMP–glycogen complex, K_a' = dissociation constant of phosphite from an enzyme–phosphite–AMP–glycogen–glucose-1-P complex, K_s = dissociation constant of glucose-1-P from an enzyme–AMP–Glycogen–glucose-1-P complex, and K_s' = dissociation constant of glucose-1-P from an enzyme–AMP–glycogen–glucose-1-P–phosphite complex. [b] The PLP–enzyme was prepared by incubating apophosphorylase with a 1.2-fold excess of PLP at 30 °C for 30 min. The reaction mixture was then extensively dialyzed against a buffer solution containing β-glycerophosphate (40 mM), EDTA (2 mM), and DTT (8 mM) at pH 6.8. The V_{max} value is at least 95% of that of the native unresolved phosphorylase. [c] Chang & Graves (1985). [d] Chang et all. (1983). [e] Parrish et al. (1977).

Enzymatic activities of 6-FDPL-reconstituted phosphorylase in the presence of different concentrations of glucose-1-P (from 10 to 60 mM) and phosphite (from 3 to 20 mM) were assayed. Double-reciprocal plots of initial rates vs. concentration of glucose-1-P in the presence of different amounts of phosphite (data not shown) converge on the *x* axis as obtained with 6-FPAL- and pyridoxal-reconstituted phosphorylase (Chang & Graves, 1985; Chang et al., 1983), suggesting a sequential mechanism. The kinetic parameters derived from this graph are shown in Table II. In the same table, kinetic parameters of phosphorylase reconstituted with PLP, 6-FPLP, 6-FPAL,

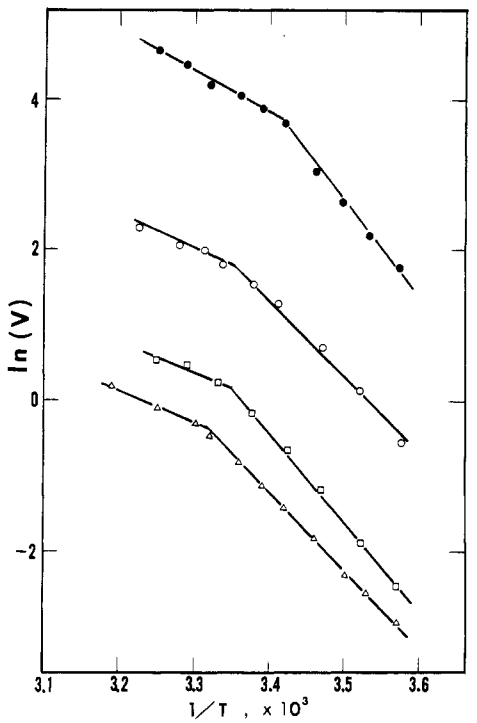

FIGURE 3: Arrhenius plots of the activities of phosphorylase reconstituted with PLP (●), pyridoxal (○), DPL (□), and 6-FDPL (△) at different temperatures. The reaction mixture for the native phosphorylase contains 25 mM glucose-1-P, 1 mM AMP, and 1% glycogen. The reaction mixture for the pyridoxal–enzyme contains 60 mM glucose-1-P, 1 mM AMP, 1% glycogen, and 5 mM phosphite. The reaction mixture for the DPL- and 6-FDPL–enzymes contains 75 mM glucose-1-P, 1% glycogen, 25 mM phosphite, and 1 mM AMP.

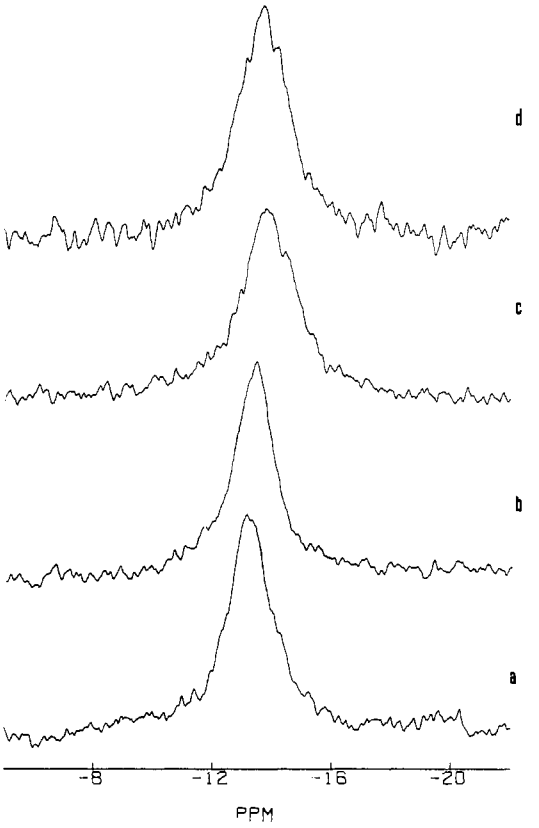

FIGURE 4: ^{19}F NMR spectra of 6-FDPL-reconstituted phosphorylase (30 mg/mL) (a) in the presence of 40 mM β-glycerophosphate, 5 mM DTE, and 2 mM EDTA, pH 6.8, (b) in the presence of 25 mM phosphite and 1 mM AMP, (c) in the presence of 25 mM phosphite, 1 mM AMP, and 15 mM glucose-1-P, and (d) in the presence of 1 mM AMP, 25 mM phosphite, and 150 mM glucose-1-P. All spectra were recorded at 30 °C.

and pyridoxal are also listed for comparison. The dissociation constants of glucose-1-P toward 6-FDPL–phosphorylase, K_s and K_s', respectively, are similar to those of 6-FPAL–enzyme, whereas the dissociation constants of phosphite, K_a and K_a' values, of the former enzyme are about 9 times higher than those of the latter enzyme. This comparison shows that the substitution of the 5′-OH group of the enzyme-bound 6-FPAL with a hydrogen does not affect the binding of glucose-1-P to the enzyme but weakens the binding of phosphite and lowers the V_{max}.

Parrish and Graves (unpublished results) found that phosphorylase reconstituted with DPL can be partly activated by including phosphite in the assay solution. To complete the kinetic study of this reconstituted enzyme, activities of the DPL–enzyme in the presence of different concentrations of glucose-1-P (from 10 to 75 mM) and phosphite (from 3 to 25 mM) were assayed at 30 °C. Double-reciprocal plots of initial rates vs. concentrations of glucose-1-P in the presence of different amounts of phosphite show a pattern similar to that of 6-FDPL-reconstituted phosphorylase, straight lines converging at the x axis. Kinetic parameters are evaluated and listed in Table II. The dissociation constants, K_s, K_s', K_a, and K_a', are virtually identical with their counterparts of 6-FDPL–enzyme. However, the V_{max} value of DPL–enzyme is 4 times higher than that of 6-FDPL–enzyme.

To investigate the possibility that phosphorylase reconstituted with 6-FDPL may undergo a catalytic process different from that of the native phosphorylase or 6-FPAL–enzyme, the pH and temperature dependencies of the enzymatic activities of both the native and the reconstituted enzymes were studied. The apparent optimal pHs for the activities of these enzymes are almost identical, at around 6.3 and 6.4. These results show that the functional groups involved in the general acid or base catalysis of these enzymes are likely to be identical. The Arrhenius plots of the activities of enzymes at different temperatures are shown in Figure 3. Curves of both the PLP– and 6-FDPL–enzymes show a biphasic pattern, but the transition temperature of the 6-FDPL–enzyme is significantly higher than that of the PLP-reconstituted enzyme. However, the Arrhenius plot of the 6-FDPL-reconstituted enzyme is almost identical with that of 6-FPAL-reconstituted enzyme (Chang et al., 1985). On the basis of these comparisons, it is likely that, in the presence of a sufficient amount of substrates and phosphite, phosphorylases reconstituted with 6-FPAL and 6-FDPL share a common catalytic mechanism. The Arrhenius plots of phosphorylases reconstituted with 5′-DPL and pyridoxal are also shown in Figure 3. These plots show a pattern similar to that of 6-FDPL–enzyme. Above the transition temperatures, around 28 °C, the activation energies of all the enzymes examined are indistinguishable. To test the possibility that the variations of enzymatic activities of these enzymes at different temperatures are due to the change of the affinities of substrate or activator toward the enzyme, kinetic parameters of phosphorylase reconstituted with pyridoxal were obtained at 7 °C. The dissociation constants of substrates and phosphite are listed along with those obtained at 30 °C in Table II for comparison. Obviously, a temperature change from 30 to 7 °C does not affect the dissociation constants of ligands to the pyridoxal–enzyme. This observation indicates that temperature is a determining factor for chemical reaction rates of the enzyme-bound substrates but not for the affinities of ligands toward the enzyme.

^{19}F NMR spectra of phosphorylase reconstituted with 6-FDPL (30 mg/mL) under different conditions are shown in

Figure 4. The free enzyme in a buffer solution containing 40 mM β-glycerophosphate, 2 mM EDTA, and 5 mM DTE at pH 6.8 shows a peak at −13.2 ppm (relative to the signal of 0.03 mM trifluoroacetate at pH 6.8) with a half-height width of 380 Hz (Figure 4a). When phosphite (25 mM) and AMP (1 mM) are included in the enzyme solution, neither the chemical shift nor the line width of the protein signal is affected (Figure 4b). When 15 mM glucose-1-P was included in the solution, the protein signal was shifted to −13.6 ppm, with a half-height width of 580 Hz (Figure 4c). When the glucose-1-P concentration in the sample is increased to 75 mM, the signal is further shifted to −13.8 ppm, and the line width becomes 500 Hz. The protein signal stays at −13.8 ppm even when the glucose-1-P concentration is further increased to 150 mM, whereas the line width becomes 480 Hz (Figure 4d). The gradual change of chemical shift and line width of the ^{19}F NMR signal of the 6-FDPL–enzyme–AMP–phosphite complex in the presence of varied amounts of glucose-1-P may be described by a two-site exchange model. In this model, the chemical shift of the enzyme–AMP–phosphite complex is different from that of the same complex with bound glucose-1-P, and these two species undergo an exchange process at an intermediate rate. Therefore, the variation of chemical shift is a function of the concentration of glucose-1-P and can be described by a saturation curve, whereas the line width shows a bell-shaped change: the maximal line width is observed when half of the protein becomes ligand bound and the remaining half is still free of ligand [a more detailed description of this model can be found in Chang et al. (1985)]. Because the chemical shift of the 6-FDPL–enzyme–AMP–phosphite complex in the presence of 75 mM glucose-1-P is not affected by further increase of ligand concentration, to 150 mM, the chemical shift value, 14.1 ppm, of 6-FDPL–enzyme under these conditions is likely to correspond to that of a 6-FDPL–enzyme–AMP–glucose-1-P–phosphite complex. This analysis shows that the binding of glucose-1-P to a 6-FDPL–enzyme–AMP–phosphite complex shifts the protein signal 0.6 ppm upfield. This value, however, is much less than the chemical shift change, 2.1 ppm, induced by the binding of glucose-1-P toward a 6-FPAL–enzyme–AMP–phosphite or a 6-FPLP–enzyme–AMP complex (Chang et al., 1985).

Discussion

Results of this study show that phosphorylases reconstituted with 6-FDPL and with 6-FPAL are indistinguishable in many aspects, such as the kinetic mechanism, the Arrhenius plot (Figure 3), the apparent pH optimum for the enzymatic activity, and the affinities of glucose-1-P to the protein (Table II). The binding of phosphite to 6-FDPL–enzyme is weaker than that to the 6-FPAL–enzyme, and the V_{max} value of the former enzyme is lower than that of the 6-FPAL–protein (Chang et al., 1985). The similarities between these enzymes indicate that they share a common catalytic process. The different K_m values may be explained by either the 5′-OH group of the coenzyme in 6-FPAL–enzyme participating in the formation of the binding site for phosphite or the same OH group interacting with the bound phosphite, probably by forming a hydrogen bond. Either of these mechanisms tightens the binding of phosphite to 6-FPAL–enzyme. However, when the 5′-OH group of the coenzyme is replaced by a hydrogen atom, as in 6-FDPL– or DPL–enzyme, none of these mechanisms exists and, as a result, leads to a weaker binding of phosphite toward these enzymes. Because the activation energy of 6-FDPL–enzyme above 30 °C is virtually identical with that of 6-FPAL–enzyme, the lower activity of the 6-FDPL–enzyme may be explained by its lower activation entropy. When the characteristics of phosphorylase reconstituted with DPL are compared with those of phosphorylase reconstituted with pyridoxal (Chang et al., 1983), the substitution of the 5′-OH group of the coenzyme in pyridoxal enzyme with a hydrogen was also found to weaken the binding of phosphite to the protein and lower the V_{max} value but did not affect other properties of the enzyme. The same explanations for the differences between 6-FDPL– and 6-FPAL–enzymes may also hold for those between DPL– and pyridoxal–enzymes. Kinetic parameters of the apophosphorylase reconstituted with PLP, 6-FPLP, 6-FPAL, 6-FDPL, pyridoxal, or 5′-DPL are listed in Table II. The variation of the K_m values for glucose-1-P toward these enzymes is between 1- and 3-fold, whereas the maximal difference among their V_{max} values is around 100-fold. Because the activation energies of these enzymes are virtually identical above 30 °C, the different V_{max} values are likely to arise from different activation entropies of the catalytic processes of these enzymes. The differences of the entropy of activation of different enzymes, $\Delta S^{\ddagger}$, shown in Table II, are calculated by the following equations. The free energy of activation, $\Delta F^{\ddagger}$, can be expressed by

$$\Delta F^{\ddagger} = -RT \ln k \quad (1)$$

where k is the rate constant, R is the gas constant, and T is the temperature. The $\Delta F^{\ddagger}$ term can then be expressed by

$$\Delta F^{\ddagger} = E_a - RT - T\Delta S^{\ddagger} \quad (2)$$

where E_a is the activation energy obtained as the slope of the Arrhenius plot. Combining eq 1 and 2, the entropy term can be expressed as

$$\Delta S^{\ddagger} = R \ln k + B \quad (3)$$

where B is a function of E_a and T. Because the activation energies of the enzymes listed in Table II are identical above 30 °C, the B term in eq 3 becomes a constant. Therefore, the difference between $\Delta S^{\ddagger}$ of the reconstituted enzyme with that of the PLP–enzyme can be calculated by

$$\Delta S^{\ddagger}(\text{reconstituted enzyme}) - \Delta S^{\ddagger}(\text{PLP–enzyme}) = R \ln \frac{k(\text{reconstituted enzyme})}{k(\text{PLP–enzyme})} \sim R \ln \frac{V_{max}(\text{reconstituted enzyme})}{V_{max}(\text{PLP–enzyme})} \quad (4)$$

Two generalizations can be deduced by analyzing the variation of the activation entropies among these enzymes. First, a specific modification of PLP or its analogues induces a characteristic decrease of the activation entropy of catalysis. For instance, the substitution of the 6-hydrogen of the coenzyme molecule in phosphorylase containing PLP, pyridoxal, or DPL by a fluorine atom decreases the activation entropy of these enzymes from the unsubstituted forms by a constant value of around 2.6 eu. The absence of covalent bonding between the pyridine ring and the 5′-phosphate of the coenzyme in phosphorylase containing pyridoxal or 6-FPL causes the activation entropy of these enzymes to be decreased from the values obtained for PLP– and 6-FPLP–enzymes by 2.9 eu. Furthermore, the entropy difference between the PLP– and DPL–enzymes as well as the entropy difference between the 6-FDPL– and 6-FPAL–enzymes is around 6.5 eu. A second conclusion is the change of the activation entropy of the enzyme reconstituted with a parent compound from that of the enzyme containing an analogue with modifications on more than one site of the parent compound is the sum of the entropy changes induced by individual modifications of the parent compound. For example, the entropy difference between 6-FPAL– and the PLP–enzyme is 5.5 eu, which is close to the

sum of the entropy changes induced by the substitution of the 6-hydrogen with a fluorine, 2.6 eu, and that induced by the cleavage of the covalent bonding between the 5′-phosphate and the 5′-OH of the coenzyme, 2.9 eu. The entropy difference between 6-FDPL and the native enzymes, 9.0 eu, is close to the sum of the entropy changes induced by the fluorination of the coenzyme (2.6 eu) and the entropy difference between DPL and native enzyme (6.3 eu). This analysis clearly shows that the influences of different modifications at the pyridine nitrogen or the 5′-position of the coenzyme molecule on the activation entropy are independent from one another. The physical meaning for the change of activation entropy has been clearly demonstrated by the kinetic studies of chymotrypsin (Bender et al., 1964; Jencks, 1969). On the basis of the study of the deacylation reaction of different acylchymotrypsins, the variation of activation entropy was found to be related to the similarities of the average orientation of the bound substrate with the orientations of the transition state of substrates. In the transition state, the reacting groups of the substrate are thought to be ideally aligned with respect to the catalytic groups of the enzyme. Because the substrates used in the activity assays of the enzymes listed in Table II are identical, the changes of activation entropy of different reconstituted phosphorylases are likely to be due to certain changes of the construction of the active site that result in the lower probability of the bound substrate to be utilized during catalysis.

Although the catalytic processes of phosphorylase reconstituted with 6-FDPL and 6-FPAL, in the presence of saturating amounts of phosphite and substrates, are virtually identical, these enzymes show significantly different ^{19}F NMR spectroscopic properties. The binding of glucose-1-P toward a 6-FPAL–enzyme–AMP–phosphite complex shifts the protein signal 2.1 ppm upfield, but the same process induces only a 0.6 ppm upfield shift of the signal for a similar protein complex containing 6-FDPL. For the 6-FPAL–enzyme, a protein-bound phosphite has been found to be required for the chemical shift change of the protein signal. In the absence of phosphite, the chemical shift of a 6-FPAL–enzyme–AMP–glucose-1-P complex is virtually identical with that of an enzyme–AMP complex (Y. C. Chang and D. J. Graves, unpublished results). These observations clearly show that the 5′-OH group of the coenzyme in a 6-FPAL–enzyme–phosphite–AMP complex plays important roles in determining the chemical shift of the protein signals. Two explanations may account for how the 5′-OH group and phosphite affect the chemical shift of the 6-fluorine nucleus of the protein-bound coenzyme. The first possibility is that the 5′-OH group, together with a bound phosphite, is required for a structural change of the coenzyme binding site induced by the binding of ligands. This change may alter the relative position between the coenzyme fluorine nucleus with respect to the neighboring amino acid residues and thus lead to a change of the chemical shift of the ^{19}F signal. The substitution of the 5′-OH group in 6-FPAL–enzyme with a hydrogen prevents such a structural change, and, therefore, the fluorine resonance cannot be significantly affected by the binding of glucose-1-P. The second possibility is that both the protein structure of the coenzyme binding site and the configuration of the coenzyme, which is a trans,trans configuration in the phosphorylase *b*–IMP–glucose-1-P crystal (Sansom et al., 1984), are affected by the binding of glucose-1-P. As a result, the distances between the ring fluorine in 6-FPLP– or 6-FPAL–enzyme with the neighboring amino acid residues as well as with the 5′-protons are changed. The observed chemical shift change of 6-FPLP– or 6-FPAL–enzyme is due to a combination of these changes. Among these distance changes, the change between the 5′-protons and the 6-fluorine may influence the chemical shift of the protein signal more effectively because it has been demonstrated that the chemical shift of a macromolecule-bound fluorine nucleus is strongly influenced by its interactions with neighboring protons; the magnitude of the effect of H–F interactions on the ^{19}F NMR chemical shift is inversely proportional to the sixth power of the distance between the protons with the target nucleus (Hull & Sykes, 1976). The positive NOE effects between the fluorine of these fluorinated compounds with the 5′-protons also show that the fluorine and the 5′-protons can be brought within a short distance to form strong dipole–dipole interactions. However, a similar reorientation of the coenzyme ring does not change the average distance between the 5′-protons with the 6-fluorine of the coenzyme in 6-FDPL–enzyme because of the symmetrical nature of the 5′-methyl group and because this methyl group probably undergoes a rapid rotation. Therefore, the binding of glucose-1-P toward a 6-FDPL–enzyme–phosphite–AMP complex does not induce a 1.5 ppm upfield shift of the fluorine signal as the result of the change of the interactions between the 5′-protons and the fluorine. Nevertheless, the fluorine signal of 6-FDPL–enzyme is still under the influence of the structural changes of the protein and is shifted 0.6 ppm upfield. The verification of these two possible explanations has to await future X-ray crystallographic studies.

X-ray diffraction studies of phosphorylase *b* in the presence of IMP and glucose-1-P show that the glycine loop residues 133–136 lie between the pyridine ring of the coenzyme and the bound substrate, glucose-1-P (Johnson et al., 1980). The difference electron density map between phosphorylase *b* liganded with heptulose 2-phosphate and that complexed with glucose-1-P shows a different orientation of the glycine loop residues and disturbances around the pyridine ring of the coenzyme (McLaughlin et al., 1984). On the basis of these observations, along with the different NMR spectral properties of 6-FPAL– and 6-FDPL–enzymes observed in this study, the first effect of the binding of glucose-1-P toward phosphorylase may be shifting the orientation of the glycine loop residues. This structural change may affect the orientation of the coenzyme ring, using the 5′-phosphate group as an anchoring point. According to the activation entropy changes of phosphorylase containing various coenzyme analogues, the interactions between the coenzyme and protein are important for the construction of the active-site region. As the coenzyme is surrounded by residues of different domains, the reorientation of the coenzyme ring may further affect the arrangement of the remaining parts of the protein. Because the active site of phosphorylase is also made of amino acid residues of different domains, such a structural change may, in turn, influence the fine adjustment of the orientation of catalytic groups in the active site to improve the efficiency of catalysis.

In conclusion, the coenzyme PLP in phosphorylase is an indispensable structural determinant for an active enzyme. One of its major functions in the catalytic process of phosphorylase may be coordinating the orientation of different domains of the protein by forming specific contacts with residues of different domains and mediating the structural changes of varied parts of the protein to the active-site region. These changes are necessary for efficient catalysis.

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DNA Structure Equilibria in the Human c-*myc* Gene†

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ABSTRACT: We have employed analytical S1 nuclease analysis to identify sites with altered DNA secondary structure in the human c-*myc* gene. We have mapped several sites of that kind in vitro at one-base resolution but have focused our attention on one particularly stable conformational isomer which occurs approximately 270 base pairs upstream from the preferred transcription origin. We have analyzed the kinetics of that conformational equilibrium as a function of supercoil density and enzyme concentration and find that DNA structure in this region is adequately modeled as a two-state equilibrium between an undistorted (S1 nuclease insensitive) and a distorted (S1-sensitive) state. We find that at fixed supercoil density, S1 nuclease cleavage at this DNA segment can be altered in vitro by a DNA sequence change as far away as 1500 bases. We also find that the S1 nuclease cleavage at this site can be dramatically enhanced by the binding of small RNA molecules. On the basis of an analysis of S1 cutting kinetics and an analysis of DNA sequence at the S1 cleavage site, we conclude that RNA may bind directly to DNA, thereby shifting the underlying conformational equilibrium. Together, these data suggest that as a class, short RNA molecules could serve as site-specific regulatory elements in the *myc* gene and elsewhere.

Recently, on the basis of the use of chemical and enzymatic probes, sites have been identified in cloned genes which appear to have assumed an atypical DNA secondary structure. Sites of that kind have been identified in the chicken β^A-globin gene (Schon et al., 1983; Nickol & Felsenfeld, 1983; Wang & Hogan, 1985), in the *Drosophila* HPS70 gene (Mace et al., 1983), in the sea urchin histone gene cluster (Hentschel, 1982), and in a variety of synthetic DNA inserts (Pullyblank et al., 1985; Kohwi-Shigematsu & Kohwi, 1985).

Several laboratories are attempting to rationalize those data in structural terms, and some generalizations have been made.

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