Pyridoxal Phosphate Site in Glycogen Phosphorylase *b*: Structure in Native Enzyme and in Three Derivatives with Modified Cofactors†

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ABSTRACT: The detailed environment of the essential cofactor pyridoxal 5′-phosphate in glycogen phosphorylase *b*, resulting from crystallographic refinement at 1.9-Å resolution, is described. The pyridoxal ring is buried in a nonpolar site containing three aromatic rings while the 5′-phosphate group is highly solvated and makes only three direct contacts to the protein. The pyridine nitrogen interacts via a water with protein atoms [main chain carbonyl oxygen (Asn-133) and OH of tyrosine (Tyr-90)]. The crystal structures of three active derivatives of phosphorylase reconstituted with 5′-deoxypyridoxal 5′-methylenephosphonate (PDMP), 6-fluoropyridoxal 5′-phosphate (6-FPLP), and pyridoxal (PL) in place of the natural cofactor have been determined at 2.5-Å resolution. The results for PDMP-phosphorylase show a closer proximity of the phosphonate group to the NZ atom of a lysine (Lys-574) than that observed in the native enzyme, consistent with ^{31}P NMR studies that have shown a change in ionization state of the phosphonate group compared to the native cofactor phosphate. The replacement of the polar 5′-ester linkage by a CH_2 group results in a small shift of a water and its hydrogen-bonded tyrosine (Tyr-648). In 6-FPLP-phosphorylase the fluorine is accommodated with no significant change in structure. It is suggested that substitution of the electronegative fluorine at the 6-position may result in lower activity of 6-FPLP-phosphorylase through a strengthening of hydrogen-bonded interactions to the pyridine nitrogen N1. In PL-phosphorylase cocrystallized with 6.5 mM phosphite and 50 mM glucose, the phosphite anion binds to a site that is close to but distinguishable from the 5′-phosphate site of the coenzyme (P–P distance = 1.0 Å). In the presence of glucose, phosphite binding provides significant stability to the crystal structure of PL-phosphorylase (T state) through a number of polar interactions. The implications of these results on the role of PLP in phosphorylase are discussed.

Glycogen phosphorylase[1] (EC 2.4.1.1) catalyzes the reversible transfer of a glucosyl group from the nonreducing end of an α-1,4-glucosidic polysaccharide to orthophosphate to form α-D-glucose-1-P. The enzyme from rabbit muscle exists in two major dimeric conformational states (T and R), depending upon the binding of specific allosteric effectors (Graves & Wang, 1972). It is completely dependent on the coenzyme pyridoxal 5′-phosphate (PLP) for activity. Removal of PLP from the enzyme produces an inactive apoenzyme, which is polydispersed in solution and is not as stable as the holoenzyme, but both the enzymatic activity and the aggregation state can be restored on incubation of apoenzyme with PLP (Hendrick et al., 1966; Shaltiel et al., 1969). Phosphorylase contains stoichiometric amounts of PLP (Baranowski et al., 1957), which is bound to Lys-680 via a Schiff base. X-ray crystallographic studies have established that the coenzyme is buried in the center of the enzyme molecule close to the catalytic site in a cleft between the C- and N-terminal domains (Weber et al., 1978; McLaughlin et al., 1984; Johnson et al., 1987).

The role of the coenzyme in the phosphorylase reaction has been extensively investigated by using a number of PLP analogues. These studies have demonstrated that the functional groups at the 2-, 3-, 4-, and 6-positions of PLP are not essential for catalysis (Fischer et al., 1958; Shaltiel et al., 1969; Chang & Graves, 1985). The role of the pyridine nitrogen (N1) has been rather more difficult to establish (Bresler & Firsov, 1968). Recent results with phosphorylase *b* reconstituted with 6-fluoropyridoxal 5′-phosphate (6-FPLP) have shown that the enzyme is active with the N1 in the neutral form and that the state of ionization of the pyridine N1 is unlikely to be affected by the binding of substrates (Chang & Graves, 1985; Chang et al., 1986). The 5′-phosphate group has been studied by using a large number of PLP analogues modified in position 5, and it is now recognized to play an important role in phosphorylase action (Kastenschmidt et al., 1968; Shaltiel et al., 1969; Pfeuffer et al., 1972a,b; Vidgoff et al., 1974; Shimomura & Fukui, 1978; Yan et al., 1979; Klein et al., 1982). Cofactor analogues with a single negative charge in the 5′-side chain such as pyridoxal 5′-phosphate monomethyl ester (PLPME) do not restore enzyme activity, while 5′-deoxypyridoxal 5′-methylenephosphonate (PDMP) with a pK_a = 7.35 restored 25% of the normal activity, sug-

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PL, pyridoxal; PDMP, 5′-deoxypyridoxal 5′-methylenephosphonate; 6-FPLP, 6-fluoropyridoxal 5′-phosphate; PLPME, pyridoxal 5′-phosphate monomethyl ester; heptenitol, 2,6-anhydro-1-deoxy-D-*gluco*-hept-1-enitol; heptulose-2-P, 1-deoxy-D-*gluco*-heptulose 2-phosphate; glucose-1-P, α-D-glucopyranose 1-phosphate; glucose-1,2-P, α-D-glucopyranose cyclic 1,2-phosphate; glycogen phosphorylase, 1,4-α-D-glucan:orthophosphate α-glucosyltransferase (EC 2.4.1.1); BES, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance.

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gesting that the state of ionization of the 5′-phosphate group of the natural coenzyme (pK = 6.2) has an important role in catalysis. This evidence, combined with ^{31}P NMR spectroscopic and X-ray crystallographic studies, led to two main proposals for the role of the coenzyme 5′-phosphate in the catalytic mechanism, i.e., that it functions (1) as an acid–base (Feldmann & Hull, 1977; Helmreich & Klein, 1980; Klein et al., 1984) or (2) with the phosphorus atom as an electrophile [review by Madsen and Withers (1986)]. The acid–base mechanism has been supported by studies on phosphorylase catalysis of glycosidic substrates which yield a glucosyl residue on protonation (Klein et al., 1982, 1984; Palm et al., 1983; Klein et al., 1986) and by the direct interaction observed between the cofactor 5′-phosphate and the product phosphate of heptulose-2-P in the crystallographic observations (McLaughlin et al., 1984; Hajdu et al., 1987). The electrophilic mechanism has also received support from a number of different experiments (Takagi et al., 1982; Chang et al., 1983; Soman et al., 1983) using phosphorylase b reconstituted with pyridoxal (PL) and model compounds synthesized to mimic the normal enzymatic reaction.

One important result from the studies of PL-phosphorylase b was that phosphate analogues such as phosphite, fluorophosphate, and thiophosphate can activate PL-enzyme to different extents, indicating that these anions may bind at the subsite occupied by the 5′-phosphate of the coenzyme in native PLP-phosphorylase (Parrish et al., 1977; Chang et al., 1983). Results from studies of PL-phosphorylase b with vanadates, molybdates, and tungstates which are potent inhibitors of the enzyme (Chang et al., 1983; Soman et al., 1983) and recent kinetic and ^{19}F NMR experiments of phosphorylase b reconstituted with 6-fluorinated PL or PLP analogues (Chang et al., 1987) suggest that the 5′-phosphate of the cofactor may also have an important role in holding other groups in the correct orientation for catalysis. It is therefore of key importance to establish as precisely as possible the structure and stereochemical constraints of the enzyme active site in considering possible catalytic mechanisms.

In this paper, we report studies on the crystallization and crystal structures of PDMP-, 6-FPLP-, and PL-phosphorylase b (Figure 1). Since the crystal forms of these enzyme derivatives were found to be isomorphous to the native enzyme crystal, the results of the X-ray analyses allowed a comparison with those of the native crystalline PLP-phosphorylase b.

Materials and Methods

Rabbit phosphorylase b was isolated from skeletal muscle according to Fischer and Krebs (1962) except that 2-mercaptoethanol was used instead of L-cysteine in all steps. The enzyme was recrystallized at least 4 times; the fifth crystals were dissolved in 50 mM β-glycerol phosphate, 50 mM 2-mercaptoethanol, and 1 mM EDTA buffer, pH 6.8 (35 °C), and passed through a Sephadex G-25 column equilibrated with the same buffer to remove bound AMP. Apophosphorylase b was prepared by the method of Withers et al. (1982b). Assays of this apoenzyme routinely showed no activity and could be reconstituted with a 8-fold excess of PLP to a specific activity of 60 μmol/(min·mg). Phosphorylases reconstituted with PL, PDMP, 6-FPLP, and PLPME were prepared by incubating apoenzyme with a 50-fold excess of PL, 4-fold excess of PDMP, or 2-fold excess of 6-FPLP and PLPME in 40 mM β-glycerolphosphate, 30 mM 2-mercaptoethanol, and 0.8 mM EDTA buffer, and pH 6.8 and 35 °C for 35 min. Unbound PLP analogues were removed by ammonium sulfate precipitation of the protein derivatives and dialysis according to Chang and Graves (1985). 6-FPLP-phosphorylase b (80 mg) was further crystallized with AMP and magnesium acetate. The white crystalline pellet was dissolved in a minimum volume of 20 mM β-glycerol phosphate, 20 mM 2-mercaptoethanol, and 0.2 mM EDTA buffer (pH 6.8) and dialyzed overnight (4 °C) against the same buffer in the presence of charcoal (100 mg) to remove AMP. Protein concentration was determined from absorbance measurements at 280 nm by using an extinction coefficient ($\epsilon^{1\%}_{10mm}$) of 13.2 (Kastenschmidt et al., 1968). Calculations of phosphorylase b molarity were based on a molecular weight of 97 434 for monomer b (Johnson et al., 1987). For the analysis of enzyme-bound PLP analogues the methods of Baranowski et al. (1957) and Wada and Snell (1961) were used. Phosphorylase activity was measured at pH 6.8 and 30 °C in the direction of glycogen synthesis. Unless otherwise stated, the enzymes (5 μg/mL for the native, 20 μg/mL for PDMP- and 6-FPLP-reconstituted, and 35 μg/mL for PL-reconstituted phosphorylases) were assayed with 1% glycogen, 20 mM glucose-1-P, and 1 mM AMP (and 7.5 mM phosphite in the case of PL-enzyme) in 25 mM β-glycerol phosphate, 25 mM 2-mercaptoethanol, and 0.5 mM EDTA in a final volume of 0.5 mL. Enzyme and glycogen were preincubated for 15 min at 30 °C before initiating the reaction with glucose-1-P. Inorganic phosphate release in the enzymatic reaction was measured by using the method of Fiske and Subbarow (1925). Initial velocities were calculated from the pseudo-first-order rate constants according to Engers et al. (1970).

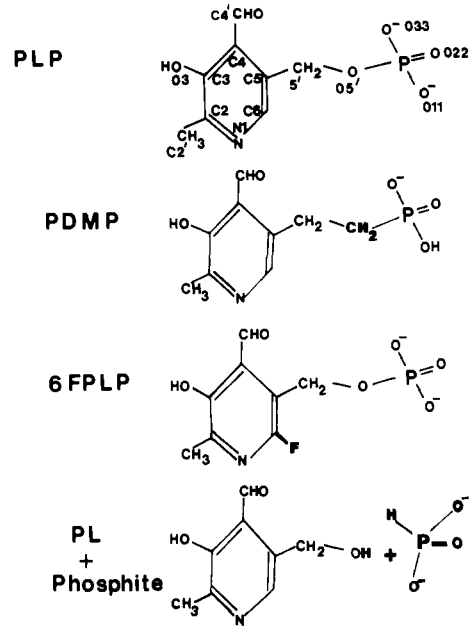

FIGURE 1: The structures of pyridoxal 5′-phosphate (PLP) and its analogues used in this study.

Imidazole, L-cysteine, sodium β-glycerol phosphate, BES, IMP, spermine tetrahydrochloride, α-D-(+)-glucose, pyridoxal 5′-phosphate, pyridoxal hydrochloride, sodium thiophosphate, sodium dithiophosphate, (aminomethyl)phosphonic acid, and sodium selenite were products of Sigma Chemical Co. Tetrasodium pyrophosphate was obtained from BDH Chemicals. Disodium fluorophosphate was purchased from Alfa Products. The syntheses of PLPME and 6-FPLP have been described by Pfeuffer et al. (1972a) and Chang and Graves (1985), respectively.

Crystals of rabbit muscle glycogen phosphorylase b grown under conditions previously described by Johnson et al. (1974) are tetragonal, space group $P4_32_12$ with unit cell dimensions $a = b = 128.5$ Å and $c = 116.3$ Å, and contain one phosphorylase b subunit per asymmetric unit, and the two subunits

Table I: Summary of Data Processing and Refinement Statistics for PDMP-, 6-FPLP-, and PL-Reconstituted Phosphorylase *b*

enzyme derivative	resolution (Å)	no. of reflections measured	no. of unique reflections	merging R_m[a]	fractional change in F	R factor[b]	ΔBL[c] (Å)
PDMP-phosphorylase *b*	2.5	74333	21871	0.070	0.096	0.147	0.015
6-FPLP-phosphorylase *b*	2.5	45501	18919	0.113	0.140	0.139	0.019
PL-phosphorylase *b*	2.5	51065	19314	0.117	0.183	0.146	0.015
PL-phosphorylase *b* soaked with 5 mM pyrophosphate	2.4	98321	32351	0.074	0.122	0.180	0.016

[a] $R_m = \sum_i\sum_h|\bar{I}(h) - I_i(h)|/\sum_i\sum_h I(h)$, where $I_i(h)$ is the ith measurement of the intensity of the reflection h and $\bar{I}(h)$ is the mean of these measurements. [b] The crystallographic R factor is defined as $R = \sum||F_o| - |F_c||/\sum|F_o|$, where F_o and F_c are the observed and the calculated structure factors, respectively. [c] ΔBL is the root mean square deviation from ideal bond lengths.

of the dimer are related by the twofold axis at $z = {}^1/_2$. Crystals of phosphorylase *b* reconstituted with PLP analogues were grown under conditions in which the counterion Mg^{2+} was replaced with spermine (Oikonomakos et al., 1985). Before crystallization, the enzyme solutions were passed through a Sephadex G-25 column equilibrated with 10 mM BES buffer (pH 6.7) containing 0.1 mM EDTA and 0.02% NaN_3 and crystallized under the following conditions: (1) PDMP-phosphorylase *b* was crystallized from a concentration of 10 mg/mL enzyme of specific activity 10–12 μmol/(min·mg) with 2 mM IMP, 2 mM spermine, and 5 mM glucose. (2) PL-phosphorylase *b* crystallized from concentrations of 13–21 mg/mL of specific activity 5–7 μmol/(min·mg) as above except that 6.5 mM phosphite and 50 mM glucose were present. (3) 6-FPLP-phosphorylase *b* crystallized from a concentration of 20 mg/mL of specific activity 9–11 μmol/(min·mg) as in the case of PDMP-enzyme except that the buffer was 5 mM BES, 5 mM β-glycerol phosphate, 0.5 mM EDTA, and 0.01% NaN_3 (pH 6.7). Dithiothreitol (3 mM) was also present in all crystallization media. The crystals which appeared within a few days were found to be identical with the tetragonal crystal form grown in the presence of 2 mM IMP and 10 mM magnesium acetate (Johnson et al., 1974).

Three-dimensional data to 2.5-Å resolution for PDMP-, 6-FPLP-, and PL-phosphorylase *b* crystals were collected by using an Arndt–Wonacott oscillation camera at the Synchrotron Radiation Source at a wavelength of 1.488 Å at Daresbury (Station 7.2). Data to 2.4-Å resolution for PL-enzyme crystals soaked in a solution at pH 6.7 containing 5 mM $Na_4P_2O_7{\cdot}10H_2O$, 10 mM IMP, 50 mM glucose, 10 mM BES, and 0.1 mM EDTA, for 2 h at 22 °C, were collected as above at a wavelength of 0.86 Å on the Wiggler line at Daresbury (Station 9.6). The design of the two stations has been described by Helliwell et al. (1982, 1986). The data were processed by using a modified version (D. I. Stuart, unpublished work) of the MOSCO data processing programs (Nyborg & Wonacott, 1977) implemented on a PDP11/70 computer. The inter- and intrapack scaling were done on a VAX11/750 computer by using the CCP4 program package ROTAVATA and AGROVATA. Difference Fourier syntheses were computed by using the calculated phases resulting from the refined native structure to 1.9-Å resolution. The coefficients for the difference Fourier syntheses were $m(F_d - F_p)$ where F_d and F_p are the structure factor amplitudes for the derivative protein and the native protein, respectively, and m is the figure of merit associated with the native phases (Sim, 1959).

Binding sites and the electron density maps were examined in detail with the aid of a modified (J. W. Pflugrath, M. Saper, R. Hubard, and P. R. Evans) program FRODO (Jones, 1978, 1985) implemented on an Evans and Sutherland PS300 picture system on line to a VAX11/750 computer. The atomic coordinates of the phosphite anion were taken from the crystal structure analysis of $CuHPO_3{\cdot}2H_2O$ (Handlovic, 1969). The coordinates for α-D-glucose based on neutron diffraction data were taken from Brown and Levy (1979).

The crystal structure of phosphorylase *b* (Weber et al., 1978; Sansom et al., 1985) has been refined by the Konnert and Hendrickson restrained parameter crystallographic least-squares procedure (Hendrickson & Konnert, 1985) implemented on a fast array processor (Furey et al., 1982). At the present stage of the analysis, the crystallographic R factor for 61344 reflections [$(I) > 3\sigma(I)$] at 1.9-Å resolution is 0.187 (K. R. Acharya et al., unpublished results). The current model contains some 7398 atoms including 692 water molecules, and the root mean square deviation from ideal covalent bond lengths is 0.018 Å. An additional Ile residue inserted after Ile-307 has increased the numbering of residues by 1 from 308 onward. Thus, the Lys to which PLP is linked via a Schiff base is now Lys-680 and not -679 as given previously (Titani et al., 1977). Seven further small changes in amino acid sequence as indicated by the cDNA sequence of rabbit muscle phosphorylase (Nakano et al., 1986) have also been incorporated.

The structures of the derivative proteins were refined by the restrained parameter least-squares method by using as starting coordinates those of the native structure with conformational changes and additional ligands as indicated from the difference and $(2F_d - F_p)$ Fourier syntheses. The restraints were similar to those used for the refinement of the native structure. Possible hydrogen bonds were noted if the donor–acceptor distances were less than 3.3 Å and if the bond was linear to within 40°. Atoms were considered to be in van der Waals contacts with the protein atoms if their distances were less than 4.0 Å.

The data processing and refinement statistics are shown in Table I.

Results

Crystallization. The inclusion of spermine rather than magnesium acetate in the crystallization medium resulted in large, well-ordered tetragonal crystals within a few days. The method worked well for native, $NaBH_4$-reduced, and PDMP- and 6-FPLP-reconstituted phosphorylase *b*. Phosphorylase *b* reconstituted with PL or PLPME could not be crystallized in the tetragonal form. Instead, clusters of small, possibly monoclinic crystals appeared in the crystallization tubes. However, successful crystallization of PL-enzyme took place in the presence of 6.5 mM phosphite and 50 mM glucose. Under conditions similar to those employed for the crystallization of PL-phosphorylase *b*, fluorophosphate, phosphate, and sulfite were found to be effective in inducing crystallization of tetragonal crystal form of PL-enzyme, and selenite caused protein precipitation, while approximately square plates were grown with sulfate, thiophosphate, dithiophosphate, (aminomethyl)phosphonate, pyrophosphate, phosphonoacetate, and molybdate. These plates however have been too small for the crystallographic characterization. In general, conditions that favored T-state phosphorylase resulted in tetragonal crystals,

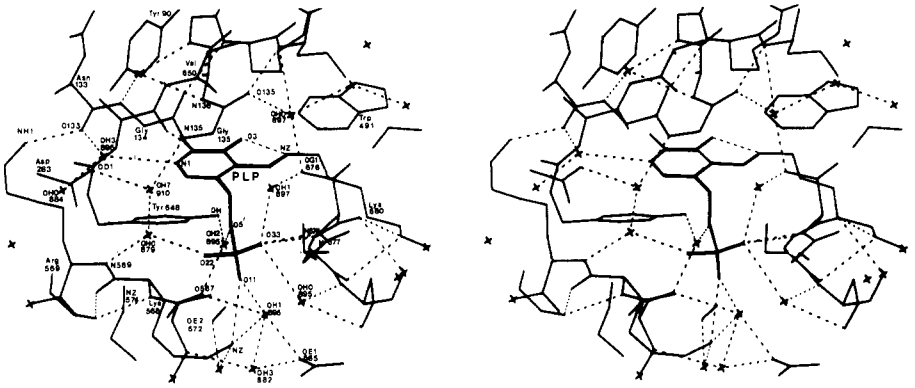

FIGURE 2: A stereo drawing of the interactions between PLP and phosphorylase *b* residues of the catalytic site. Hydrogen bonds less than 3.3 Å are indicated. Water molecules are shown as crosses.

Table II: Kinetic Properties of Glycogen Phosphorylase *b* Reconstituted with PLP and PLP Analogues

enzyme	V_{max} [μmol/(min·mg)]	K_m for glucose-1-P (mM)
PLP-phosphorylase *b*	74.5[a]	4.2[a]
PDMP-phosphorylase *b*	23.8[b]	3.1[b]
6-FPLP-phosphorylase *b*	17.8[c]	7.4[c]
PL-phosphorylase *b* + phosphite	15.4[d]	22[d]

[a] Determined at 30 °C and pH 6.8 in a reaction mixture consisting of 25 mM β-glycerolphosphate, 25 mM 2-mercaptoethanol, 0.5 mM EDTA, 1% glycogen, 1 mM AMP, and varied concentrations of glucose-1-P (2–60 mM) from double-reciprocal plots (see Materials and Methods). [b] Data from Vidgoff et al. (1974). [c] Data from Chang and Graves (1985). [d] Data from Chang et al. (1983).

while conditions that favored R state (e.g., PL, PLPME, or PL plus pyrophosphate) gave small, possibly monoclinic crystals.

Kinetic Properties. The kinetic properties of native and derivative phosphorylases *b* are summarized in Table II. PDMP-phosphorylase shows a reduction in V_{max} with no significant change of K_m for glucose-1-P. 6-FPLP-phosphorylase shows a further reduction in V_{max}. The increase in K_m with respect to glucose-1-P is probably due to the different assay conditions used by Chang and Graves (1985). In fact, the K_m for glucose-1-P for the native enzyme in their kinetic experiments was reported to be 8 mM (Chang et al., 1987). PL-phosphorylase activated with phosphite shows the least activity and exhibits a significant increase in K_m for the substrate.

Structure of the Pyridoxal Phosphate Site in Native Phosphorylase b. The overall structure of phosphorylase *b* and the general environment of the cofactor have been described previously (Weber et al., 1978; Sansom et al., 1985; Johnson et al., 1987). The long polypeptide chain is folded in two domains: domains 1 (residues 19–484) and 2 (residues 485–841). The first 19 and the last residue from the N and C terminal, respectively, cannot be located and are assumed to be mobile. Apart from these, the chain can be traced continuously from residue 19 to residue 841.

The PLP is buried in the center of the molecule at a site some 15 Å from the surface where domains 1 and 2 come together and is close to the substrate binding site. Calculations of solvent contact areas (Sansom et al., 1985) show that the cofactor is approximately 90% buried and only one of the phosphate oxygen atoms is accessible to bulk water. The details of the contacts to the enzyme are shown in Figure 2 and Table III.

Lys-680 is the fifth residue of α-helix 21 (residues 676–686). [Helix 21 is equivalent to α-E of the nucleotide binding domain in the terminology of Rossmann et al. (1974).] The plane of the pyridoxal ring lies above the β-sheet. There are three aromatic rings in the vicinity: Tyr-648, Tyr-90, and Trp-491. The side chain of Tyr-648 makes the most extensive contacts (Table III). It stacks against the A face with the plane of the

Table III: Hydrogen Bonds and van der Waals Contacts to Pyridoxal Phosphate in Phosphorylase *b*[a]

	Hydrogen Bonds			
atom	first coordination	second coordination	third coordination	fourth coordination
N1	OH3.896	OH Tyr-90	NH1 Arg-569	OD1 Asn-133
		OH0.884	N Lys-608	
O3	NZ Lys-680			
O5′	OH2.895	O Val-567	OH1.895 ⟶	see below for atom O11
		OH Tyr-648		
O11	NZ Lys-568			
	OH1.895	OE1 Gln-665		
		OH3.882	N Val-567	
			O Val-565	N Glu-664
		O Val-567	OH2.895 ⟶	see above for atom O5′
O22	OH0.879	N Arg-569		
		OH7.910	N Gly-135	
			OD1 Asp-283	
O33	N Thr-676			
	N Gly-677			
	OH1.897	OG1 Thr-676		
	OH0.885	N Asn-678		
		ND2 Asn-678		
		N Thr-676		

	van der Waals Contacts	
atom	no. of contacts	protein atoms
N1	2	CA Gly-134; CE2 Tyr-648
C2	3	CE2, CZ, OH Tyr-648.
C2′	9	OH Tyr-90; CE2, CZ, OH Tyr-648; CA, C, O Arg-649; CA, CG2 Val-650
C3	1	OH Tyr-648
O3	4	CH2 Trp-491; OH Tyr-648; CZ Arg-649; CB Ala-653
C4	1	OH2.895
C4′	1	OG1 Thr-676
C5	1	OH2.895
C5′	5	CA, N Gly-135; OG1 Thr-676; OH2.895; OH1.897
O5′	3	CA, N Gly-677; OH0.879
P	5	NZ Lys-568; N Gly-677; OH0.879; OH0.885, OH2.895
O11	6	O Val-567; CB, CE Lys-568; CA Gly-677; OH0.885; OH2.895
O22	1	CB Lys-568
O33	4	CA Gly-675; CA, C, OG1 Thr-676
C6	5	CA, C Gly-134; OH0.879; OH2.895; OH3.896
total	53	

[a] Water molecules are listed as OH*n.m*, indicating that the water is the *n*th atom in residue *m*. This was a convenient way to group the large number of atoms for the refinement and graphics programs.

tyrosyl residue inclined 36° to that of the pyridoxal ring. The side chain of Tyr-90 is inclined 58° to the pyridoxal ring and makes a few contacts to some atoms from the B face. The indole ring of Trp-491 is inclined 66° to the pyridoxal and packs against the edge of the pyridoxal ring, making van der Waals contacts to O3 and the lysine-680 side chain on the A face. (The A and B faces are those viewed with the numbering system clockwise and anticlockwise, respectively.) Val-650 packs against the B face so that the cofactor ring is sandwiched between nonpolar residues. In addition, the following residues also make van der Waals interactions: Gly-134, Gly-135, Arg-649, Ala-653, and Thr-676.

The N1 atom makes no direct polar contacts with protein atoms but interacts via a water molecule (OH3.896) to a hydrogen bond network involving a second water molecule (OH0.884), Asn-133, and Tyr-90. The C2′ methyl group makes the largest number of van der Waals contacts (Table III) and appears to be a key feature in localizing the pyridoxal ring. The close contacts at the C2′ methyl group explain why ω-methylpyridoxal 5′-phosphate in which the $-CH_3$ group is replaced by the bulkier $-CH_2CH_3$ is not able to activate apophosphorylase (Shaltiel et al., 1969). However, 2-norpyridoxal 5′-phosphate in which the $-CH_3$ group is substituted by an –H atom can reactivate apoenzyme by 65%, an example whereby a site may exclude a bulkier substituent but is more tolerant to a smaller substituent (Fersht, 1985). The Schiff base is planar and trans (torsion angles CE–NZ–C4′–C4 and NZ–C4–C4′–C5 are 179° and −178°, respectively) as is found in the single-crystal structure of pyridoxal phosphate oxime (Barrett & Palmer, 1969). There is a hydrogen bond between the 3′-OH and the Schiff base nitrogen. The aliphatic part of Lys-680 is shielded by Trp-491. The C6 position is relatively open with van der Waals contacts only to residue Gly-134 and three water molecules. Examination of the model with van der Waals spheres generated by FRODO shows in fact there is a small cavity adjacent to this atom.

The torsion angles for the phosphate ester are C4–C5–C5′–O5′ = 94° and C5–C5′–O5′–P = 167°. Initially, it was thought that the conformation was trans–trans as observed in single-crystal studies, but the conformation resulting after high-resolution refinement at 1.9 Å shows that the C5′–O5′ bond is more nearly perpendicular to the pyridoxal ring. The O5′ oxygen is hydrogen bonded to a water molecule (OH2.895), which in turn is hydrogen bonded to main chain O Val-567 and OH Tyr-648. The phosphate group is highly solvated (Figure 2, Table III). One phosphate oxygen (O11) interacts with a basic group (Lys-568) and with a water molecule that is involved in an extensive network of hydrogen bonds. The second oxygen (O22) is the most exposed and is linked via a water to the main chain NH of Arg-569 and via a second water to Asp-283 and main chain NH Gly-135. In the enzyme–product complex with heptulose 2-phosphate (Hajdu et al., 1987) this oxygen makes a direct contact to the product phosphate. The third oxygen (O33) makes two direct protein contacts to the main chain NH groups of Thr-676 and Gly-677 at the start of the α-21 helix. The span provided by the lysine side chain and the aromatic ring allows the phosphate of the cofactor to interact with the amino terminus of the Lys-680 α-21 helix. This characteristic phosphate helix dipole interaction (Hol et al., 1978) is likely to be strong in the buried environment. In addition, O33 makes contact to two further water molecules which are involved in additional hydrogen bond networks.

PDMP-phosphorylase b. The resulting difference Fourier synthesis for PDMP-reconstituted phosphorylase *b* showed

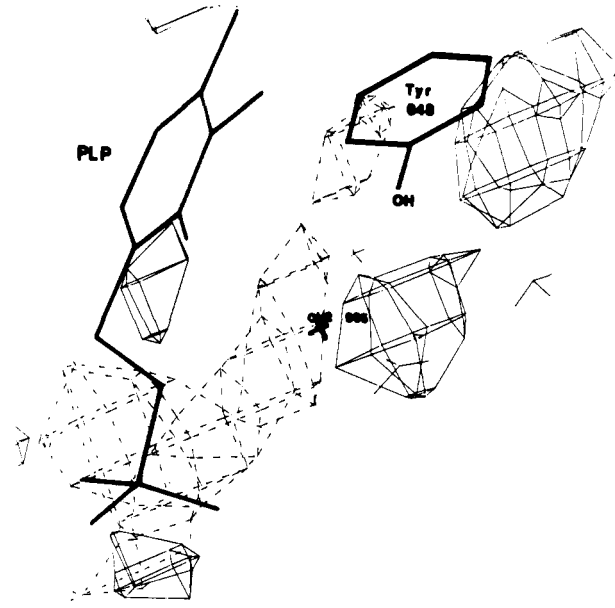

FIGURE 3: Difference Fourier synthesis in the vicinity of the catalytic site for PDMP-phosphorylase *b*. Difference electron density is contoured at 510 arbitrary units (3 times the root mean square density over the map). Positive and negative densities are represented by full and dotted lines, respectively. The atomic coordinates of the native structure are displayed.

significant peaks at the cofactor and the catalytic sites and no disturbance elsewhere (Figure 3). Weak binding of glucose (present at a concentration of 5 mM in the crystallization medium) was observed. Yan et al. (1979) have determined a K_i value of 12 mM for glucose inhibition of PDMP-phosphorylase *a*. The constructed model for PDMP was based on the PLP model by substituting O5′ by C and assuming C5′–C and C–P bond length values of 1.54 and 1.81 Å, respectively. The angles C5′–C–P was adjusted so as to produce a value such as expected from tetrahedral geometry about C. In the native PLP-phosphorylase *b*, O5′ makes a hydrogen bond with OH of Tyr-648 through the water OH2.895. The substitution of O5′ by a methylene group ($-CH_2-$) in PDMP-phosphorylase *b* results in removal of this hydrogen bond. In the refined atomic coordinates of PDMP-enzyme, OH2.895 and OH of Tyr-648 were shifted 0.9 and 0.6 Å, respectively, from their initial positions in the native structure. The pyridoxal ring was also translated about 0.4 Å to follow the movement of the tyrosine. The phosphorus atom of PDMP was shifted only 0.3 Å from the position observed for PLP. However, the amino group NZ of Lys-574 was shifted 0.7 Å. Thus, the NZ of Lys-574 comes close to the 5′-phosphonate oxygen O22 (3.4 Å), a feature different from that in the native PLP-phosphorylase *b*, were the separation is 4.5 Å (Figure 2). O11 interacts with the NZ of Lys-568 (O11–NZ distance = 2.6 Å) as in the case of PLP-phosphorylase *b*, and the hydrogen bond networks with which the 5′-phosphonate group is connected are similar to those of the native structure (Table III). Apart from these movements, there are no indications of other changes in PDMP-phosphorylase *b* structure.

6-FPLP-phosphorylase b. The electron density for the fluorine atom in position 6 of the coenzyme ring is easily identified in both the difference Fourier (Figure 4) and the refined map. In constructing a model for 6-FPLP, we assumed a C6–F6′ bond length of 1.4 Å. The fluorine atom makes contacts with residues Ca of Gly-134 (3.1 Å), C of Gly-134 (3.6 Å), N of Gly-135 (3.6 Å), and three water molecules, OH3.896 (3.3 Å), OH0.879 (3.5 Å), and OH7.910 (3.8 Å). These water molecules are involved in an extensive network

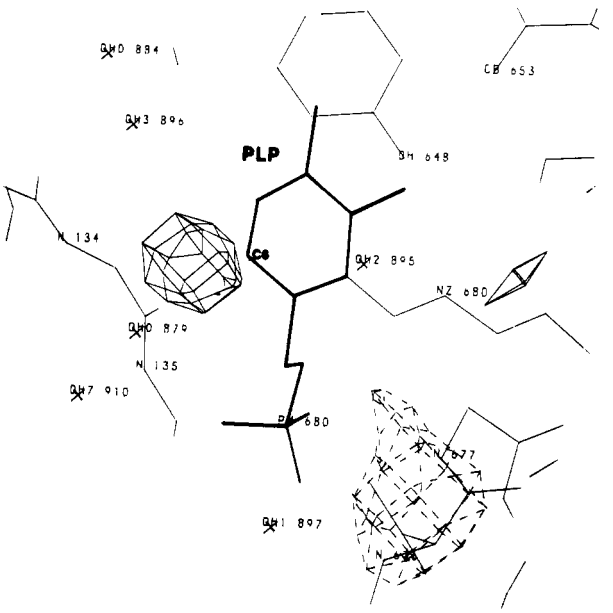

FIGURE 4: Difference Fourier synthesis of the catalytic site for 6-FPLP-phosphorylase *b*. Density is contoured at 550 arbitrary units (3 times the root mean deviation in the map). The atomic coordinates of the PLP-phosphorylase *b* are displayed.

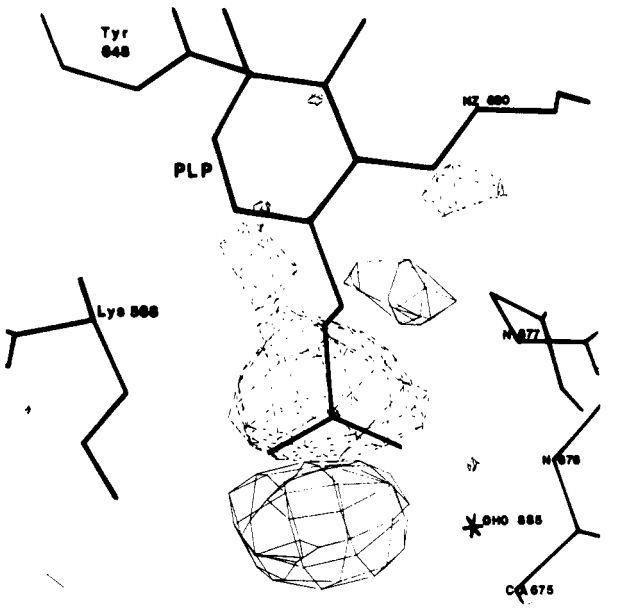

FIGURE 5: Difference electron density map of PL- versus native PLP-phosphorylase *b* in the region of the active site. Density is contoured at 200 arbitrary units (4 times the root mean deviation in the map). The atomic coordinates of the native enzyme are displayed.

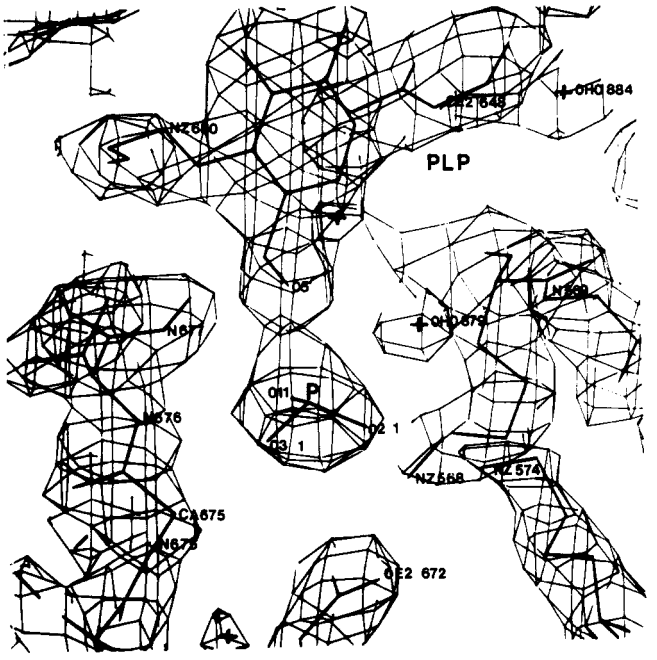

FIGURE 6: Electron density in the region of the active site of PL-phosphorylase *b*, where phosphite is bound. The map was calculated by using as coefficients $2F_o - F_c$. The refined atomic coordinates for the PL-phosphorylase *b*–phosphite–glucose complex are shown.

of hydrogen bonds in the native structure (Table II). Murray-Rust et al. (1983), from a detailed examination of the literature on the possible modes of interaction of C–F bonds with other groups, have concluded that fluorine in a C–F bond can act as weak proton acceptor in forming hydrogen bonds. A water-mediated coordination involving a C–F bond (3.0 Å) and a Ca^{2+} ion in calcium 2-fluorobenzoate has also been reported (Karipides & Miller, 1984). In our results, distances of 3.3 and 3.5 Å between F6′ and water molecules OH3.896 and OH0.879 are considered rather long to represent strong hydrogen bonds.

The difference Fourier synthesis for 6-FPLP-phosphorylase *b* showed another positive peak (height 650 units; maximum height for fluorine peak was 850). This peak was found in the vicinity of residues Leu-139, Asn-484, and Thr-676 and also very close to the position occupied by the C6 of glucose. Binding of glucose at the catalytic site was not observed. At the present time, it is difficult to characterize this peak. A possible interpretation is that it represents some "contamination" resulting from the treatment of 6-FPLP-phosphorylase *b* with charcoal (see Materials and Methods). Since, the enzyme solution used in our crystallization experiments was further purified by gel filtration, it is likely that this "contamination" is tightly bound to the enzyme. In addition, the difference map showed a negative density (with no associated positives), indicating possibly some small disturbances in residues Gly-675 and Thr-676.

PL-phosphorylase b. The interpretation of the structural results was based upon difference Fourier and refined maps resulting from two different data sets (Table I): PL-phosphorylase *b* crystals soaked in (i) their mother liquor (6.5 mM phosphite, 50 mM glucose, and 10 mM BES, pH 6.7) or (ii) 5 mM pyrophosphate, 10 mM IMP, 50 mM glucose, and 10 mM BES, pH 6.7. The latter buffer was chosen because pyrophosphate alone (1–20 mM in 10 mM BES) caused cracking of the crystals. Both maps showed the same features, and a double difference Fourier map, calculated by using as coefficients $F_{PL+pyrophosphate} - F_{PL}$, was clean at the catalytic site with no indication of pyrophosphate binding. Since, however, the resolution was higher in the "pyrophosphate" experiment (2.4 Å) and the quality of the data better than in the "native PL-enzyme" data set, the former map was used to analyze most of the structural changes.

Replacement of PLP in phosphorylase by PL resulted in a negative peak which appeared in the position of the 5′-phosphate of the native PLP-enzyme and a positive peak representing the binding of phosphite (Figure 5). Crystallographic refinement (Figure 6) showed that the phosphite anion is located close to but separate from the 5′-phosphate binding site of the native enzyme (P–P separation = 1.0 Å). The peak is sufficiently strong to indicate a high affinity of PL-phosphorylase *b* for phosphite (in the presence of glucose). Other significant features of the difference map included (1) a strong positive density corresponding to glucose binding,

associated with negative densities, indicating movement of His-377 and displacement of a water molecule (OH9-904) and (2) shifts of three water molecules, OH7.910, OH1.895, and OH0.879, located at the active site.

The mode of binding and the contacts that glucose makes with PL-phosphorylase *b* in the presence of phosphite are almost identical with those previously reported for glucose phosphorylase *a* complex (Sprang et al., 1982; Street et al., 1986). Ultracentrifugation and ^{31}P NMR experiments have shown that glucose is unable to bind to the PL-phosphorylase *b* (or binds weakly) in the absence of phosphite (Withers et al., 1982a). They also showed that PL-phosphorylase *b* is an allosteric enzyme whose R state is stabilized by pyrophosphate and whose T state is stabilized by glucose and phosphite. Attempts to wash out phosphite and glucose from PL-phosphorylase *b*–phosphite–glucose crystals resulted in crystals showing a disordered diffraction pattern, indicating that both phosphite and glucose binding provide significant stability to the tetragonal crystal form. In the present results at 2.4-Å resolution, we cannot define the positions of the phosphite oxygens and must infer them from stereochemistry. Thus, from the orientation of phosphite oxygens as deduced from the crystallographic refinement, oxygen O1 makes no direct interactions with protein residues.

It is interesting however that it interacts with O1G and O4G of glucose via two water molecules, OH0.914 and OH7.910, respectively. Phosphite oxygen O2 makes a direct interaction with NZ of Lys-568, a water molecule (OH0.879), and possibly NZ of Lys-574 and OE2 of Glu-672. The third oxygen, O3, makes a direct interaction with main chain NH of Thr-676 and two indirect interactions to ND2 of Asn-678 and carbonyl O of Ser-674, both through a water molecule (OH0.885). The position of the phosphite ion is approximately 0.5 Å closer to the position observed for the phosphates in the enzyme–substrate complexes with both heptulose-2-P and glucose-1-P (Hajdu et al., 1987).

Discussion

PDMP-phosphorylase b. PDMP is the only PLP analogue containing a modified 5′-phosphate that is able to confer significant activity on apophosphorylase *b* (Table II). ^{31}P NMR experiments (Klein et al., 1984) indicate that the state of ionization of the phosphonate component changes on going from inactive to active phosphorylase in a way that is different from that of the native enzyme. In native enzyme, PLP-phosphorylase, the ^{31}P NMR results indicate that the 5′-phosphate group is monoanionic in T-state phosphorylase and dianionic in R-state phosphorylase (Helmreich & Klein, 1980). In PDMP-phosphorylase similar studies indicate that in the T state the 5′-phosphonate group is dianionic and in the R state it is monoanionic. These differences cannot be attributed to the change in pK_a values of the two cofactors [pK_a(PLP) = 6.2; pK_a(PDMP) = 7.35]. PLP- and PDMP-phosphorylases exhibit similar pH dependencies for activity (Vidgoff et al., 1974). Part of the challenge for the structural results is to explain these changes in ionization. With our present understanding of electrostatic effects in protein molecules [e.g., Rogers (1986)] it would be rash to state definitely the likely state of ionization of the cofactor phosphate, on the basis of its structural environment. Nevertheless, the structural results for the T-state phosphorylase *b* structure are consistent with a monoanion or monoanion/dianion mixture. The cofactor 5′-phosphate interacts with one basic group (NZ of Lys-568) and with the helix dipole of the α-21 helix. The latter could contribute about 0.5 of a positive charge (Hol et al., 1978) so that the total positive charge in the immediate neighborhood of the phosphate is 1.5. Other ionic groups in the vicinity are Lys-574 (4.5 Å) and Glu-672 (4.8 Å), where distance refers to the closest approach of charged group to the cofactor 5′-phosphate oxygens. Asp-283 (6.6 Å), however, interacts with the cofactor 5′-phosphate oxygens by a route that involves two water molecules. The presence of Asp-283, which is also hydrogen bonded through its other oxygen to His-571 and Asn-284, would therefore tend to diminish the net positive charge. In order to preserve electrostatic neutrality, the 5′-phosphate group should be either a monoanion or a monoanion/dianion mixture.

In PDMP-phosphorylase, as a result of the different geometry of the phosphonate linkage and movement of Lys-574, the phosphonate oxygens are placed over 1 Å closer to the NZ of Lys-574. This closer proximity to a positively charged group could explain the apparent change in state of ionization of the PDMP-phosphorylase *b* from that observed for PLP-phosphorylase *b*.

The presence of the nonpolar phosphonate linkage in PDMP caused a movement of an internal bound water and the side chain of Tyr-648 to which the water is hydrogen bonded. The protein appears to be able to accommodate these shifts of 0.9 Å, which correspond to a shift from hydrogen bonding to van der Waals contact of the water, without any further adjustment.

6-FPLP-phosphorylase b. The study with 6-FPLP analogue demonstrates the power of the difference Fourier synthesis in that a single fluorine atom can be detected among over 7000 protein and water molecules. The structural results show that fluorine can be accommodated in this position with essentially no disturbance of the structure. The pyridoxal ring has altered its position slightly, but the shifts are too small to change the van der Waals contacts or hydrogen bonds.

Solution studies have shown that, except for the lower activity, most of the properties of phosphorylase such as pH optimum, reconstitution energy, K_m values for glucose-1-P and AMP, and cooperative binding of AMP and glucose, are retained in 6-FPLP-enzyme (Chang & Graves, 1985). The question therefore remains why 6-FPLP-phosphorylase is less active than the native enzyme. Chang et al. (1986, 1987) in their ^{19}F NMR studies have pointed out the likely importance of the cofactor in coordinating the correct conformation of the protein during the catalytic cycle. The presence of the electronegative fluorine at position 6 changes the p*K* of the ring nitrogen from 8.7 in free PLP to <1 in 6-FPLP (Korytnyk & Kravastava, 1973). Chang et al. (1985) concluded that the lower activity of 6-FPLP-phosphorylase was due to a change in the interactions at N1. The structural results show that in fact the interactions in T-state phosphorylase do not change as such. The spectroscopic evidence (Shaltiel & Cortijo, 1970; Johnson et al., 1970; Feldman & Helmreich, 1976) shows that, despite the high p*K* of N1 in free PLP, in the buried environment of the phosphorylase cofactor site the N1 is neutral. It participates in a hydrogen bond network through a water molecule to a number of important residues (Table II). The link via O Asn-133 to NH1 Arg-569 seems especially important in view of the conformational changes observed for the arginine side chain (Hajdu et al., 1987). Thus the lower p*K* of N1 in the 6-FPLP-phosphorylase could result in a strengthening of this network, making it less easy for the arginine to move. The weak link via the water to tyrosine-90 is also interesting. Tyr-90 is one of the few residues from domain 1 (the control domain) that makes direct contact with the cofactor. In the nonregulatory *Escherichia coli* maltodextrin phosphorylase (Palm et al., 1985) and the potato

phosphorylase (Nakano & Fukui, 1986), Tyr-90 is replaced by Leu but all other groups of atoms in contact with the cofactor are conserved. Leu-90 could serve the same role as the Tyr for van der Waals contacts, but it could not participate in this weak hydrogen bond to the water which is linked to the ring nitrogen.

PL-phosphorylase–Phosphite–Glucose Complex. Many studies on the catalytic mechanism of phosphorylase have exploited the ability of PL-phosphorylase to be activated by a number of dianions (Parrish et al., 1977; Chang et al., 1983). In general, these observations are in agreement with corresponding studies with modified cofactors with the exception of fluorophosphate. Fluorophosphate has a pK_a of 4.8 and can exist only as a dianion at pH at which phosphorylase is active. Pyridoxal 5′-fluorophosphate phosphorylase is inactive (Klein et al., 1982; Withers et al., 1982b), but pyridoxal phosphorylase plus fluorophosphate exhibits 12% activity (Parrish et al., 1977). The former result is consistent with a role for the cofactor phosphate as a proton donor in catalysis. The latter result, however, does not support a role as a direct proton donor but could be consistent with the electrophilic hypothesis. Klein et al. (1984) have suggested that in studies with dianion activated PL-phosphorylase the mobile dianion might perform a different role from that in native PLP-phosphorylase.

The present structural results show that, in T-state PL-phosphorylase *b*, the phosphite dianion occupies a site which is 1.0 Å (P–P distance) from that occupied by the 5′-phosphate of PLP. In the presence of glucose this site is a tight binding site for phosphite and cannot be replaced by pyrophosphate, nor can it be washed out of the crystals. The electrophilic hypothesis for catalysis demands very specific interactions between the phosphate or phosphite group and the enzyme in order to stabilize the constrained dianion. The different position of the phosphite ion and the different contacts made to the enzyme compared to the native cofactor show a degree of flexibility that is not in accord with this hypothesis.

The pyridoxal of PL-phosphorylase is remarkably similar to the pyridoxal of PLP-phosphorylase. The electron density indicates that even the 5′-OH group occupies a similar position to its counterpart in PLP.

In conclusion, studies with modified cofactors show that on the one hand the cofactor site is remarkably tolerant to substitutions and deletions since it can accommodate 6-FPLP, PDMP, or PL and phosphite with only minor adjustments of the site. On the other hand, these small adjustments and changes in electronic properties have significant effects on the catalytic efficiency of the enzyme. For PDMP-phosphorylase there are small but significant changes in the environment of the phosphonate group that could account for reduced activity. 6-FPLP-phosphorylase shows no significant structural changes, but the reduction in maximum velocity may be due to the change in pK of the ring nitrogen. The lowered pK could lead to an increase in strength of those interactions that stabilize the T-state conformation. The more substantial chemical change of replacement of PLP by PL and phosphite results, not surprisingly, in a greater shift between the phosphate and phosphite positions than any of the shifts observed with the other derivatives. Indeed, it is remarkable that the enzyme can tolerate a shift of 1.0 Å in an essential catalytic group and exhibit a reduction in rate by a factor of about 5. In triosephosphate isomerase a similar shift in a catalytic group achieved in a mutant enzyme in which an active site glutamate was changed to an aspartate resulted in a reduction in rate by a factor of about 1000 (Raines et al., 1986). Thus in phosphorylase the different position and the slightly different environment of the phosphite ion when compared with those of the native PLP indicate a certain tolerance of the enzyme for the disposition of a key catalytic group.

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