Phosphonate and α-Fluorophosphonate Analogue Probes of the Ionization State of Pyridoxal 5′-Phosphate (PLP) in Glycogen Phosphorylase†

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ABSTRACT: To investigate the role of the essential cofactor pyridoxal phosphate in rabbit muscle glycogen phosphorylase catalysis, two phosphonate analogues of pyridoxal phosphate, 5′-deoxypyridoxal 5′-methylenephosphonic acid and 5′-deoxypyridoxal 5′-difluoromethylenephosphonic acid, have been prepared and reconstituted into apophosphorylase *b*. UV/Vis spectroscopic and ^{31}P and ^{19}F NMR studies confirmed the successful reconstitution and revealed significant changes in phosphate environment upon nucleotide activation. Both such reconstituted enzymes had activities of approximately 25%−30% of that observed in the native enzyme, while K_m values were similar to those of the native enzyme. Very similar dependences upon pH of V_{max}, K_m, and V_{max}/K_m were found for the two reconstituted enzyme derivatives and the native enzyme despite the considerable difference in phosphonic acid pK_a values. These results suggest that pyridoxal phosphate does *not* function as an essential acid/base catalyst in glycogen phosphorylase; rather they suggest that the cofactor phosphate moiety remains dianionic throughout catalysis and functions as an essential dianion. Mechanistic implications of these findings are discussed.

Glycogen phosphorylase catalyzes the reversible phosphorolysis of glycogen, generating glucose-1-phosphate[1] which can then be metabolized. Despite extensive structural information on both the R- and T-state forms of glycogen phosphorylase (Sprang & Fletterick, 1979; Barford et al., 1991; Sprang et al., 1991), in addition to decades of mechanistic studies [for reviews see Madsen and Withers (1986) and Palm et al. (1990)] the catalytic mechanism remains unclear. Of particular interest is the role of the essential cofactor, PLP, in catalysis. Over the years, in an effort to test every part of the PLP molecule for its role in catalysis, a number of derivatives have been synthesized and tested for their ability to bind to and reactivate the apoenzyme [for a review see Madsen and Withers (1986)]. While PLP requires its aldehyde functionality for binding to phosphorylase, catalytic activity is only restored if the cofactor phosphate moiety is capable of forming a dianion.

Largely on the basis of ^{31}P NMR studies coupled with evidence from kinetic studies with glycosylic substrates lacking a phosphate moiety, it has been suggested that the coenzyme phosphate moiety in phosphorylase functions as a Bronsted acid/base catalyst, protonating the phosphate moiety of the substrate glucose-1-phosphate upon binding and thus assisting in the leaving group departure (Helmreich, 1992; Palm et al., 1990; Klein et al., 1982, 1984). However, studies with pyridoxal-reconstituted phosphorylase, an enzyme derivative which is only active in the presence of activator anions which bind in place of the missing coenzyme phosphate moiety, cast doubt upon this mechanism. Parrish and co-workers (1977) were able to demonstrate that both fluorophosphate (pK_2 = 4.8) and phosphite (pK_2 = 6.6) were equally effective as activators of pyridoxal-phosphorylase. The relatively low second pK_a value for fluorophosphate essentially ensures that it binds to the protein in a dianionic state, and possibly excludes this phosphate analogue from participation in a proton shuttle mechanism. Further, pH−activity profiles determined for both fluorophosphate- and phosphite-activated pyridoxal-phosphorylase were essentially identical (Withers et al., 1982a), yet large differences might be expected if these phosphate analogues were involved in an essential proton transfer since the two phosphate analogues have such different pK_a values. While these results suggest that PLP is *not* involved in an essential proton transfer, they are still consistent with the cofactor phosphate acting in an electrophilic role (Madsen & Withers, 1984) or, alternatively, as an indispensable structural element which functions to orient active site residues for catalysis (Chang et al., 1983, 1987). Nonetheless, concerns have been raised as to how well the pyridoxal-phosphorylase enzyme, activated by noncovalently bound phosphate analogues, reflects the behavior of the native PLP cofactor in phosphorylase (Klein et al., 1984). Klein et al. (1984) have argued that slight differences in binding due to the lack of the pyridoxal-phosphate covalent bond make it unlikely that any activating anion, regardless of its pK_a, can replace the PLP phosphate moiety as a proton shuttle. Alternatively, but without justification, Klein and co-workers suggest that these phosphate mimics force pyridoxal-phosphorylase to follow a slightly different mechanism using alternative amino acid side chains as general acid−base catalysts (Klein et al., 1984).

Two phosphonate analogues of PLP which might be particularly suited to further probe the role of the cofactor

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[1] Abbreviations: glucose-1-phosphate, α-D-glucopyranosyl phosphate; PLP, pyridoxal 5′-phosphate; αG1CP, α-D-glucose-1-methylene phosphonate; 5-CH_2PLP, pyridoxal 5-methylene phosphonic acid; 5-CF_2PLP, pyridoxal 5-difluoromethylene phosphonic acid; AMP, adenosine 5′-monophosphate; AMPS, adenosine 5′-*O*-thiomonophosphate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TEA, triethanolamine; THF, tetrahydrofuran; NMR, nuclear magnetic resonance.

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phosphate moiety in phosphorylase would be pyridoxal 5-methylene phosphonic acid (5-CH_2PLP) and pyridoxal 5-difluoromethylene phosphonic acid (5-CF_2PLP). The former compound has been prepared previously (Hullar, 1969) and shown to reactivate apophosphorylase to approximately 25% of the activity found with the native enzyme (Vidgoff et al., 1974). Further, X-ray crystallographic studies of glycogen phosphorylase reconstituted with 5-CH_2PLP have demonstrated that this phosphonate analogue binds to the enzyme in an essentially identical mode to that of the native cofactor (Oikonomakos et al., 1987), and some ^{31}P NMR studies have been performed (Klein et al., 1984). Since 5-CH_2PLP and 5-CF_2PLP should possess significantly different phosphonic acid pK_a values, a study of the pH-dependence of the kinetic parameters should provide valuable insight into their possible roles as acid/base catalysts. In addition, since the latter analogue has two NMR active nuclei, ^{19}F and ^{31}P, whose chemical shift could be expected to be sensitive to phosphonate ionization state, NMR studies of the reconstituted enzyme derivative might also provide insight into the cofactor ionization state. This paper describes the investigation of the pH-dependence of the kinetic parameters for the two reconstituted phosphorylase derivatives, as well as ^{31}P and ^{19}F NMR studies of phosphorylase reconstituted with 5-CF_2PLP.

EXPERIMENTAL PROCEDURES

Syntheses

Pyridoxine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). αG1CP was synthesized as described previously (Street & Withers, 1995) by Dr. Ian Street. THF was distilled from sodium and benzophenone. 4′,3-*O*-Isopropylidene-5′-deoxy-5-chloropyridoxal was synthesized from pyridoxine hydrochloride using procedures established by Korytnyk and Ikawa, (1970).

The compound 5-CH_2PLP was prepared in a similar manner to that previously reported by Hullar (1969), except that the Wittig reaction in which tetraethyl methylenediphosphonate was condensed with α[4],3-*O*-isopropylideneisopyridoxal, was replaced by a direct displacement reaction in which the lithio anion of dimethyl methylphosphonate (Corey & Volante, 1976) was reacted with the free base of 4′,3-*O*-isopropylidene-5′-deoxy-5′-chloropyridoxol (Korytnyk & Ikawa, 1970). This modification allows the fully saturated analogue to be prepared directly, avoiding the need for a subsequent reduction step, and moreover, could be adapted to the synthesis of 5-CF_2PLP, the difluorophosphonate analogue. Thus, to dimethyl methylphosphonate (2 mL, 19.0 mmol) in dry THF (20 mL) under nitrogen with stirring at −78 °C was added *n*-butyllithium (12 mL, 19.2 mmol). After 45 min at this temperature the generated dimethyl lithiomethylphosphonate carbanion was transferred by cannula into a stirred suspension of 4′,3-*O*-isopropylidene-5′-deoxy-5′-chloropyridoxol (1.4 g, 6.16 mmol) and a catalytic amount of tetra *n*-butylammonium iodide in THF (20 mL) at −78 °C. The mixture was stirred at −78 °C (30 min) and then allowed to warm to room temperature. The THF was evaporated *in vacuo* followed by an extractive workup (CH_2Cl_2−H_2O), drying over magnesium sulfate and evaporation of solvent under reduced pressure to yield a crude oil. This fully protected form of 5-CH_2PLP, dimethyl 2-(4′,3-*O*-isopropylidene-2-methyl-5-pyridyl)ethylphosphonate, was purified at this stage by silica-gel flash chromatography, or after the following deprotection step. ^{1}H NMR data ($CDCl_3$, 300 MHz): δ 7.88 (s, 1 H), 4.82 (s, 2 H), 3.77 (d, 6 H, $J_{H,P}$ = 10 Hz), 2.72 (m, 2 H), 2.39 (s, 3 H), 2.01 (m, 2 H), 1.55 (s, 6 H). Mass spectral data *m/z*: 315; expected mass, 315. The fully protected form of 5-CH_2PLP was then deprotected and oxidized essentially as described by Hullar (1969), except for the final purification of 5-CH_2PLP which was carried out using AG-1 × 8 resin (Cl^- form, 2 cm × 10 cm) from which 5-CH_2PLP was eluted with a linear gradient of H_2O/0.01 M HCl, 0.02 M LiCl. Thin-layer chromatography: R_f = 0.5, 13:3:3 (butanol, formic acid, water). ^{1}H NMR data (D_2O, solution pH ≈ 7, 300 MHz): aldehyde species, δ 10.48 (s, 1 H), 8.17 (s, 1 H), 3.34 (m, 2 H), 2.66 (s, 3 H), 2.01 (m, 2 H); hemi-acetal, δ 8.02 (s, 1 H), 6.52 (s, 1 H), 3.05 (m, 2 H), 2.58 (s, 3 H), 2.01 (m, 2 H). ^{31}P NMR data (D_2O, 122.5 MHz): δ 24.4 (s, broad). Mass spectral data, FAB (*p*-nitrobenzylalcohol matrix) [*m* + 1], 252; expected mass for the mono lithium salt of 5-CH_2PLP [*m* + 1], 252. The free acid form of CH_2PLP was prepared by elution of a solution through a column of Bio-Rex 70 (H^+ form, 0.7 cm × 12 cm) ion-exchange resin. After evaporation *in vacuo* the free acid was obtained as a yellow solid. Elemental analysis required $C_9H_{12}NO_5P + 1.2H_2O$: C, 40.51; H, 5.40; N, 5.25; found, C, 40.35; H, 5.60; N, 4.94.

5-CF_2PLP was prepared exactly as 5-CH_2PLP with minor modifications. Butyllithium (10 mmol) in dry THF (10 mL) and diisopropylamine (10.3 mmol) were stirred at −20 °C (20 min) and then cooled to −78 °C at which time diethyl difluoromethylphosphonate (Bergstrom & Shum, 1988) (10.3 mmol) in dry THF (10 mL) was added dropwise. After stirring the solution at −78 °C (30 min) to generate the lithio carbanion of diethyl difluoromethylphosphonate, 4′,3-*O*-isopropylidene-5′-deoxy-5′-chloropyridoxol (1.56 g, 6.9 mmol) (Korytnyk & Ikawa, 1970), suspended in a minimal volume of dry THF, was added with stirring. The reaction mixture was allowed to warm to room temperature, and the solvent was evaporated *in vacuo* to leave a brown oil. The crude oil was redissolved (CH_2Cl_2), washed with saturated sodium bicarbonate, dried over magnesium sulfate, and filtered, and the solvent evaporated under reduced pressure. The fully protected form of 5-CF_2PLP, diethyl 1,1-difluoro-2-(4′,3-*O*-isopropylidene-2-methyl-5-pyridyl)ethylphosphonate, was purified by silica-gel flash chromatography. ^{1}H NMR data ($CDCl_3$, 400 MHz): δ 7.95 (s, 1 H), 4.83 (s, 2 H), 4.25 (m, 4 H), 3.25 (dt, 2 H, $J_{H,F}$ = 18, 18, $J_{H,P}$ = 5.0 Hz), 2.42 (s, 3 H), 1.52 (s, 6 H), 1.32 (t, 6 H, J = 7.0 Hz). ^{31}P NMR data ($CDCl_3$, 81 MHz): δ 6.20 (t, $J_{P,F}$ = 105 Hz). ^{19}F NMR data ($CDCl_3$ 188 MHz): δ −34.50 (d, $J_{F,P}$ = 105 Hz). The fully protected form of 5-CF_2PLP was deprotected and purified as described above for 5-CH_2PLP. Thin-layer chromatography: R_f = 0.45, 13:3:3 (butanol, formic acid, water). ^{1}H NMR data (D_2O, solution pH ≈ 10, 400 MHz): δ 10.30 (s, 1 H), 7.45 (s, 1 H), 3.65 (t (br), 2 H, $J_{H,F}$ = 20 Hz), 2.36 (s, 3 H); ^{31}P NMR data (D_2O, 81 MHz): δ 6.00 (t, $J_{P,F}$ = 86 Hz); ^{19}F NMR data (D_2O, 188 MHz): δ −34.70 (d, $J_{F,P}$ = 86 Hz). The monolithium salt of 5-CF_2PLP was prepared as a yellow solid by use of Bio-Rex 70 (H^+ form, 0.7 cm × 12 cm) ion-exchange resin to generate the free acid, to which was added lithium hydroxide. Mass spectral data, FAB (glycerol matrix) [*m* + 1], 288; expected mass for the monolithium salt of 5-CF_2PLP [*m* + 1], 288. Elemental analysis required $C_9F_2H_9LiNO_5P$ + LiCl +

$3H_2O$: C, 28.20; H, 3.92; N, 3.65; found, C, 28.23; H, 3.55; N, 3.26.

NMR Studies

^{31}P NMR spectra were recorded at 28 °C on a Bruker MSL 200 spectrometer operating at 81 MHz using a 10 mm probe. ^{31}P NMR resonances are given in the δ scale and are referenced to 85% phosphoric acid ($\delta = 0.00$ ppm), with signals occurring downfield of this position being assigned positive values. A spectral width of 10 000 Hz was employed, with a 20−30° pulse angle and a repetition time of 1.5 s. Exponential line-broadening (25 Hz) was used prior to Fourier transformation, and all line width data have been corrected for this. Sample size was 1.3−1.5 mL in a 10 mm NMR tube, with enzyme concentrations between 0.9 and 1.0 mM calculated for the phosphorylase monomer. ^{19}F NMR spectra of glycogen phosphorylase *b* reconstituted with 5-CF_2PLP were recorded at 28 °C on a Bruker AC-200E spectrometer operating at 188 MHz with a 5 mm probe. A spectral width of 30 000 Hz was employed with a 35−40° pulse angle and a repetition time of 0.8−1.1 s. Exponential line-broadening (75 Hz) was used prior to Fourier transformation, and all line width data have been corrected for the line-broadening factor. Sample size was 0.4−0.5 mL, with enzyme concentrations between 0.6 and 0.8 mM calculated for the phosphorylase monomer. ^{19}F signals are reported in the δ scale and are referenced against trifluoroacetic acid ($\delta = 0.00$ ppm), signals occurring downfield of the reference being assigned positive δ values. Protein NMR experiments were conducted without proton decoupling. All D_2O used in the protein NMR experiments was pre-treated with Chelex 100 to remove divalent metal ions.

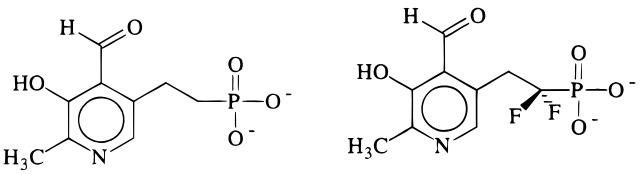
^{31}P NMR spectra for the titrations of 5-CH_2PLP and 5-CF_2PLP were recorded on a Varian XL-300 spectrometer operating at 121 MHz at room temperature. Titrations were performed on 5-CH_2PLP (28 mM) or 5-CF_2PLP (23 mM) in a solution of 50% D_2O and 100 mM KCl. The pH of the solution was adjusted with a small volume (1−5 μL) of concentrated HCl or NaOH (2 M), after which the spectra were accumulated (384 scans). Generally a 4 Hz line-broadening factor was used in data processing. Values of pK_a were calculated with the aid of the GraFit computer program (Leatherbarrow, 1990).

Native glycogen phosphorylase *b* was freed from AMP by extensive dialysis against TEA buffer. Enzyme samples were concentrated using a Millipore CL filter (30 000 MW cutoff), and then dialyzed against a small volume (~10 mL) of D_2O-TEA buffer (pH 6.8), or diluted with D_2O-TEA buffer and then further concentrated, such that final D_2O concentrations were 50%−60%. The D_2O present in the buffer was used for field/frequency lock. Solutions of effectors in TEA buffer (pH 6.8) were added directly to the enzyme solution to give the final concentrations shown in the legends of Figures 3 and 4.

Kinetic Studies

Rabbit muscle was obtained from Pel-Freez Biologicals. Rabbit liver glycogen (type III) was purchased from Sigma Chemical Co. and was purified with AG-1 × 8 (200-400 mesh, Cl^- form) ion-exchange resin. Glycogen was assayed by the method of Dische (Ashwell, 1957). Ammonium sulfate (ultrapure grade) was obtained from Schwarz/Mann Biotech. AMP, AMPS, glucose-1-phosphate, and all buffer chemicals were purchased from Sigma Chemical Co. Rabbit muscle phosphorylase *b* was prepared by the method of Fischer and Krebs (1962) using DTT instead of cysteine and recrystallized at least three times before use. Protein concentrations were determined from absorbance measurements at 280 nm using an absorbance index $E_{280}^{0.1\%} = 1.32$ mL mg^{-1} cm^{-1} (Buc & Buc, 1968).

Chart 1: Phosphonic Acid Analogues of PLP



5-CH_2PLP 5-CF_2PLP

Apo-phosphorylase *b* was prepared according to Withers et al. (1982b). Reconstitutions were performed at 37 °C in the dark over a period of 45 min with a 10−25-fold molar excess of the 5-CH_2PLP or 5-CF_2PLP. The reconstituted enzyme was precipitated with an equal volume of saturated $(NH_4)_2SO_4$ (pH 6.8), pelleted by centrifugation at 4 °C, redissolved, and dialyzed overnight against buffer at 4 °C. The UV−vis spectra of each reconstituted enzyme showed an absorption band at 333 nm representing the Schiff base linkage between the cofactor and Lys-680 (Feldmann & Helmreich, 1976).

Initial reaction rates in the direction of glycogen synthesis were determined by the Fiske-Subbarow phosphate analysis as described by Engers et al. (1970). TEA buffer (50 mM TEA HCl, 100 mM KCl, 1 mM EDTA, 1 mM DTT, pH 6.8) was employed for all kinetic experiments and reaction mixtures were 0.5 mL containing 1 mM AMP, 1% glycogen, and studies were performed for 5 min at 30 °C. In the case of pH variation, reactions were conducted at pH values ranging from 5.78 to 7.55 for 5-CH_2PLP-phosphorylase and from 5.55 to 7.55 for 5-CF_2PLP-phosphorylase. The enzyme concentration in each reaction was 9.1 and 7.0 μg mL^{-1} for the 5-CH_2PLP- and 5-CF_2PLP-phosphorylase enzymes, respectively. The final pH values were recorded for reaction mixtures containing all substrates, effectors and enzyme. Equilibrium constants for the phosphorylase reaction at each pH value were kindly supplied by Dr. N. B. Madsen. Control experiments were completed to ensure that irreversible pH-dependent inactivation of phosphorylase reconstituted with 5-CH_2PLP or 5-CF_2PLP did not occur over the pH range studied. All values of V_{max} and K_m were calculated by fitting the rate data to the nonlinear form of the Michaelis−Menten equation with the aid of the GraFit computer program (Leatherbarrow, 1990). Apparent pK_a values were also calculated with the aid of the GraFit computer program (Leatherbarrow, 1990).

RESULTS

Kinetic Studies

The methylene (5-CH_2PLP) and difluoromethylene (5-CF_2-PLP) phosphonic acid analogues of PLP in Chart 1 were prepared and reconstituted into apo-glycogen phosphorylase *b*. Both such reconstituted enzymes were catalytically active

Table 1: Kinetic Parameters for Glycogen Phosphorylase *b* Reconstituted with 5-CH_2PLP and 5-CF_2PLP[a]

enzyme	K_m (mM)[b]	V_{max} (μmol/min/mg)	% reactivation
native enzyme	2.0 ± 0.1	62.4 ± 1.3	100
5-CH_2PLP-enzyme	0.70 ± 0.03	16.4 ± 0.1	26
5-CF_2PLP-enzyme	2.1 ± 0.2	20.0 ± 0.5	32

[a] Reactions were conducted at pH 6.8 in TEA buffer over a range of glucose-1-phosphate concentrations (1−20 mM) as described in Experimental Procedures. Enzyme concentrations were 1.94, 8.02, and 6.24 μg mL^{-1} for the native, 5-CH_2PLP-, and 5-CF_2PLP-phosphorylase enzymes, respectively. [b] The Michaelis constant for glucose-1-phosphate.

and the kinetic parameters for each reconstituted enzyme are presented in Table 1 along with those for the native enzyme. As can be seen, V_{max} for 5-CH_2PLP-phosphorylase is only approximately 4 times lower than that for the native enzyme while affinity for glucose-1-phosphate, as reflected in the Michaelis constant, was somewhat higher. These results are in excellent agreement with those reported previously for the same enzyme derivative (Vidgoff et al., 1974). Reconstitution of apo-phosphorylase *b* with 5-CF_2PLP reactivated the apoenzyme to a level consistently higher than 5-CH_2-PLP, with a V_{max} value only 3 times lower than that of the native enzyme. The Michaelis constant for glucose-1-phosphate, however, was greater than for 5-CH_2PLP-phosphorylase, now equal to that observed with the native enzyme.

The pH-dependence of the kinetic parameters (V_{max}, K_m, and V_{max}/K_m) for phosphorylase reconstituted with 5-CH_2-PLP and 5-CF_2PLP was determined by calculating the V_{max} and K_m at each pH studied, and is shown in Figure 1. Data were fit to an equation for two essential ionizations using the program GraFit (Leatherbarrow, 1990), and the pK_a values obtained are shown in Table 2. The pH profiles are very similar for the two enzyme systems. This is particularly evident for the pH-dependence of K_m and V_{max}/K_m (Figure 1b,c). The apparent pK_a values for the acidic (5-CH_2PLP = 6.2; 5-CF_2PLP = 6.2) and basic limbs (5-CH_2PLP = 7.0; 5-CF_2PLP = 7.1) describing the pH-dependence of pK_m for the two enzyme systems are the same within experimental error. Similarly, the pH-dependences of log V_{max}/K_m for the two reconstituted enzymes are essentially superimposable, the apparent pK_a values for the acidic (5-CH_2PLP = 6.5; 5-CF_2PLP = 6.3) and basic limbs (5-CH_2PLP = 7.1; 5-CF_2-PLP = 6.9) being the same within experimental error. The apparent pK_a values for the basic limb of the log V_{max} plot were 7.9 and 7.6 for 5-CH_2PLP- and 5-CF_2PLP-phosphorylase enzymes, respectively (Figure 1a). The calculated pK_a of 7.9 for 5-CH_2PLP-phosphorylase, however, is quite approximate since the pH optimum (pH 7.0), and the limited pH range in which phosphorylase is active, restricts data collection in the basic limb region for this enzyme derivative. Only within the acidic limb region of the log V_{max} plot was a significant difference observed between pH-profiles for the two reconstituted enzyme species; the apparent pK_a values for the two enzyme systems differing by approximately 0.8 pH units (5-CH_2PLP-enzyme = 6.4; 5-CF_2PLP-enzyme = 5.6). This difference in pK_a is also reflected in a difference in pH-optima of about 0.5 units between the two enzyme systems, optimal pH values of 7.0 and 6.5 being observed for 5-CH_2PLP- and 5-CF_2PLP-phosphorylase, respectively.

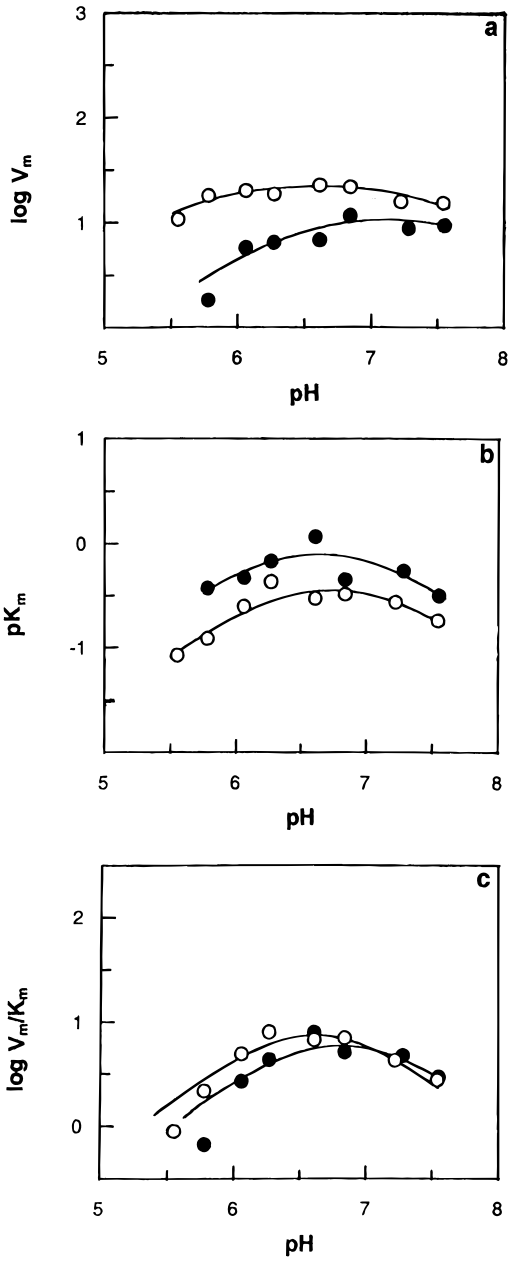


FIGURE 1: pH-dependences of V_{max}, K_m, and V_{max}/K_m for 5-CH_2-PLP- (●) and 5-CF_2PLP-phosphorylase *b* (○). are shown in Table 2. The solid lines shown are the fits used to estimate the pK_a values as described in Results. pK_a values are shown in Table 2.

Table 2: pK_a Values for Modified Phosphorylases Derived from pH-Dependence of Kinetic Parameters

enzyme	pK_1 (V_m/K_m)	pK_2 (V_m/K_m)	pK_1 (V_m)	pK_2 (V_m)	pK_1 (K_m)	pK_2 (K_m)
5-CH_2PLP-enzyme	6.5	7.1	6.4	7.9	6.2	7.1
5-CF_2PLP-enzyme	6.3	6.9	5.6	7.6	6.2	7.1

NMR Studies

The catalytically relevant second pK_a value for each phosphonic acid analogue, free in solution, was determined in an effort to estimate the difference in pK_a between the two cofactor analogues once bound at the active site of the enzyme. pH titrations were followed by ^{31}P NMR spectroscopy (Schnackerz, 1986; Schnackerz & Feldmann, 1980). The titration curves obtained in this manner are shown in Figure 2. Fitting this data to a titration curve yielded two

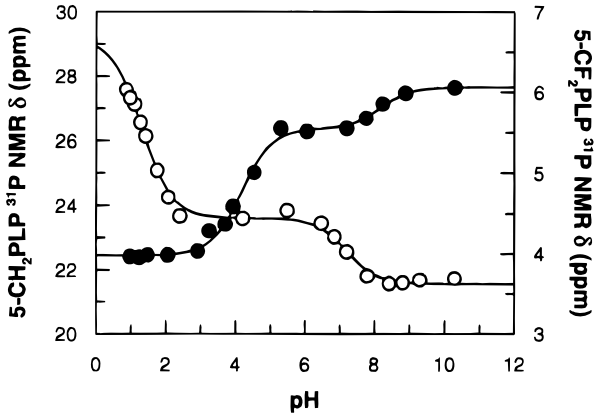


FIGURE 2: ^{31}P NMR titration of PLP phosphonate cofactor analogues. (a) 5-CH_2PLP (○); (b) 5-CF_2PLP (●). pK_a values obtained by fitting are as follows: 5-CH_2PLP, pK_1 = 1.3, pK_2 = 7.2; 5-CF_2PLP, pK_1 = 4.2, pK_2 = 8.2. ^{31}P NMR titrations were carried out at room temperature [D_2O (50%), 100 mM KCl] in the presence of 5-CH_2PLP (28 mM) or 5-CF_2PLP (23 mM).

ionizations for each PLP analogue: 5-CF_2PLP, pK_1 = 4.2 ± 0.1 and pK_2 = 8.2 ± 0.1; 5-CH_2PLP, pK_1 = 1.3 ± 0.1 and pK_2 = 7.2 ± 0.2. Interestingly, the changes in ^{31}P NMR chemical shift as a function of pH for 5-CH_2PLP and 5-CF_2-PLP were in opposite directions for the two cofactors. Such "inversions" of chemical shift response to ionization are not uncommon, with similar phosphates and phosphonates, for example, often titrating in the opposite sense (Vogel & Bridger, 1982). The second phosphonate ionization observed for 5-CH_2PLP (pK_2 = 7.2) is in good agreement with that determined previously (pK_a = 7.3−7.4) for 5-CH_2PLP (Miura et al., 1989; Schnackerz & Feldmann, 1980; Vidgoff et al., 1974). Since the first pK_a value for the phosphonate moiety of 5-CF_2PLP is below the limit of this pH titration, the observed pK_a value of 4.2 is assigned as the pK_2 value for the phosphonic acid moiety of 5-CF_2PLP, in general agreement with earlier studies of similar phosphonic acids possessing the same difluoromethylene functionality (Blackburn et al., 1987; Bigge et al., 1989). The second ionization observed for 5-CF_2PLP (pK_a = 8.2 ± 0.2), which produced only a small change in the ^{31}P NMR chemical shift, closely matches the expected pK_a for the pyridoxal phenolic moiety when the ring nitrogen is deprotonated (Schnackerz, 1986; Kallen et al., 1985; Vidgoff et al., 1974). Thus, it appears that the phosphonate moiety is sensitive to the ionization of the phenolic group on the pyridoxal ring, possibly through some preferred solution conformation which places the two in juxtaposition. This ionization only became visible in the 5-CF_2PLP titration because of the significantly lower phosphonic acid pK_a values: in the 5-CH_2PLP titration it is masked by the second ionization of the phosphonate.

The ^{31}P NMR spectra of 5-CF_2PLP-phosphorylase *b* in the presence of AMPS and αG1CP, a phosphonate analogue of glucose-1-phosphate, are shown in Figure 3 along with a ^{31}P NMR spectrum of native glycogen phosphorylase *b* recorded under similar AMPS activating conditions. The large resonance observed (Figure 3a) at 43.8 ppm in the ^{31}P-NMR spectrum of 5-CF_2PLP-phosphorylase *b* in the presence of activating AMPS (2 equiv) is that of free AMPS and is present as an exchange-broadened signal ($\Delta\upsilon_{1/2}$ = 99 Hz). Enzyme-bound AMPS appears as a small, broad shoulder peak located at δ ~41.1 ppm. Extensive line-broadening is also seen in the signal arising from the cofactor phosphonate, which is centered at δ ~2.2 ppm with a line width of 380 Hz. The sharp resonance observed at 1.9 ppm is due to contaminating phosphate, derived from contaminated AMPS or possibly released from the protein itself (Withers et al., 1979). On addition of αG1CP to the nucleotide-activated enzyme (Figure 3b), the free AMPS resonance shifts slightly downfield to 44.0 ppm with a significant reduction in line width ($\Delta\upsilon_{1/2}$ = 84 Hz). Correspondingly, the bound AMPS signal has shifted upfield slightly, to 40.9 ppm, providing slightly better resolution of the free and bound forms of the nucleotide. These changes show that a decrease in the AMPS exchange rate has accompanied αG1CP binding. The cofactor resonance for 5-CF_2PLP-phosphorylase *b* shifted slightly downfield to 2.4 ppm and slightly narrowed ($\Delta\upsilon_{1/2}$ = 365 Hz) upon addition of αG1CP. The resonance for contaminating phosphate shifted slightly upfield to 1.8 ppm, with an increase in signal intensity and a considerable reduction in line width (best estimate using 10 Hz line-broadening). These effects on contaminating phosphate are consistent with its initial involvement in an exchange process at the active site of the enzyme, binding to the vacant phosphate site normally occupied by glucose-1-phosphate,

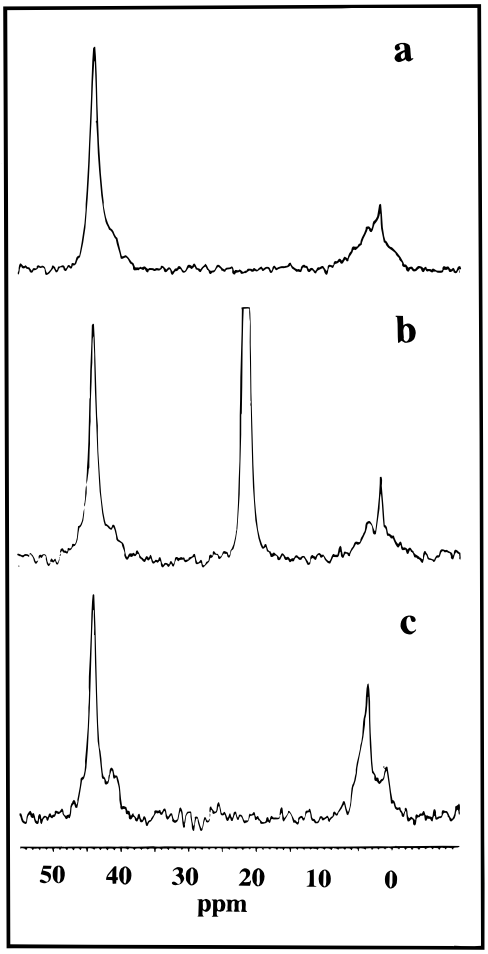


FIGURE 3: ^{31}P NMR spectra of glycogen phosphorylase *b* reconstituted with 5-CF_2PLP and in its native form. ^{31}P NMR spectra were recorded at 28 °C in TEA buffer (pH 6.8) containing D_2O (50%−60%). (a) Signal averaged over 33675 transients, reaction mixture contained 5-CF_2PLP-phosphorylase *b* (0.92 mM) AMPS (1.8 mM). (b) Signal averaged over 21600 transients, reaction mixture contained 5-CF_2PLP-phosphorylase *b* (0.90 mM), AMPS (1.7 mM), and αG1CP (3.4 mM). (c) Signal averaged over 4760 transients, reaction mixture contained native glycogen phosphorylase *b* (1.0 mM) and AMPS (2.4 mM).

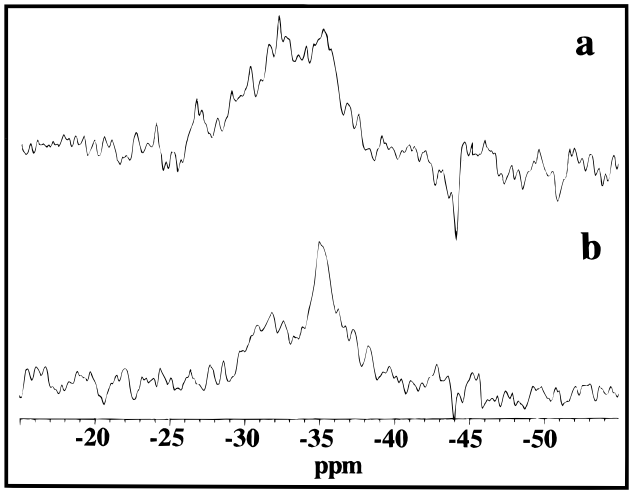


FIGURE 4: ^{19}F NMR spectra of glycogen phosphorylase b reconstituted with 5-CF_2PLP. ^{19}F NMR spectra were recorded at 28 °C in TEA buffer (pH 6.8) containing D_2O (50%−60%). (a) Signal averaged over 55 824 scans, sample contained 5-CF_2PLP-phosphorylase *b* (0.84 mM). (b) Signal averaged over 54 399 scans, sample contained 5-CF_2PLP-phosphorylase *b* (0.80 mM) and AMP (1.8 mM). Chemical shifts are referenced to TFA.

but then being displaced from the active site upon αG1CP addition. The resonance due to αG1CP is observed as a single exchange-averaged signal, in which both the chemical shift (21.4 ppm) and the line width ($\Delta\upsilon_{1/2} = 40$ Hz) represent a weighted average of the free and enzyme-bound species. The ^{31}P NMR spectrum of native glycogen phosphorylase, collected under similar conditions of AMPS activation, is shown in Figure 3c. The free AMPS signal is observed at 43.9 ppm ($\Delta\upsilon_{1/2} = 63$ Hz); the enzyme-bound AMPS signal is clearly resolved at 41.1 ppm ($\Delta\upsilon_{1/2} = 143$ Hz). The resonance for the PLP cofactor is also well defined at $\delta \sim 4$ ppm, with a small resonance at 0.8 ppm, representing the two forms of the enzyme (Feldmann & Hull, 1977). The sharp resonance at 3.6 ppm is commonly observed in spectra of the native enzyme (Withers et al., 1979) and assigned as AMP.

^{19}F NMR spectra of 5-CF_2PLP-phosphorylase *b* in the absence of activating AMP are shown in Figure 4a. The cofactor signal is observed as an extremely broad resonance ($\Delta\upsilon_{1/2} \approx 710$ Hz) centred at $\delta \sim -32$ ppm. Upon addition of AMP (Figure 4b), the cofactor ^{19}F signal appeared as two broad components, shifted slightly upfield to $\delta \sim -34$ ppm, with a considerable reduction in total line width ($\Delta\upsilon_{1/2} \approx$ 610 Hz).

DISCUSSION

It is well-known that apo-glycogen phosphorylase can only be reactivated by cofactor analogues capable of forming a dianion (Madsen & Withers, 1986). In this study, two phosphonic acid analogues of PLP were reconstituted into apo-glycogen phosphorylase *b* in an effort to address the specific question of whether or not PLP acts as an essential Brønsted acid/base catalyst. 5-CH_2PLP and 5-CF_2PLP were synthesized and used because of the large difference in their phosphonic acid pK_a values, yet very similar structures, the van der Waals radius for fluorine (1.35 Å) being only slightly greater than that for the proton it replaced (1.20 Å). However, unlike the C−H bond, the C−F bond has a very significant polarity and the potential to form weak hydrogen bonds, possibly mimicking the bridging phosphate oxygen in the native PLP cofactor. Indeed, phosphorylase reconstituted with 5-CF_2PLP behaved more like the native enzyme than 5-CH_2PLP-phosphorylase, both in terms of glucose-1-phosphate affinity and also its slightly greater activity. Given the similar activities of the two reactivated enzymes, and the tendency for 5-CF_2PLP to remain dianionic at pH 6.8, it seems likely that both analogues bind to the enzyme as dianions. This is completely consistent with recent solid-state ^{31}P-NMR studies (Challoner et al., 1993; Taguchi et al., 1993) in which the PLP phosphorus nucleus within both the R- and T-state enzyme conformations was shown to exhibit axial shielding parameters, characteristic of dianionic phosphomonoesters, and consistent with the PLP phosphate moiety being dianionic in both allosteric forms of the enzyme.

Enzymic pH profiles invariably suffer from an uncertainty of approximately 0.3 pH units (Kasvinsky & Meyer, 1977) and are often overinterpreted (Knowles, 1976; Brocklehurst, K., 1994). Isolated interpretations of individual pH-dependences are particularly hazardous. However, if the pH dependences of two minimally modified systems of significantly different inherent pK_a can be investigated, then interpretation is considerably simplified. It therefore seems likely that given the extreme difference in solution pK_2 for 5-CH_2PLP and 5-CF_2PLP ($\Delta pK_2 \approx 3$), if the phosphonic acids were indeed involved in a catalytically essential proton transfer, then the large difference in cofactor pK_a would necessarily be reflected in the pH profiles for the two, otherwise internally consistent, enzyme species. The pH-dependences of pK_m and log V_{max}/K_m for both reconstituted enzyme species are very similar, with apparent pK_a values of approximately 6.3 and 7.0 for the acid and basic limbs in each case. These pK_a values are very similar to those observed in previous pH studies of the native enzyme (Kasvinsky & Meyer, 1977; Uhing et al., 1981; Withers et al., 1982a; Zographos et al., 1995) despite the differences in the inherent pK_a values. It therefore seems unlikely that these pH profiles directly reflect cofactor ionizations, as had been assumed previously. Similarly, the pH-dependences of log V_{max} for the two reconstituted enzymes differ by only 0.3 pH units in the basic limb region. This suggests that the cofactors are not involved as essential acid catalysts since otherwise the basic limbs would be quite different, that for the 5-CF_2PLP-enzyme derivative having a much lower pK_a value. The only significant difference observed in any of the pH profiles was in the acidic limb of the log V_{max} plot, where apparent pK_a values of 6.4 and 5.6 were observed for the 5-CH_2PLP and 5-CF_2PLP-enzymes, respectively. Indeed, the native enzyme possesses an acidic limb pK_a of 5.7 under very similar conditions (Withers et al., 1982a). Since this difference in acidic limb pK_a between the two reconstituted enzymes was not observed for the free enzyme and free substrate (log V_{max}/K_m plot), it must arise from ionizations in the enzyme−substrate complex which are perturbed upon substrate binding and formation of the ternary enzyme complex. This difference in pK_a values ($\Delta pK_a = 0.8$) would seem to be too small to arise from the cofactors themselves ($\Delta pK_a = 3$). Rather, it may have its origin in the way the two cofactors interact with residues located at the active site. These results therefore strongly support earlier studies with pyridoxal-phosphorylase and added phosphate suggesting that

PLP cannot function as an essential acid catalyst in glycogen phosphorylase (Withers et al., 1982a).

^{31}P and ^{19}F NMR studies of 5-CF_2PLP-phosphorylase were able to demonstrate the presence of the enzyme-bound cofactor analogue. While extensive line-broadening was observed in both cases, this was not unexpected since the two fluorine nuclei, once bound at the active site of the enzyme are non-equivalent, coupling to one another, and also to the phosphorus nucleus, with very large *J*-values. Results from ^{31}P NMR studies demonstrated that, although binding of αG1CP to 5-CF_2PLP-phosphorylase *b* stabilizes the R-state, as reflected in lowered AMPS exchange rates, these exchange rates are generally greater for 5-CF_2PLP-phosphorylase *b* than for native enzyme, suggesting that 5-CF_2PLP-phosphorylase may exist in a less activated state than the native enzyme (Withers et al, 1981). Interpretation of the ^{31}P NMR spectra in terms of the cofactor ionization state was avoided since only a small change in chemical shift was observed and, moreover, since previous studies have clearly demonstrated the difficulties (Gorenstein, 1975; Schinzel, 1992; Schnackerz et al., 1989) and hazards (Challoner et al., 1993) of using solution state ^{31}P NMR isotropic chemical shifts to identify changes in ionization state.

The original intention had been that ^{19}F NMR chemical shifts would provide independent insights into the ionization state of the phosphonate moiety. Unfortunately the very large line widths observed for the enzyme-bound cofactor made this difficult. Such large line widths are a consequence of a combination of two broad overlapping and spin-coupled ^{19}F signals possessing different chemical shifts. Indeed, similarly large line widths have been observed in previous ^{19}F NMR studies of phosphorylase reconstituted with 6-fluoropyridoxal phosphate (Chang et al., 1986), a system with a single fluorine bonded to the cofactor and lacking any large *J*-coupling. The ^{19}F NMR resonance for the enzyme-bound cofactor appears approximately 3 ppm downfield of that for the cofactor free in solution, consistent with a change to a relatively hydrophobic environment (Sykes & Hull, 1978; Gerig, 1989; Percival & Withers, 1992). Some narrowing of this line was observed upon addition of nucleotide activator (AMP), but not enough to significantly help. Finally, studies of the pH-dependence of the ^{19}F NMR resonance from 5-CF_2PLP surprisingly revealed almost complete insensitivity of the chemical shift to pH change within this range. Thus ^{19}F NMR chemical shifts were not able to provide insight into the ionization state of the cofactor.

The results presented in this study contradict earlier proposals that PLP functions as an essential acid/base catalyst (Helmreich, 1992) and in conjunction with the solid-state ^{31}P NMR studies (Challoner et al., 1993; Taguchi et al, 1993) suggest that the phosphate moiety remains fully deprotonated throughout. This conclusion is consistent with PLP functioning as an essential dianionic moiety, possibly in an electrophilic role (Madsen & Withers, 1984), or as an indispensable structural element (Chang et al., 1983). An alternative role for a dianionic cofactor phosphate might be in destabilisation of the ground state enzyme−substrate complex through electrostatic interactions [see Jencks (1975) for the general case]. This could result in catalysis if the enzyme/substrate binding energy were used to enforce the close binding of the substrate and cofactor phosphate moieties. The electrostatic destabilisation could then be released at the transition state by a change in position or charge of the substrate phosphate. A somewhat different mode of ground state destabilisation of glucose-1-phosphate in phosphorylase catalysis has been suggested previously on the basis of X-ray crystallographic studies of phosphorylase *b* in the presence of various glucose-1-phosphate derivatives (Martin et al., 1990; Johnson et al., 1990). These studies show that the bound substrate phosphate occupies a position under the C-2 hydroxyl group of the sugar ring, distinct from the more stable rotamer in which the phosphate moiety is oriented away from the sugar ring trans to C-2 (O'Connor et al., 1979). It was suggested (Martin et al., 1990) that this substrate conformation prevents exo-anomeric stabilization of the sugar−phosphate bond, inducing a ground-state destabilization and also positioning the substrate phosphate for effective proton transfer from the cofactor phosphate (Martin et al., 1990). Results from the present study suggest that while the "under the ring" conformation may serve to destabilize the substrate, glucose-1-phosphate, PLP does not act as an essential acid catalyst, thus juxtaposition of the substrate and cofactor phosphate moieties may serve to provide further destabilization by electrostatic means. Destabilization of the ground-state enzyme−substrate complex could be somewhat relieved at the transition state by any movement of the phosphate leaving group away from the cofactor phosphate. Indeed, X-ray structural studies have found additional phosphate binding sites directly adjacent to that which binds the substrate phosphate (Sprang et al., 1992; Mitchell et al, 1996). While this new role in ground-state destabilization is another alternative, consistent with all the evidence, the exact role of the cofactor phosphate in glycogen phosphorylase is not known and is sure to remain a topic of continuing controversy.

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