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# Characterization of the Pyridoxal Phosphate Site in Glycogen Phosphorylase *b* from Rabbit Muscle†

Shoji Shimomura and Toshio Fukui*

ABSTRACT: The cofactor site in glycogen phosphorylase *b* from rabbit muscle (EC 2.4.1.1) has been characterized by circular dichroism and electronic absorption studies on the reconstitution of the apoenzyme with pyridoxal 5′-phosphate (natural cofactor) and its analogues modified at the 5′ position, i.e., 5′-sulfate, 5′-phosphate monomethyl ester, 5′-phosphate monobenzyl ester, 5′-deoxy-5′-sulfonate, 5′-diphosphate, and 5′-diphosphate β-monophenyl ester, and $P^1$,$P^2$-bis(5′-pyridoxal) diphosphate (bis-PLP). All the cofactor analogues bind to the apoenzyme in the same (or similar) binding mode as pyridoxal 5′-phosphate, though these reconstituted enzymes show no enzyme activity. The asymmetric environment of the pyridoxal bound at the cofactor site is not altered by introducing a bulky substituent to the 5′ position. The size of the 5′ substituent of pyridoxal seems not to impose any restriction on the rate of reconstitution. Therefore, the 5′-phosphate locus of the cofactor site is a wide space or of flexible structure enough to adapt a large group therein. The rate of reconstitution markedly increases with the increments in the number of anions at the 5′ position of pyridoxal. A cationic group or groups interacting with the phosphate group should be present at the cofactor binding site. Two ionizable groups with p*K*s of 6.0 and 8.0 affect the rate of reconstitution, and are assigned to the 5′-phosphate group of the cofactor and to the ε-amino group of Lys-679 (the original cofactor-binding residue) in apophosphorylase, respectively. The optical properties of the bis-PLP cross-linking two ε-amino groups of Lys-573 and Lys-679 indicate that the environment around Lys-573 is similar to 70–80% dioxane in water, while around Lys-679 to more than 95% dioxane. The presence of the hydrophobic region adjacent to the 5′-phosphate locus of the cofactor site is also suggested by the finding that the cofactor derivatives having a benzene ring on the 5′-phosphate group can rapidly bind to the apoenzyme.

Pyridoxal 5′-phosphate (PLP[1]) is the cofactor present in all known α-glucan phosphorylases (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1). Removal of the cofactor from rabbit muscle phosphorylase produces the inactive apoenzyme which, in turn, restores its original activity on incubation with PLP (see Fischer et al., 1970; Graves & Wang, 1972). The cofactor binds to Lys-679 in rabbit muscle phosphorylase (Titani et al., 1977). The amino acid sequence around the lysine residue linking to the cofactor is highly conserved in phosphorylases from rabbit muscle, yeast, and potato tubers (Lerch & Fischer, 1975; Nakano et al., 1978), while these phosphorylases have different regulatory properties. The results of reconstitution studies using various PLP analogues have demonstrated the importance of the 5′-phosphate group of PLP in phosphorylase action (Shaltiel et al., 1969b; Pfeuffer et al., 1972a,b; Vidgoff et al., 1974; Parrish et al., 1977). The results of recent X-ray crystallographic studies on rabbit muscle phosphorylase *a* have shown that PLP is buried inside the protomer and its 5′-phosphate group is located adjacent to the substrate site (Sygusch et al., 1977). Close proximity of the two sites has also been suggested from pyrophosphate inhibition of PL-reconstituted phosphorylase *b* (Parrish et al., 1977). These findings lead one to speculate that the cofactor, especially its 5′-phosphate group, participates in catalysis, while no definitive evidence for its catalytic role has so far been obtained.

Although it has been shown from spectroscopic and fluorescence studies that the pyridoxal moiety of the PLP in phosphorylase is in a highly hydrophobic environment (Shaltiel & Cortijo, 1970; Johnson et al., 1970), detailed characterization of the cofactor site, e.g., what kind of amino acid residues are present, and whether the 5′-phosphate moiety of the cofactor is also in a hydrophobic environment or not, is lacking. We have previously been using circular dichroism (CD) to study the interaction of the allosteric activator (5′-AMP) and the cofactor with rabbit muscle phosphorylase, and found that

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[1] Abbreviations used: CD, circular dichroism; UV, ultraviolet; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal 5′-phosphate; SA, salicylaldehyde; PL, pyridoxal; PLSN, 5′-deoxypyridoxal-5′-sulfonate; PLS, pyridoxal 5′-sulfate; PLP-Me, pyridoxal 5′-phosphate monomethyl ester; PLP-Bz, pyridoxal 5′-phosphate monobenzyl ester; PLDP, pyridoxal 5′-diphosphate; PLDP-Phe, pyridoxal 5′-diphosphate β-monophenyl ester; bis-PLP, $P^1$,$P^2$-bis(5′-pyridoxal) diphosphate; 3-*O*-MePLP, 3-*O*-methylpyridoxal 5′-phosphate.

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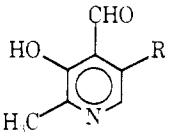
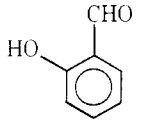
CD is a useful tool to detect selectively the enzyme-bound PLP or its analogues and to investigate the specific interaction between an inactive PLP analogue and apophosphorylase (Shimomura & Fukui, 1976, 1977). In this paper, we report some characteristics of the cofactor site in rabbit muscle phosphorylase *b*, based on the results of a comparison of reactivities toward apophosphorylase between PLP and its analogues. The cofactor analogues used here are listed in Diagram I. Five

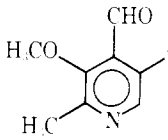
DIAGRAM I



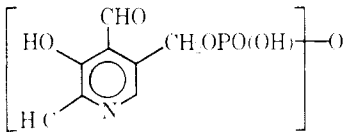
PLP, R = $CH_2OPO_3H_2$
PL, R = $CH_2OH$
PLSN, R = $CH_2SO_3H$
PLS, R = $CH_2OSO_3H$
PLP-Me, R = $CH_2OPO_2(OCH_3)H$
PLP-Bz, R = $CH_2OPO_2(OCH_2C_6H_5)H$
PLDP, R = $CH_2OPO_2(OPO_3H_2)H$
PLDP-Phe, R = $CH_2OPO_2(OPO_2(OC_6H_5)H)H$



SA



3-*O*-MePLP



bis-PLP

compounds (PLSN, PLP-Bz, PLDP, PLDP-Phe, and bis-PLP) among them were first tested for possible interaction with phosphorylase, and their synthetic procedures are described herein. Bis-PLP has recently been shown to be an affinity labeling reagent of the cofactor site in phosphorylase *b* (Shimomura et al., 1978), and was used here as a probe for microenvironment in the vicinity of the cofactor site.

## Experimental Procedures

*Enzymes.* Phosphorylase *b* was prepared from frozen rabbit muscle using the procedure of Fischer & Krebs (1958). The enzyme was recrystallized three times. Apophosphorylase *b* was prepared according to Shaltiel et al. (1966), and passed over a Sephadex G-25 column equilibrated with 0.1 M sodium 2-glycerophosphate and 20 mM 2-mercaptoethanol (pH 7.0) or with 0.1 M sodium 2-glycerophosphate and 5 mM EDTA (pH 7.0). Phosphorylase activity was assayed in the direction of glycogen synthesis. The assay medium contained 75 mM glucose 1-phosphate, 1% glycogen, 1 mM 5′-AMP, 25 mM 2-glycerophosphate, and 25 mM 2-mercaptoethanol (pH 7.0). Inorganic orthophosphate released in the reaction was measured by the method of Fiske & Subbarow (1925). Enzyme concentration was determined spectrophotometrically using an absorbance index $A^{1\,cm}_{280nm} = 13.2$ for a solution containing 10 mg/mL (Kastenschmidt et al., 1968). Molecular weight of 97 300 for phosphorylase *b* monomer was used (Titani et al., 1977).

*Optical Measurements.* CD measurements were carried out with a Jasco J-20 spectropolarimeter equipped with a CD attachment, which was calibrated with *d*-camphor-10-sulfonic acid (Cassim & Yang, 1969). Molar ellipticity ($[\theta]_M$) was obtained in units of deg $cm^2$ $dmol^{-1}$. Absorption spectra were measured with a Hitachi 124 spectrophotometer. Molar absorbance ($\epsilon$) was obtained in units of $M^{-1}$ $cm^{-1}$. The temperature was controlled by means of a thermostated cell holder, to which water at a constant temperature was circulated.

*Reconstitution Experiments.* Titrations of apophosphorylase *b* with the PLP analogues were performed as described previously (Shimomura & Fukui, 1977). The CD and absorption spectra and the enzyme activities of the reconstituted phosphorylases were measured after standing overnight at room temperature and in the dark. Time course of the reconstitution process was followed by CD. Second-order rate constant for the reaction was calculated according to Florentiev et al. (1970). The reaction was started by addition of the apophosphorylase solution to the solution of PLP or its analogue. In the experiments of the pH dependence, a small volume of the apoenzyme solution (pH 7.0) was added to the solution of PLP or its analogue adjusted to a desired pH, and the final pH was measured after the time course of the reconstitution had been followed.

*Dissociation Experiments.* Dissociation of the cofactor analogues from the reconstituted enzymes was followed by the exchange reaction between the enzyme-bound analogue and free PLP. In these experiments, PLP analogue–phosphorylase was mixed with a large molar excess of PLP to trap the free apoenzyme released from the reconstituted enzyme and thus to prevent the reverse reaction with the PLP analogue. A solution of 9.7 $\mu$M apophosphorylase reconstituted with 0.5 mM PL, 77 $\mu$M PLSN, 80 $\mu$M PLS, 72 $\mu$M PLP-Me, or 18 $\mu$M PLDP-Phe was diluted twofold with a solution of 1 mM PLP. After incubation for 20 h, the recovery of the enzyme activity was measured at 25 °C. The activities recovered were 12, 4, 6, 1 and 1 $\mu$mol/(mg min), respectively. The apoenzyme itself incubated with 0.5 mM PLP under the same conditions had an activity of 51 $\mu$mol/(mg min), half the specific activity of the intact holoenzyme, indicating that the excess of PLP partly inhibited the enzyme activity, as already was shown by Avramovic-Zikic & Madsen (1972). Therefore, degrees of the recovery of the enzyme activity were calculated on the basis of the activity of PLP–phosphorylase under the inhibited conditions. The exchange between the enzyme-bound SA and free PLP was monitored by CD using a wavelength at 340 nm, as the CD spectrum of SA–phosphorylase differs from that of PLP–phosphorylase (Shimomura & Fukui, 1977). Apophosphorylase (21 $\mu$M) was reconstituted with 0.2 mM SA, and then the CD change was followed after the addition of PLP at a final concentration of 0.1 mM. It obeyed the pseudo-first-order rate equation, and the rate constant was obtained to be $9.7 \times 10^{-5}$ $s^{-1}$.

*Absorption Spectra of Model Compounds.* The adduct of PLP with 1-hexylamine was used as a model compound of the Schiff base (Shaltiel & Cortijo, 1970). PLP (1.1 mM) was reacted with an excess of 1-hexylamine (0.91 M) in water at pH 7.0, and diluted tenfold in dioxane–water mixtures, and then the absorption spectra were measured. Pyridoxamine 5′-phosphate was used as a model of the $N^6$-(phosphopyridoxyl)-L-lysyl residue. The pyridoxamine 5′-phosphate (2.4 mM) solution at pH 7.0 was diluted 20-fold in dioxane–water mixtures, and then the absorption spectra were measured.

*Materials.* PLP, PL·HCl, SA, pyridoxine hydrochloride, phenyl phosphate, and dicyclohexylcarbodiimide were purchased from Nakarai Chemicals, Ltd. Diphenyl phosphochloridate was obtained from Tokyo Kasei Kogyo Co., Ltd. Pyridoxamine 5′-phosphate was generously supplied by Dr. Hiroshi Wada. 3-*O*-MePLP was a generous gift of Dr. Ernst J. M. Helmreich. PLS was synthesized according to Yang et al. (1974). PLP-Me was synthesized by the method of Pfeuffer

TABLE I: Thin-Layer Chromatography of PLP Analogues on Silica Gel ($R_f$ Values).[a]

| PLP analogues | solvent system A | solvent system B |
|---|---|---|
| PLP | 0.50 | 0.38 |
| PL | 0.59 | 0.78 |
| PLSN | 0.58 | 0.70 |
| PLS | 0.66 | 0.71 |
| PLP-Me | 0.57 | 0.61 |
| PLP-Bz | 0.71 | 0.67 |
| PLDP | 0.24 | 0.18 |
| PLDP-Phe | 0.52 | 0.55 |
| bis-PLP | 0.48 | 0.51 |

[a] Solvent A: 1-butanol–99% formic acid–water (13:3:3, v/v), and used immediately after mixing. Solvent B: 1-butanol–pyridine–water (6:4:3, v/v). The phenylhydrazine reagent described by Wada & Snell (1961) was used for detection of aldehyde.

et al. (1972a). PLP-Bz was synthesized from PLP and benzyl alcohol by a procedure similar to the synthesis of PLP-Me. Thin-layer chromatography on silica gel (Kieselgel 60 $F_{254}$; E. Merck AG, Darmstadt) was performed using two solvent systems, and the results of the PLP analogues snythesized are summarized in Table I. The syntheses of the PLP analogues were performed in the dark as far as possible.

*Synthesis of PLDP.* PLDP was synthesized from PLP using diphenyl phosphochloridate by the anion-exchange method of Michelson (1964). PLP·$H_2O$ (0.53 g, 2 mmol) was suspended in $CHCl_3$ (20 mL) and triethylamine (0.28 mL), and stirred until a clear solution was obtained. After evaporation, the triethylammonium PLP was redissolved in $CHCl_3$ (15 mL) and triethylamine (0.4 mL). Diphenyl phosphochloridate (0.52 mL, 2.5 mmol) was added with stirring, and then the solution was kept at room temperature for 3 h under anhydrous conditions. After removal of the solvent in vacuo, the yellow syrup was shaken with dry ether to remove the excess diphenyl phosphochloridate, and then the ether was removed by decantation. The remaining syrup was a crude pyridoxal 5′-diphosphate $\beta$-diphenyl ester ($R_f$ value, 0.77, with the solvent A). To this syrup was added tri-1-butylammonium orthophosphate (6 mmol) in dry pyridine (10 mL). After standing at room temperature overnight, the solution was evaporated to remove the pyridine. The residue was dissolved in water (20 mL) and extracted with ether (50 mL). The aqueous layer was diluted with water (500 mL) and applied to a Dowex 1-X8 ($Cl^-$) column (1.6 × 16 cm). The column was washed with 0.01 N HCl (200 mL) and eluted with a linear gradient of 750 mL of 0.01 N HCl and 750 mL of 0.01 N HCl–0.05 M LiCl. After PLP and bis-PLP had been eluted, PLDP was eluted at about 0.03 M LiCl. The eluate was neutralized with tri-1-butylamine and concentrated to a small volume. The yellow lithium salt of PLDP (0.36 g) was precipitated by addition of ethanol. The UV spectra showed $\lambda_{max}$ 295 ($\epsilon$ 7450) and 334 ($\epsilon$ 1400) in 0.1 N HCl, $\lambda_{max}$ 328 ($\epsilon$ 2750) and 388 ($\epsilon$ 5050) in 0.1 M sodium phosphate buffer (pH 7.0), and $\lambda_{max}$ 308 ($\epsilon$ 1250) and 389 ($\epsilon$ 6300) in 0.1 N NaOH. Anal. Calcd for $C_8H_9O_9NP_2$·2Li·$H_2O$: C, 26.91; H, 3.11; N, 3.92. Found: C, 26.49; H, 3.07; N, 3.99.

*Synthesis of Bis-PLP.* Bis-PLP was, in principal, a byproduct in the synthesis of PLDP. The triethylammonium PLP (4 mmol) was dissolved in $CHCl_3$ (10 mL) and triethylamine (0.5 mL). Diphenyl phosphochloridate (0.8 mL) was added with stirring, and the mixture stood for 2 h. Commercial (not anhydrous) pyridine (10 mL) was added and then left at room temperature overnight. Under these conditions, the pyridoxal 5′-diphosphate $\beta$-diphenyl ester was partly hydrolyzed by traces of water to PLP, and then the unhydrolyzed ester was attacked by the phosphate group of the PLP being converted to bis-PLP. The reaction mixture was evaporated to remove the pyridine, mixed with water (20 mL), and then extracted with $CHCl_3$ and ether. The aqueous layer was evaporated to remove the ether and then mixed with *p*-aminoacetophenone (0.68 g, 5 mmol) dissolved in 0.33 N HCl (15 mL). The Schiff base derivative formed was precipitated after standing for 3 h, collected by filtration, washed with water, and then dissolved in 0.3 N NaOH (10 mL). The *p*-aminoacetophenone was repeatedly extracted with ether. The aqueous layer containing bis-PLP and a small amount of PLP was diluted with water (2 L), and then applied to a Dowex 1-X8 ($Cl^-$) column (1.6 × 16 cm). The column was washed with water and eluted with 0.01 N HCl (1 L). Bis-PLP was eluted following PLP. Appropriate fractions (about 500 mL) were combined, neutralized with 1 N LiOH, and then concentrated to a small volume. The yellow lithium salt of bis-PLP (0.38 g) was precipitated by addition of acetone. The UV spectra showed $\lambda_{max}$ 294 ($\epsilon$ 14 000) and 335 ($\epsilon$ 2500) in 0.1 N HCl, $\lambda_{sh}$ 330 ($\epsilon$ 3900) and $\lambda_{max}$ 391 ($\epsilon$ 9400) in 0.1 M phosphate buffer (pH 7.0), and $\lambda_{max}$ 308 ($\epsilon$ 1700) and 391 ($\epsilon$ 12 000) in 0.1 N NaOH. Anal. Calcd for $C_{16}H_{16}O_{11}N_2P_2$·2Li·3$H_2O$: C, 35.44; H, 4.09; N, 5.17. Found: C, 34.65; H, 4.12; N, 5.19.

*Synthesis of PLDP-Phe.* PLDP-Phe was synthesized from PLP and phenyl phosphate using dicyclohexylcarbodiimide as a condensing reagent. PLP·$H_2O$ (1 g, 3.8 mmol) and phenyl phosphate (1.6 g, 11 mmol) were dissolved in dry pyridine (50 mL) and triethylamine (2.2 mL), and then dicyclohexylcarbodiimide (6.4 g, 31 mmol) was added. The reaction mixture was stirred at room temperature and under anhydrous conditions overnight. The pyridine was removed in vacuo, and water (20 mL) was added to the residue. After 1 h with stirring, the precipitated urea was removed by filtration. The solution was extracted twice with ether. The aqueous layer was mixed with *p*-toluidine (1 g, 9.3 mmol) dissolved in hot 0.3 N acetic acid (30 mL). The solution was concentrated until the orange precipitate of the Schiff base derivative of PLDP-Phe appeared, and then cooled overnight. The Schiff base derivative was filtered, washed with cold water, and dissolved in 0.3 N NaOH (10 mL). The *p*-toluidine liberated was extracted with ether. The aqueous layer was applied to a Dowex 50-X8 ($H^+$) column (3 × 20 cm). The product was eluted with water. The eluate was neutralized with 1 N NaOH and concentrated to a small volume. The yellow sodium salt of PLDP-Phe (0.45 g) was precipitated by addition of acetone. The UV spectra showed $\lambda_{max}$ 295 ($\epsilon$ 6800) and 336 ($\epsilon$ 1150) in 0.1 N HCl, $\lambda_{sh}$ 330 ($\epsilon$ 1950) and $\lambda_{max}$ 391 ($\epsilon$ 5700) in 0.1 M phosphate buffer (pH 7.0), and $\lambda_{max}$ 306 ($\epsilon$ 600) and 391 ($\epsilon$ 6550) in 0.1 N NaOH. Anal. Calcd for $C_{14}H_{13}O_9NP_2$·2Na·2$H_2O$: C, 34.8; H, 3.6; N, 2.9; P, 12.8. Found: C, 34.7; H, 3.5; N, 3.0; P, 11.9.

*Synthesis of PLSN.* The pyridoxal oxazolidine derivative (0.63 g, 2 mmol) obtained from PL·HCl and D,L-ephedrine hydrochloride was suspended in dry benzene (8 mL). $SOCl_2$ (1 mL) was added dropwise with stirring, and then the reaction mixture was cooled. The yellow solid product was collected by filtration, washed with dry benzene, and then extracted with water (10 mL × 2). The turbid solution was neutralized with 1 N NaOH and immediately extracted with $CHCl_3$ (30 mL × 2). The $CHCl_3$ layer was evaporated to remove the $CHCl_3$. The oily residue was a crude 5′-deoxypyridoxal 5′-chloride ($R_f$ value, 0.85, with the solvent A). An alkyl chloride is converted to the alkylsulfonate by the Strecker reaction. To the residue containing 5′-deoxypyridoxal 5′-chloride, 3 mL of 2 M $Na_2SO_3$

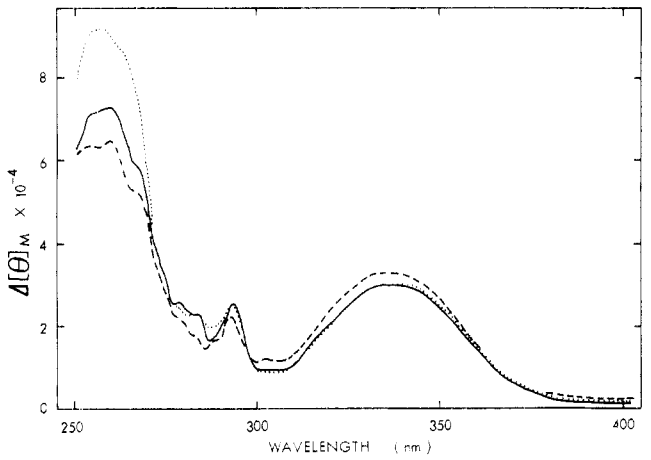



FIGURE 1: Difference CD spectra of apophosphorylase *b* in the presence of PLP analogue vs. in its absence. Apophosphorylase at 19 $\mu$M in the presence of 40 $\mu$M PLSN (···), PLDP (—), and PLDP-Phe (---) in 0.1 M sodium 2-glycero-P and 20 mM 2-mercaptoethanol buffer at pH 7.0 and at 25 °C.

TABLE II: Titration of Apophosphorylase *b* with PLP and Analogues.[a]

| cofactor used for reconstitution | $\Delta[\theta]_\infty \times 10^{-4}$ 259 nm | 335 nm | $\Delta\epsilon_\infty \times 10^{-3}$ 335 nm | $g \times 10^3$ 335 nm |
|---|---|---|---|---|
| PLP[b] | 6.5 | 2.5 | 4.9 | 1.6 |
| SA[b] | 5.3 | 2.6[d] | | |
| PL[b] | 7.2 | 2.8 | 5.1 | 1.7 |
| PLSN | 8.2 | 2.7 | 5.2 | 1.5 |
| PLS | 6.9 | 3.1 | 5.3 | 1.8 |
| PLP-Me | 6.5 | 3.1 | 4.8 | 1.9 |
| PLP-Bz | 5.7 | 2.8 | 5.0 | 1.7 |
| PLDP | 6.8 | 2.8 | 5.3 | 1.6 |
| PLDP-Phe | 5.9 | 3.1 | 5.1 | 1.9 |
| bis-PLP[c] | 5.3 | 3.0 | 8.1 | 1.1 |

[a] The medium for the titration contained 9.6–10.0 $\mu$M apophosphorylase, 0–20 $\mu$M PLP or PLP analogue, 0.1 M sodium 2-glycero-P, and 20 mM 2-mercaptoethanol at pH 7.0. $\Delta[\theta]_\infty$ and $\Delta\epsilon_\infty$ are the maximum changes in molar ellipticity and in molar absorbance, respectively. *g* is the Kuhn dissymmetry factor (Kuhn, 1958) and was calculated as in the previous report (Shimomura & Fukui, 1977). [b] From the data of Shimomura & Fukui (1977). [c] From the data of Shimomura et al. (1978). [d] The value at 312 nm.

was added with stirring at room temperature. After 30 min, the turbid solution was mixed with water (10 mL) and ether (20 mL). The aqueous layer was applied to a Dowex 50-X8 ($H^+$) column (3 × 20 cm), eluted with water. The eluate was concentrated to a small volume in vacuo. PLSN (81 mg) was crystallized by addition of cold acetone. The UV spectra showed $\lambda_{max}$ 298 ($\epsilon$ 6300) and 339 ($\epsilon$ 2350) in 0.1 N HCl, $\lambda_{sh}$ 335 ($\epsilon$ 3150) and $\lambda_{max}$ 370 ($\epsilon$ 3850) in 0.1 M phosphate buffer (pH 7.0), and $\lambda_{sh}$ 310 ($\epsilon$ 750) and $\lambda_{max}$ 389 ($\epsilon$ 6500) in 0.1 N NaOH. Anal. Calcd. for $C_8H_9O_5NS \cdot {}^1/_2H_2O$: C, 40.0; H, 4.2; N, 5.8; S, 13.3. Found: C, 40.2; H, 3.8; N, 5.9; S, 12.9.

## Results

*Mode of Binding of the Cofactor Analogues.* Phosphorylase exhibits two absorption maxima at 333 and 415 nm in the above 300-nm region, where the latter absorbance is as low as 8% of the former (Kent et al., 1958). The species absorbing around 415 nm is well defined as to the Schiff base of the enzyme-bound PLP. On the other hand, the absorption around 333 nm can be interpreted by two different modes of binding of PLP; a substituted aldimine and an enolimine form of the Schiff base (Johnson & Metzler, 1970). The results of a comparison of absorption spectral and fluorescence properties between the enzyme-bound PLP and the model compounds have provided evidence for the latter binding mode, in which PLP binds to phosphorylase as an imine and exists in equilibrium between enolimine (333 nm) and ketoenamine (415 nm) forms (Shaltiel & Cortijo, 1970; Johnson et al., 1970; Feldman & Helmreich, 1976; Cortijo et al., 1976).

We have recently found positive maxima at 251 nm in addition to those at 335 nm on the difference absorption and difference CD spectra of holo- vs. apophosphorylases (Shimomura & Fukui, 1977). On the basis of a comparison of the intensity and the shape of the UV-absorption and CD spectra between phosphorylase and the several PLP adducts, we have concluded that the absorption and CD around 251 nm originated from the enzyme-bound PLP, and that the 251-nm band having stronger intensity than the 335-nm band is a characteristic of an enolimine form of the Schiff base. PL, when bound to apophosphorylase, shows similar absorption and CD spectral changes to PLP in the above 240-nm region. It therefore follows that PL binds at the cofactor site with essentially the same binding mode as PLP. SA also binds to the apoenzyme in the form of the neutral tautomeric Schiff base like PLP (Shimomura & Fukui, 1977).

Incubation of apophosphorylase *b* with PLSN, PLS, PLP-Me, PLP-Bz, PLDP, or PLDP-Phe resulted in a CD spectral change of the apoenzyme, indicating that these compounds can interact with the apoenzyme. Figure 1 shows some typical difference CD spectra of the apoenzyme in the presence of PLP analogue vs. in its absence. The peaks in the difference CD were found at 250–260, 292–294, 335–338, and around 420 nm. Then, the titrations of apophosphorylase with these analogues were performed using CD and absorption at two wavelengths of 259 and 335 nm (data not shown). The ellipticities at 259 and 335 nm of apophosphorylase increased in parallel with the amount of the PLP analogues added, and reached plateaus at a PLP analogue/enzyme monomer ratio of 1.01 to 1.07. The increments of the absorbance at 335 nm also had refraction points at an equimolar concentration. Thus, PLSN, PLS, PLP-Me, PLP-Bz, PLDP, and PLDP-Phe form equimolar complexes with apophosphorylase under these conditions. The analogues have strong affinities for the apoenzyme, their dissociation constants ($K_d$) being estimated to be $\leq 10^{-8}$ M from the CD titration curves.

Table II summarizes the parameters obtained from the titrations. The ellipticities, absorbances and Kuhn's dissymmetry factors (Kuhn, 1958) at 335 nm of PLP analogue–phosphorylases were all in good agreement with those of PLP–phosphorylase. The ellipticities at 259 nm were also similar for these reconstituted enzymes. All PLP analogue–phosphorylases showed as small absorbances and positive ellipticities around 420 nm as PLP–phosphorylase. Increases in the ellipticity around 293 nm in apophosphorylase were induced by the binding of PLP and all the analogues used; the changes may be due to the perturbation of the asymmetric environment of the protein chromophores (tryptophan or possibly tyrosine). Thus, PLSN, PLS, PLP-Me, PLP-Bz, PLDP, and PLDP-Phe bind to apophosphorylase essentially in the same mode as PLP and PL.

*Activities of PLP Analogue–Phosphorylases.* PL–phosphorylase showed about 5% activity of native phosphorylase in a high substrate concentration (75 mM glucose 1-phosphate). The result could be expected from the finding by Parrish et al. (1977). The other analogues (SA, PLSN, PLS, PLP-Me, PLP-Bz, PLDP, and PLDP-Phe) did not activate the

TABLE III: Rate Constants of Reconstitution.

| cofactor for reconstitution | $k_{on}$[a] ($M^{-1}$ $s^{-1}$) | $E_a$[b] (kcal/mol) |
|---|---|---|
| PLP | 420 | 19.4 |
| SA | 4.0[c] | 16.9[c] |
| PL | 0.32 | |
| PLSN | 37 | 20.6 |
| PLS | 97 | 19.6 |
| PLP-Me | 36 | 21.5 |
| PLP-Bz | 170 | 22.8 |
| PLDP | 2800 | 17.3 |
| PLDP-Phe | 950 | 22.4 |
| bis-PLP | 2800 | 28.3 |

[a] The rate constants of reconstitution were measured in 0.1 M sodium 2-glycero-P and 20 mM 2-mercaptoethanol buffer at pH 7.0 and at 25 °C. The ellipticity changes at 335 nm were used. [b] The activation energies for the reconstitution process. [c] Monitored at 312 nm.

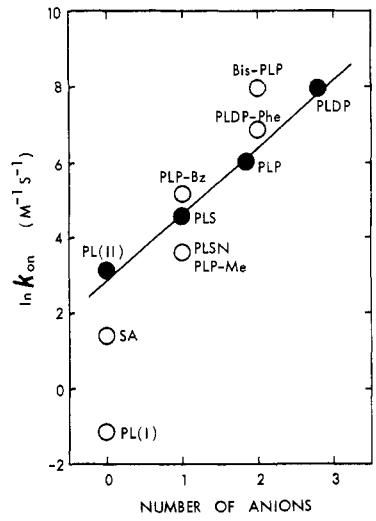



FIGURE 2: Plots of ln $k_{on}$ of apophosphorylase *b* for PLP and its analogues against the number of anions at the 5′ position of pyridoxal. The rates were monitored by the ellipticity changes at 335 nm, for the binding of SA at 312 nm, in 0.1 M sodium 2-glycero-P and 20 mM 2-mercaptoethanol buffer at pH 7.0 and at 25 °C. The abbreviations of PL (I) and PL (II) indicate the ln $k_{on}$ values observed experimentally and calculated on the basis of an assumption that only the aldehyde form of PL can react with apophosphorylase, respectively. The number of anions indicates the number of ions dissociable at pH 7.0. The values for PLP and PLDP were calculated assuming a p$K_2$ of 6.2 of the 5′-phosphate group and a p$K_2$ of 6.4 of the 5′-pyrophosphate group, respectively.

apoenzyme at all. The possible formation of PLDP from PLP as an active intermediate of the glycogen synthesis and degradation mediated by native phosphorylase was eliminated by the result that no enzyme activity was induced after preincubation of PLDP-phosphorylase with substrates and activator.

*Rates of Reconstitution with the Cofactor and Its Analogues.* The reconstitution of apophosphorylase *b* with PLP is a slow process (measurable in an order of minutes), as judged from the recovery of the enzyme activity (Illingworth et al., 1958; Hedrick et al., 1966). CD changes induced by the binding of PLP and its analogues to apophosphorylase were also sufficiently slow to be able to follow the time course of the reconstitution process, when the molar ratios of cofactor (analogue) to enzyme monomer were less than ten in most cases. Plots of the time course of the CD change showed good linearities on the second-order kinetic treatment to the extent of 70–95% saturations. The apparent second-order rate constants obtained were almost independent of the concentration of apophosphorylase and of the cofactor or its analogue. An appropriate mechanism for the reconstitution process might be the pathway with the formation of intermediate. The possibility of the formation of an intermediate may be examined from the dependence of the apparent rate of reconstitution on the concentration of the cofactor. If the rate was not proportional to the cofactor concentration, the reaction would contain the step of the formation of an intermediate. On the other hand, if the concentration of the cofactor was very low, the reaction would follow a simple bimolecular rate equation, in which the observed second-order rate constant is a complex containing all the rate constants of each step. We could not follow the time course of the reconstitution at the higher concentrations of the cofactor enough to detect the possible step of the formation of an intermediate, because of the limited response of a CD apparatus. Then, we used the rate constant ($k_{on}$) determined under the conditions where the reaction followed a bimolecular rate equation, for a parameter describing the overall rate of reconstitution. The results obtained at 25 °C are summarized in Table III. The $k_{on}$ values for the PLP analogues markedly varied dependent on the kind of substitutents at the 5 position of the cofactor. Figure 2 demonstrates that the rate constant increases with the increments in the number of anions at the 5 position, i.e., tri- > di- > mono- > non-anion.

An Arrhenius plot of ln $k_{on}$ against (temperature)$^{-1}$ gave a straight line in the range from 10 to 25 °C in each apophosphorylase–cofactor (analogue) system (data not shown). The activation energy for PLP (19.4 kcal/mol) obtained from the slope of the Arrhenius plot agrees well with the value obtained from the recovery of the enzyme activity (22.3 kcal/mol; Hedrick et al., 1966). However, the dependence of the activation energy on the kind of substituents at the 5 position was not so large as that of the rate of reconstitution (Table III).

As the $K_d$ value for SA is $3.2 \times 10^{-5}$ M (Shimomura & Fukui, 1977), the dissociation rate constant ($k_{off}$) for SA is calculated using an equation of $k_{off} = K_d k_{on}$ to be $1.3 \times 10^{-4}$ $s^{-1}$. This value is well consistent with the exchange rate constant determined from the CD change between the enzyme-bound SA and free PLP ($9.7 \times 10^{-5}$ $s^{-1}$). The $k_{off}$ value for PL is likewise calculated to be $8.3 \times 10^{-6}$ $s^{-1}$. From this value, the degree of the exchange of the enzyme-bound PL with free PLP is expected to be 55% after 20 h, being comparable with the recovery of the enzyme activity (23%) obtained experimentally. The degrees of the recovery of the enzyme activity for PLSN, PLS, PLP-Me, and PLDP-Phe–phosphorylases were all less than 10%, suggesting that the $k_{off}$ values for those PLP analogues are $\leq 10^{-6}$ $s^{-1}$. Therefore, these analogues almost irreversibly bind to apophosphorylase under these conditions.

*Effect of 2-Mercaptoethanol on the Reconstitution.* The buffer used in the above experiments contained 20 mM 2-mercaptoethanol to stabilize the apoenzyme (Shaltiel et al., 1969a), although the compound reacts with the 4-aldehyde group of the pyridoxal ring forming a thiomercaptal (Buell & Hansen, 1960). Shaltiel et al. (1969a) demonstrated that the reconstitution of apophosphorylase *b* with PLP is inhibited in the presence of a large excess of such aldehyde reagents as D-cysteine, cysteamine, hydroxylamine, and sodium cyanide. Only L-cysteine and 2-mercaptoethanol among various aldehyde reagents can allow the binding of PLP to apophosphorylase. However, information on the reconstitutions in the absence of L-cysteine and in the presence of saturated concentration of 2-mercaptoethanol has not been available. Then, we examined if the substitution at the 4 position with a group of suitable configuration is essential for the reconstitution. Figure

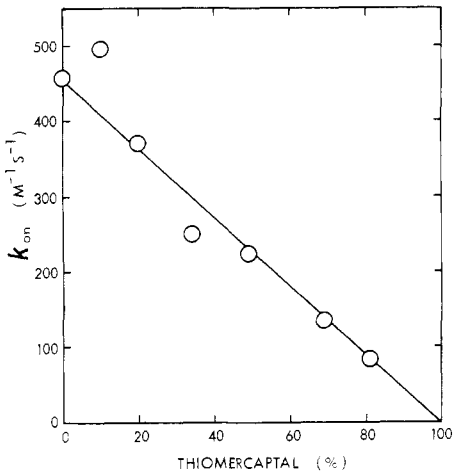



FIGURE 3: Effect of 2-mercaptoethanol on the rate constant of reconstitution of apophosphorylase *b* with PLP. The reaction mixture contained 9.5 μM apophosphorylase, 24 μM PLP, 0.1 M sodium 2-glycero-P, 5 mM EDTA, and a variable concentration of 2-mercaptoethanol at pH 7.0 and at 25 °C. The content (%) of the thiomercaptal was obtained from the titration of PLP with 2-mercaptoethanol using the decrease in the absorbance at 388 nm.

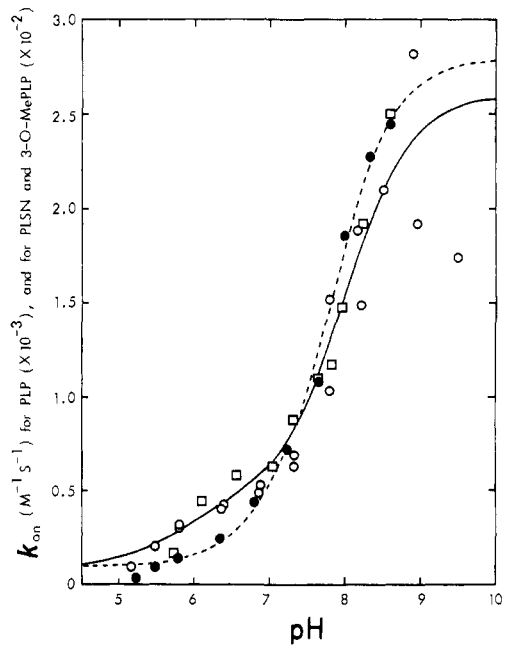



FIGURE 4: Effect of pH on the rate constants of reconstitution of apophosphorylase *b* with PLP and its analogues. The reaction mixtures contained 9.3–9.4 μM apophosphorylase and 11–45 μM PLP (○), 9.3–9.4 μM protein and 32–160 μM PLSN (●), and 18 μM protein and 16–200 μM 3-*O*-MePLP (□) in 0.1 M sodium 2-glycero-P and 5 mM EDTA buffer. The pHs of the buffers were adjusted with HCl or NaOH, and the final pHs were measured after the rates had been followed. The reactions were followed at 335 nm for the bindings of PLP and PLSN, and at 297 nm for 3-*O*-MePLP at 25 °C. The solid and the dashed lines were calculated using the two p*K* values of 6.0 and 8.0, and the p*K* value of 7.8, respectively.

3 shows that 2-mercaptoethanol inhibits the reconstitution process. The rate of reconstitution in the presence of 20 mM 2-mercaptoethanol was 1/1.5 to that in its absence. The dissociation constant of the thiomercaptal into PLP and 2-mercaptoethanol was obtained to be 35 mM from the titration experiment using the decrease of absorbance at 388 nm. Moreover, the thiazolidine derivative of PLP with L-cysteine bound to apophosphorylase at a rate of 1/2.5 to native PLP. It is thus clear that L-cysteine and 2-mercaptoethanol are not essential for the reconstitution process of phosphorylase.

PL is present in an aqueous solution at equilibrium between aldehyde and hemiacetal forms, and the content of the former is only 1.4% of the total amount (Harris et al., 1976). As shown above, all the PLP adducts with aldehyde reagents are unfit forms for reconstitution. By analogy, the hemiacetal form of PL may be unreactive species for the reconstitution. When only the aldehyde form was assumed to bind to apophosphorylase, the rate constant of reconstitution for PL is calculated to be 23 $M^{-1}$ $s^{-1}$. This $k_{on}$ value falls on the line drawn from the relation between the number of anions at the 5 position and the rate constants of reconstitution for PLDP, PLP, and PLS (Figure 2). This linear relationship would mean that, in the reconstitution with apophosphorylase, those four compounds are homologous except for only the number of anions at the 5 position. As the other compounds have different characteristics from those four compounds in hydrophobicity and configuration at the 5 position, these differences might additionally affect the rate of reconstitution. PLDP-Phe as well as PLP-Bz showed a high reactivity to the apoenzyme, in spite of their bulky substituents. These results indicate that steric hindrance does not apparently affect the rate of reconstitution, and that the electrostatic effect is a main factor for determining the rate. PLP-Bz bound more rapidly than PLS, PLP-Me, and PLSN, though these analogues have the same number of anions at the 5 position. PLDP-Phe also showed a higher $k_{on}$ value than PLP. It is considered that a hydrophobic as well as an ionic interaction exists between the cofactor site and the PLP analogue having an apolar substituent on the 5′-phosphate group.

*Effect of pH on the Reconstitution.* pH dependences of the rates of reconstitution are shown in Figure 4. The observed rate constant for PLP pronouncedly increased with the increments in pH. In the stability pH range of apophosphorylase *b* (pH 5.5–8.5; Shaltiel et al., 1972), the rate constant for PLP changed dependent on two p*K*s of 6.0 and 8.0. PLP itself has two p*K*s in that pH range: a p*K* of 6.1–6.2 due to the 5′-phosphate group and a p*K* of 8.33–8.69 due to the pyridine nitrogen (Williams & Neilands, 1954; Metzler & Snell, 1955; Harris et al., 1976). These values are nearly equal to those obtained in the pH dependence of the rate constant of reconstitution. On the other hand, the rate constant for the PLSN–phosphorylase system showed only a p*K* of 7.8. This value is also similar to the p*K* of PLSN itself; PLSN would have a single p*K* of about 8 due to the pyridine nitrogen in the pH range of 5–9. These results suggest that the rates of the reconstitution are strongly affected by the ionization states of the cofactor. However, the rate constant for 3-*O*-MePLP, an active cofactor analogue binding to apophosphorylase in a similar binding mode to PLP (Shaltiel et al., 1969b; Feldmann & Helmreich, 1976), changed dependent on the same two p*K* values as PLP, although 3-*O*-MePLP has only a p*K* originating from the 5′-phosphate group in the above pH range, its p*K* value due to the pyridine nitrogen being 4.15 (Pocker & Fischer, 1969). Therefore, the p*K* of 6.0 presently observed in the pH dependence of the rate constant of reconstitution can be assigned to the 5′-phosphate group of PLP. The other p*K* of 8.0 cannot be assigned to the pyridine nitrogen, but rather to an ionizable residue of protein.

*Effect of Substrate on the Reconstitution.* Effects of substrates and inhibitor of phosphorylase on the rates of reconstitution were examined. The rate constants for PLP-Bz in the presence of 0.5% amylodextrin (degree of polymerization, 10–15), 0.1 M D-glucose, 0.25% amylodextrin–37.5 mM glucose 1-phosphate, and 75 mM glucose 1-phosphate were 1.1, 0.8, 0.7, and 0.5 times those in their absence, respectively.

The rate constant for PLP in the presence of 75 mM glucose 1-phosphate was 0.5 time that in its absence.

*Hydrophobicity of the Vicinity of the Cofactor Site.* The present finding that the 5′-phosphate subsite of the cofactor site has a wide enough space to introduce a large group therein allowed us to use PLP derivatives with a chromophore substituent on the 5′-phosphate group as "reporter". We have chosen bis-PLP, a PLP analogue containing pyridoxal as the reporter group, since the spectra of pyridoxal have been well characterized (Johnson & Metzler, 1970). Our separate report has shown that apophosphorylase *b* is inactivated with the incorporation of one mol of the analogue per mol of enzyme monomer, and that bis-PLP cross-links two different lysine residues in an enzyme monomer (Shimomura et al., 1978).

The rate constant of binding for bis-PLP was as large as that for PLDP (Table III), and, in the presence of 75 mM glucose 1-phosphate, it decreased to half that in its absence as in the cases of PLP and PLP-Bz. Since no recovery of activity was observed on incubation of bis-PLP–phosphorylase with an excess of PLP, bis-PLP is practically irreversibly bound to apophosphorylase.

The phosphorylase-bound bis-PLP showed two absorption peaks at 335 and 415 nm (Figure 5A). The latter absorbance decreased to 10% of the original by the addition of 1 mM $NaBH_4$, and no change was observed on the further addition (Figure 5A). On this treatment, however, only one of the two pyridoxal groups of the bis-PLP is reduced and covalently fixed to a lysine residue as a secondary amine; this labeled site has been identified as Lys-573 by the peptide sequence study (Shimomura et al., 1978). Since pyridoxyllysine has no absorption at 415 nm, the remaining absorption band at 415 nm after reduction must come from the pyridoxal group linked to another lysine residue. This pyridoxal group showed an absorbance at 415 nm as small as the PLP bound to native phosphorylase (Figure 5A), suggesting that they both are bound to the enzyme in the same mode of binding.

The CD spectrum of bis-PLP–phosphorylase above 250 nm resembled those of phosphorylases reconstituted with PLP and the other cofactor analogues. Figure 5C shows the CD spectrum of bis-PLP–phosphorylase in the above 300-nm region. The CD spectrum was not significantly changed by the $NaBH_4$ reduction, in contrast to the absorption spectrum, suggesting that the pyridoxal group (linked to Lys-573) of the bis-PLP has little or no optical activity. The Kuhn dissymmetry factor at 415 nm of bis-PLP–phosphorylase increased from $0.3 \times 10^{-3}$ to $1.0 \times 10^{-3}$ by the $NaBH_4$ treatment, and the value after reduction was close to that of PLP–phosphorylase ($1.4 \times 10^{-3}$). Since all the spectroscopic properties and the resistance against $NaBH_4$ of the pyridoxal group (linked to another lysine residue) of the bis-PLP resembled those of the PLP bound to native phosphorylase, this pyridoxal group should bind to apophosphorylase in the same mode of binding and in a similar asymmetric environment to the PLP. It is probably linked to the $\epsilon$-amino group of the original cofactor-binding residue, Lys-679 (Titani et al., 1977).

Then, the absorption spectra of the pyridoxal group (linked to Lys-573) of the bis-PLP can be obtained by subtracting the spectra of PLP-phosphorylase from that of bis-PLP-phosphorylase, as the spectrum of the pyridoxal group (linked to Lys-679) of the bis-PLP seems to be equal to that of the PLP. Figure 5B shows the absorption spectra thus obtained of the pyridoxal group linked to Lys-573 before and after the $NaBH_4$ treatment. The molar absorbance at 415 nm of the pyridoxal group bound to Lys-573 before the $NaBH_4$ treatment was 2300 $M^{-1}$ $cm^{-1}$; this value is less than half of that of the Schiff base adduct of PLP with a primary alkylamine in neutral pH. The

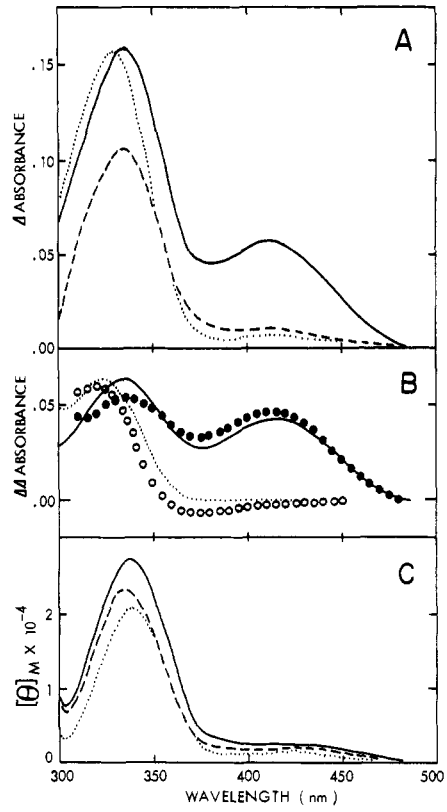



FIGURE 5: Absorption and CD spectra of bis-PLP–phosphorylase *b*. (A) Difference absorption spectra of 24.8 $\mu$M apophosphorylase in the presence of 20.5 $\mu$M bis-PLP (—), and 20.5 $\mu$M PLP (---) vs. the apoenzyme. Difference absorption spectrum of bis-PLP–phosphorylase treated with 2.3 mM $NaBH_4$ vs. the apoenzyme (···). (B) Difference absorption spectra of bis-PLP–phosphorylase vs. PLP-phosphorylase (●), and of the bis-PLP–enzyme after $NaBH_4$ treatment vs. the PLP–enzyme (○). Absorption spectra of the Schiff base derivative of PLP in a 70% dioxane–water (—), and of pyridoxamine-5′-P in an 80% dioxane–water mixture (···). The absorbances for the spectra of these model compounds were recalculated based on the concentration of 20.5 $\mu$M to compare with those of the enzyme-bound bis-PLP. (C) CD spectra of PLP–phosphorylase (---), bis-PLP–phosphorylase (—) and the bis-PLP–enzyme after $NaBH_4$ treatment (···). The samples were the same as in A. The molar ellipticity was based on the concentration of apophosphorylase. These spectra were measured at 25 °C in 0.1 M sodium 2-glycero-P and 20 mM 2-mercaptoethanol buffer at pH 7.0.

absorption spectrum of the Schiff base adduct changes dependent on the polarity of the environment (Shaltiel & Cortijo, 1970; Cortijo et al., 1976). Then, we can simulate the spectrum of the pyridoxal group bound to Lys-573 to those of the model Schiff base in 70 ± 5% dioxane–water mixtures (Figure 5B). The absorption spectrum of the pyridoxal group covalently fixed to Lys-573 with $NaBH_4$ showed a 320-nm band, and the absorbance at 320 nm was again less than half of pyridoxamine 5′-phosphate. Since the absorption spectrum of pyridoxamine 5′-phosphate also changes dependent on the polarity (Matsushima & Martell, 1967; Cortijo & Shaltiel, 1972), the spectrum of the pyridoxal group fixed to Lys-573 with $NaBH_4$ can be simulated to that of pyridoxamine 5′-phosphate in 80 ± 5% dioxane–water mixtures.

## Discussion

The results of the titrations show that all the PLP analogues used here bind to apophosphorylase in the same mode of binding as the natural cofactor PLP; they are bound mainly as an enolimine form of the Schiff base. The CD spectra of phosphorylases reconstituted with PL and its derivatives having bulky substituents at the 5′ position were quite similar to each

other. This clearly indicates that the asymmetric environments of the pyridoxal moieties of those compounds in the reconstituted enzymes are similar to each other. Thus, the structure of the pyridoxal-binding subsite of the cofactor site should not be perturbed by the binding of the 5′ substituent. This finding may be interpreted to mean (1) that the interaction between the pyridoxal moiety of the cofactor and the protein is so strong that it rigidly holds the pyridoxal-binding subsite of the cofactor site in a specific conformation, and also (2) that the phosphate-binding subsite of the cofactor site is a wide space or of flexible structure enough to adapt a large group therein. The $k_{off}$ value for PL was comparable with those for the PLP analogues with an anionic group at the 5′ position. This result suggests that the interaction between the pyridoxal moiety and the protein stabilizes the cofactor–apoenzyme complex more strongly than the interaction between the anionic group and the protein does, supporting explanation 1. On the other hand, the $k_{on}$ values for the cofactor analogues seem to change independently on the size of their substituents at the 5′ position. It is considered that such bulky substituents give little or no steric hindrance, supporting explanation 2.

The rates of reconstitution varied clearly dependent on the number of anions at the 5′ position. This finding indicates that the electrostatic effect plays an important part in the reconstitution process. The net charge of apophosphorylase *b* is negative, as the experiments of the reconstitutions were performed above the isoelectric point of apophosphorylase *b* (p*I* = 6.6; S. Shimomura, unpublished result). If the net charge of protein affects the number of collision between protein and cofactor, it would be expected that the reconstitution rate decreases with the increments in the number of anions of the cofactor. However, the result shows the opposite effect. It therefore follows that a cationic group or groups are present at the cofactor site directly interacting with the 5′-anionic group of the cofactor, and that the ionic interaction may stabilize the possible intermediate of the enzyme–cofactor complex or accelerate the rate-determining steps in the reconstitution process.

The pH dependence of the rate of reconstitution for PLP yielded inflection points indicative of two-protein dissociation with p*K*s of 6.0 and 8.0. The former is assigned to the dissociation of a proton from the monoanionic form of the phosphate group of PLP. The latter does not correspond to the deprotonation of the ring nitrogen of the pyridoxal group, and must be assigned to an ionizable group in protein. The results of model experiments for aldimine formation indicate that only the uncharged amine can react with the aldehyde (Auld & Bruice, 1967). Therefore, we consider here the p*K* of 8.0 to be attributable to the ionization of the ϵ-amino group of Lys-679 (the original cofactor-binding residue; Titani et al., 1977) in apophosphorylase. This p*K* value is, however, lower than that of the normal ϵ-amino group of a lysine residue in protein (p*K* = 9.4 to 10.6; Dixon & Webb, 1964). If the ϵ-amino group of Lys-679 in the apoenzyme was not exposed to the protein surface but present in the bottom of the cavity for the cofactor as in the holoenzyme, the observed low p*K* value in the apoenzyme could be explained on the basis of the apolar environment since the ϵ-amino group of Lys-679 in the holoenzyme is surrounded by a highly hydrophobic environment (Shaltiel & Cortijo, 1970; Johnson et al., 1970; Jones & Cowgill, 1971; Cortijo & Shaltiel, 1972; Cortijo et al., 1976; Feldmann & Helmreich, 1976; Shimomura & Fukui, 1977).

The rate of reconstitution for 3-*O*-MePLP is one order lower than that for PLP (Figure 4). We have shown that only the compound substituted by a hydroxyl group at the ortho position among the derivatives of benzaldehyde binds to apophosphorylase (Shimomura & Fukui, 1977). On the basis of the results of model experiments for aldimine formation, Auld & Bruice (1967) have shown that the phenolic hydroxyl group at the ortho position to the aldehyde group intramolecularly catalyzes the dehydration of the carbinolamine intermediate, which is perhaps a rate-limiting step for aldimine formation. Therefore, the 3-hydroxyl group of the pyridoxal may promote the formation of the Schiff base between PLP and apophosphorylase through an intramolecular catalysis.

Bis-PLP cross-links Lys-573 and Lys-679 in phosphorylase. The distance between the two ϵ-amino groups of these lysine residues, when they were cross-linked to bis-PLP, is estimated to be <17 Å on the basis of the size of this compound. The results of X-ray studies combining the data of the complete primary sequence indicated that Lys-573 is located in a loop facing to the PLP site (R. J. Fletterick & N. B. Madsen, personal communication). Therefore, the ϵ-amino group of Lys-573 may be a cation interacting with the anionic group of the cofactor or of the substrate in native phosphorylase. The absorption spectra of the pyridoxal group linked to Lys-573 are simulated by the optical properties of the model compounds in 70–80% dioxane–water mixtures. On the other hand, the optical properties of the pyridoxal group linked to Lys-679 correspond to those of >95% dioxane (Shaltiel & Cortijo, 1970; Shimomura & Fukui, 1977). Therefore, both pyridoxal groups of the bis-PLP are considered to be in hydrophobic environments. Presence of the hydrophobic region adjacent to the 5′-phosphate group of the PLP is consistent with the findings that PLP-Bz bound to apophosphorylase more rapidly than PLP-Me, and that the cofactor derivatives having a benzene ring on the 5′-phosphate group bound most rapidly among the analogues having the same number of anions at the 5 position. These results suggest that the 5′-phosphate group is also surrounded by the hydrophobic region, and so the hydrophobic environment may significantly modify the ionization capacity of the 5′-phosphate group of the cofactor.

Feldmann & Hull (1977) showed in the $^{31}P$ nuclear magnetic resonance studies that the ionization state of the 5′-phosphate group of PLP in phosphorylase *b* is the monoanionic form or possibly the fully protonated form, and it is pH independent between pH 5.8 and 8.5. We consider that these results must be ascribed either to the electrostatic effect of nearby negative charges or to a hydrophobic environment. The apparent $pK_1$ and $pK_2$ of the phosphate group of PLP increase to about 6 and 9, respectively, with the increments in the dioxane concentration to 70% or more (Pfeuffer et al., 1972b). Thus, we can explain the pH independence for the ionization of the phosphate group of PLP in phosphorylase *b* on the basis of the hydrophobic environment. The present results show that the dianionic form of the 5′-phospate group of PLP preferentially binds to the cofactor site rather than the monoanionic form. It is therefore considered that, after PLP has been bound, the dianionic form is converted to the monoanionic form owing either to the preexisting hydrophobic environment or more preferentially to the increase in hydrophobicity of the cofactor site due to a microenvironmental perturbation induced by the binding of the cofactor.

In conclusion, one of the most important features in the present studies is probably the result that the cofactor analogues with bulky substituents at the 5 position can easily bind to the cofactor site. The present and other data suggest that, when those analogues bound to enzyme, such substituents occupy the substrate site adjacent to the cofactor site. Then, it is possible to examine various aspects of the substrate site using the analogues with reporter groups designed under the proposed subjects; actually, the present experiment using

bis-PLP gave the result showing that the substrate site would be a wide space of hydrophobic nature comparable to 70–80% dioxane. It is also possible to monitor the conformational transition of the substrate site accompanied by the activation of enzyme. And, the enzyme derivatives in which the substrate site is blocked by such bulky substituents might be useful tools to examine the specific functions of the allosteric site and of the glycogen storage site (Kasvinsky et al., 1978).

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