Function of Pyridoxal 5′-Phosphate in Glycogen Phosphorylase: ^{19}F NMR and Kinetic Studies of Phosphorylase Reconstituted with 6-Fluoropyridoxal and 6-Fluoropyridoxal Phosphate†

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ABSTRACT: ^{19}F NMR spectroscopic properties of glycogen phosphorylase reconstituted with 6-fluoropyridoxal (6-FPAL) and 6-fluoropyridoxal phosphate (6-FPLP) were investigated. Analysis of the contribution of chemical shift anisotropy to the line width of the 6-FPLP–enzyme signal shows that the coenzyme molecule is tightly bound to the protein. The chemical shift of the fluorine nucleus in the free 6-FPLP protein is pH independent from pH 6 to pH 9.1. When the 6-FPLP–enzyme forms complexes with AMP, AMP plus glucose-1-P, and AMP plus inorganic phosphate, signals at −11.0, −13.1, and −10.4 ppm are observed, respectively. These different chemical shifts indicate that the protein in each complex has a distinct conformation. The exchange rate between the 6-FPLP–protein–AMP complex and the same complex with bound glucose-1-P is estimated to be 3300 ± 700 s^{-1}, and that between the 6-FPLP–protein–AMP complex and with bound inorganic phosphate is 500 ± 100 s^{-1}. The former exchange rate is 13 times faster than that of the same process for the 6-FPAL–enzyme. Analysis of the effects of temperature on the ^{19}F line shape of the 6-FPLP enzyme in the presence of ligands shows that the exchange rates between different complexes drop significantly between 20 and 10 °C. Within this temperature range, Arrhenius plots of the enzymatic activities of the native and 6-FPLP–enzymes at varied temperatures also show a pronounced curvature. The study of the temperature effect on the equilibrium between the 6-FPAL–enzyme–AMP complex and the same complex with bound glucose-1-P shows that the ligand-bound species has higher enthalpy than the ligand-free protein. Results of this study suggest that an important role of the coenzyme in phosphorylase is to minimize the activation energy of interconversion between different conformers and to keep the bound substrate well oriented for catalysis. Results also suggest an interconversion occurs between ternary complexes of different conformations in the rate-limiting step of catalysis.

Glycogen phosphorylase contains a stoichiometric amount of pyridoxal 5′-phosphate (PLP)[1] (Baranowski et al., 1957). Although the function of this coenzyme in phosphorylase is not fully understood, various studies showed that the coenzyme is an indispensible constituent for both the structural integrity and the enzymatic activity of phosphorylase (Shaltiel et al., 1966; Graves & Wang, 1972; Klein & Helmreich, 1980; Madsen & Withers, 1984). Studies of phosphorylase reconstituted with various PLP analogues show that the 5′-phosphate group of the coenzyme is necessary for the enzymatic activity (Graves & Wang, 1972; Klein & Helmreich, 1980; Madsen & Withers, 1984; Parrish et al., 1977). X-ray crystallographic studies have shown that the coenzyme phosphate is in close proximity of the phosphate group of glucose-1-P (Jenkins et al., 1981; McLaughlin et al., 1984). It was suggested that the coenzyme phosphate in phosphorylase plays a role in a general acid–base catalysis (Klein et al., 1984; Feldmann & Helmreich, 1976; Feldmann & Hull, 1977) or that this phosphate acts as an electrophile and forms a pseudopyrophosphate linkage with the phosphate of glucose-1-P (Withers et al., 1981; Takagi et al., 1982).

Kinetic studies of glycogen phosphorylase *b* indicate that its enzymatic activity is controlled by an equilibrium between at least two conformers, an active "R" conformer and an inactive "T" conformer (Helmreich et al., 1967; Kastenschmidt et al., 1968). The equilibrium is determined by the binding of various ligands to the protein such as AMP, glucose-1-P, and orthophosphate, which can stabilize the R conformer, and caffeine, glucose, and glucose-6-P, which can stabilize the T conformer. Heterotropic allosteric interactions among the binding of ligands to phosphorylase have also been observed (Graves & Wang, 1972; Klein & Helmreich, 1980; Dombradi, 1981). X-ray crystallographic studies of phosphorylase *a* complexed with glucose, glucose-1-P, or glucose cyclic 1,2-phosphate (Sprang & Fletterick, 1979; Madsen et al., 1978; Sprang et al., 1982; Withers et al., 1982b) and those of phosphorylase *b* complexed with glucose-1-P, heptenitol, or heptulose 2-phosphate (Johnson et al., 1980; McLaughlin et al., 1984) show that the substitutions of the substrate, glucose-1-P, with other ligands in the active site induce substantial changes in protein structure. However, the high-resolution structure of a fully activated phosphorylase carrying out catalysis has not yet been determined. The possibility that the coenzyme, PLP, is necessary for the allosteric transition of phosphorylase has been tested by studying the cooperative binding of ligands to phosphorylase reconstituted with different PLP analogues. Phosphorylase reconstituted with pyridoxal (Kastenschmidt et al., 1968), 5′-deoxypyridoxal (Krebs & Fisher, 1962), or pyridoxal phosphate monomethyl ester

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[1] Abbreviations: 6-FPAL, 6-fluoropyridoxal; 6-FPLP, 6-fluoropyridoxal phosphate; NMR, nuclear magnetic resonance; AMP, adenosine 5′-phosphate; glucose-1-P, glucose 1-phosphate; P_i, phosphate; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal 5′-phosphate.

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(Pfeuffer et al., 1972) is completely inactive but will bind AMP in a highly cooperative manner. These results, along with tritium–hydrogen exchange study of phosphorylase reconstituted with pyridoxal phosphate monomethyl ester (Weissbaar & Palm, 1972), indicate, at least, that the 5′-phosphate group of PLP is not needed for the allosteric transition of phosphorylase. Further kinetic studies of pyridoxal phosphorylase (Chang et al., 1983) show that the phosphate group of the coenzyme is not even important for the binding of substrate, glucose-1-P. The possibility that the rest of the coenzyme molecule is involved in the conformational transition of phosphorylase, however, cannot be ruled out.

Because phosphorylase reconstituted with 6-FPLP and 6-FPAL retains most of the structural characteristics of the native and pyridoxal-reconstituted phosphorylase, including the cooperative binding of AMP and glucose (Chang & Graves, 1985), we have used these reconstituted enzymes to study the conformational changes around the PLP binding site in phosphorylase. NMR spectroscopic techniques have been widely used in studying the conformational properties of biological macromolecules such as oligopeptides, DNA, RNA, and proteins (Govil & Hosur, 1982). Analysis of the nuclear relaxation mechanisms of these samples has given fruitful information about the mobilities or the orientations of different residues inside the whole molecule (Sykes & Weiner, 1980; Sykes, 1983). In this study, the line width due to nuclear relaxation mechanisms of the fluorine nucleus in the 6-FPLP-reconstituted phosphorylase has been analyzed. The results provide some insight into the binding of the coenzyme inside phosphorylase. Analysis of changes, induced by ligand binding, of the NMR spectrum of the fluorine nuclei and kinetic results reveal that important roles are played by the coenzyme in the conformational changes and in the orientation of the bound substrates of glycogen phosphorylase in solution.

Materials and Methods

The synthesis of 6-FPLP and 6-FPAL, enzymatic assay, and preparations of phosphorylase, apophosphorylase, and 6-FPLP- and 6-FPAL-reconstituted phosphorylases have been described in a previous study (Chang & Graves, 1985). ^{19}F NMR spectra were routinely obtained at 282.4 MHz on a Bruker WM300 spectrometer. A spectral width of 20000 Hz was employed with 28-μs (a 60° pulse) pulse width and a repetition time of 1 s. A cylindrical microcell (Wilmad) containing 1 mL of sample solution was fitted into a 10-mm NMR tube containing D_2O (50%) and trifluoroacetate (0.03 mM, pH 6.8). The D_2O outside the microcell was used for field/frequency lock, and the trifluoroacetate signal was used for chemical shift referencing. ^{19}F NMR spectrum of 6-FPLP-reconstituted phosphorylase was also obtained with a Bruker HX90 at Iowa State University. A pulse angle of 60° was applied, and the recycling time for the experiment was 1 s. A Nicolet NT 200 in the Research Resource Center at the University of Illinois at Chicago was used to record the spectrum of the free 6-FPLP–enzyme. A pulse angle of 75° was used, and recycling time was 0.5 s. Specific enzymatic activity of samples was assayed before and after experiments to make sure that the enzyme was not denatured.

A routine program from the Nicolet 1280 software, based on the original work of Gutowsky & Holm (1956), was used to analyze the ^{19}F NMR line width changes of the 6-FPLP–phosphorylase signal in the presence of varied concentrations of ligands (Figure 3). This program calculates the complete line shape by assuming a two-site exchange situation. Because the T_1 values of 6-FPLP–enzyme in the presence and absence of ligands are similar [0.9 ± 0.1 s (Y. C. Chang and D. J. Graves, unpublished results)], the peak area is proportional to the concentration of the respective species. Introduction of values for the line width, chemical shift of ligand-bound and free proteins, fractions of these species, and their estimated exchange life time, τ, allowed the simulation of a theoretical spectrum. The line-width change of these spectra calculated at varied fractions of the ligand-bound species could be fitted to the observed data by adjustment of the exchange lifetime, τ. The value of $1/\tau$ was used as the exchange rate.

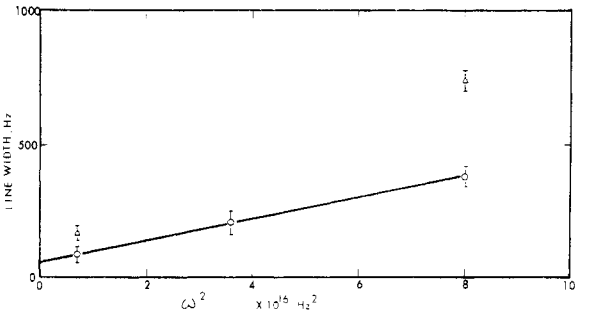

FIGURE 1: ^{19}F NMR half-height widths for 6-FPLP-reconstituted phosphorylase as a function of the square of the spectrometer frequency at 20 °C. 6-FPLP–enzyme was dissolved in buffer solution (40 mM β-glycerophosphate, 30 mM 2-mercaptoethanol, and 2 mM EDTA, at pH 6.8) either as free protein or as a complex with substrates. (○) Free 6-FPLP–phosphorylase (30 mg/mL). (△) 6-FPLP–phosphorylase (30 mg/mL) in the presence of maltopentaose (1%), AMP (1 mM), and glucose-1-P (20 mM). Line widths of these ^{19}F resonances were corrected by subtracting the line-broadening factor, 20 Hz, used for experimental data.

A 5901B Auto Viscometer (Hewlett-Packard) was used to measure the viscosities of protein samples and double-distilled and deionized water. The viscosity of protein was calculated by multiplying its viscosity relative to water by 1.002 cP, the absolute viscosity of water at 20 °C (Weast & Selby, 1966).

Oligosaccharides were identified by ascending thin-layer chromatography. It was carried out on Analtech HETLC-GHL plates by using two ascents of acetonitrile–water, 4:1 (v/v).

Results

To test the possibility that the coenzyme molecule in phosphorylase has a certain freedom of movement when the protein is dissolved in solution, the correlation time of the fluorine nucleus in phosphorylase reconstituted with 6-FPLP was estimated. ^{19}F NMR spectra of free 6-FPLP–enzyme were recorded at three different magnetic fields, 84.7, 188, and 282.4 MHz. The line-width values were plotted vs. the square of the magnetic field applied in the spectra, as shown in Figure 1. When the slope of the resulting line was analyzed, the correlation time was estimated to be 147 ns [details of calculations are documented in the supplementary material (see paragraph at end of paper regarding supplementary material)]. This value well exceeds the theoretically predicted value, 92 ns (calculations are also shown in the supplementary material). It is clear that the coenzyme molecule is tightly bound to the protein and has no internal mobility when no ligand or substrate is present. The discrepancy between the theoretically predicted and experimental τ_c's is because the former low value is calculated assuming that phosphorylase molecule is a round sphere (Vogel et al., 1982, Boere & Kidd, 1982). A similar analysis distinguishing the contributions to the line width, $\Delta\nu$, of free 6-FPLP–enzyme due to chemical shift anisotropic effects (c.s.a.) and dipole–dipole interactions (d.d.), is made of a sample containing substrates and AMP [shown as (△) in Figure 1]. The $\Delta\nu_{c.s.a.}$ was calculated to be 640 Hz. This value is well above the calculated $\Delta\nu_{c.s.a.}$ of free

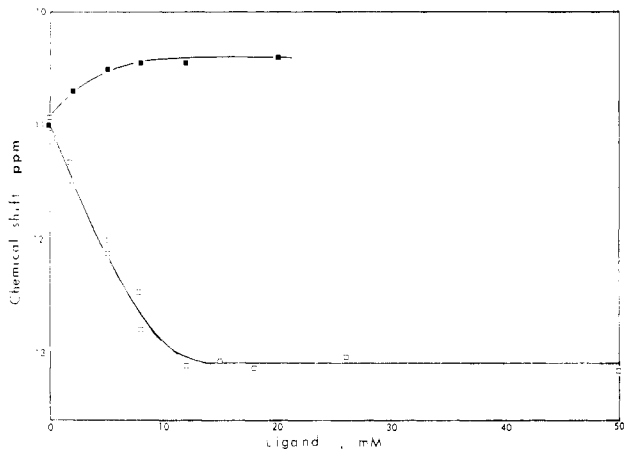

FIGURE 2: ^{19}F NMR chemical shift of 6-FPLP-reconstituted phosphorylase (30 mg/mL) in the presence of AMP (1 mM) and different concentrations of glucose-1-P (□) or inorganic phosphate (■). Each data point represents the average of at least two measurements, and the uncertainty is ±0.1 ppm.

enzyme, 310 Hz, and cannot be readily explained by any of the known mechanisms for the relaxation process of nuclei in macromolecules. The possibility existed that the larger line width was due to the formation and binding of large linear amylose chains generated in the NMR experiment. Analysis of carbohydrates showed that maltoheptaose, maltopentaose, and maltotetraose were the major species, whereas no large oligosaccharides were found. Therefore, the broad signal could not arise from the binding of carbohydrates with very high molecular weights. Because it has been shown that the active form of phosphorylase is dimeric and the presence of polysaccharide favors formation of dimers (Klein & Helmreich, 1980; Graves & Wang, 1972), the broadened signal of 6-FPLP–protein seems unlikely to be due to the aggregation of protein. It is likely that this broad signal is an exchange-broadened average of two different signals. Sykes and Scott (1972) have shown that when a nucleus undergoes an exchange with an intermediate rate between two different sites, the resulting averaged resonance shows a broader line width than the signal in either site. This extra line width is also a linear function of the square of the spectrometer frequency.

The binding of ligands such as AMP, glucose-1-P, or phosphate to glycogen phosphorylase has been investigated previously by kinetic methods and by equilibrium dialysis studies. In this work, ^{19}F NMR spectroscopy was employed to study the changes of the protein in response to ligand binding. The 6-FPLP–enzyme (30 mg/mL) at 30 °C showed a signal at −11.0 ppm (relative to the signal of trifluoroacetate) with a line width of 340 Hz. When a saturating concentration of AMP (1 mM) was included in the sample, the signal was broadened to 510 Hz, which probably is due to the formation of the tetrameric form of phosphorylase (Appleman, 1962). No appreciable change of the signal position was observed. The inclusion of either glucose-1-P or phosphate, however, changes both the chemical shift and the line width of the ^{19}F signal of the enzyme–AMP complex. Figure 2 shows that the signal of the 6-FPLP–enzyme–AMP complex gradually shifts upfield, from −11.0 to −13.1 ppm, as the glucose-1-P concentration in the solution increases from 0 to 10 mM. Above 10 mM, the chemical shift of 6-FPLP–enzyme levels off and reaches a constant value of −13.1 ppm. According to this curve, 50% of enzyme has glucose-1-P bound to it in the presence of 5 mM glucose-1-P. This is similar to the K_m value of 7.4 mM obtained by kinetic measurements (Chang & Graves, 1985). The 2 ppm upfield shift of the enzyme signal

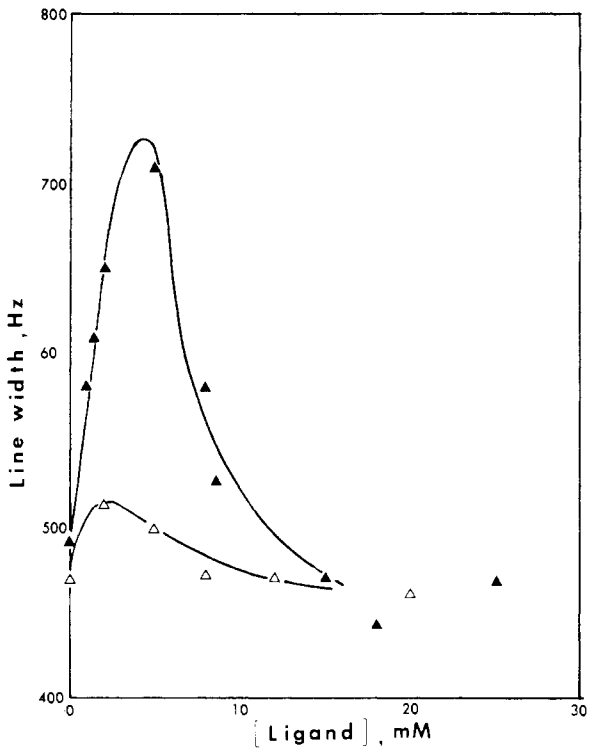

FIGURE 3: ^{19}F NMR line widths for 6-FPLP–phosphorylase–AMP complex (0.3 mM) in the presence of various concentrations of glucose-1-P (▲) or inorganic phosphate (△). Each data point represents the average of at least two measurements, and the uncertainty is ±30 Hz. Solid curves were the line-width changes obtained with computer simulation.

is characteristic for glucose-1-P binding; other compounds such as glucose and glucose cyclic 1,2-phosphate did not show a similar effect. With the increasing concentration of inorganic phosphate, from 0 to 10 mM, the ^{19}F signal of the 6-FPLP–enzyme–AMP complex shifts from −11 to −10.4 ppm, and the chemical shift reaches a plateau. According to this curve, at a concentration of 3 mM phosphate, 50% of enzyme contains bound ligand. The value represents the dissociation constant of phosphate from a protein–AMP–phosphate complex, which is lower than those obtained with kinetic and spectrophotometric studies, ranging from 10 to 20 mM (Bresler & Firsov, 1968; Avramovic & Madsen, 1968; Engers et al., 1969). The difference may be due to the higher protein concentrations used in this study. Maltopentaose, at concentrations of 2 and 20 mM, did not affect the chemical shift of the protein signal in the presence of AMP.

The width of the 6-FPLP–enzyme signal at half-height in the presence of increasing amount of glucose-1-P and phosphate shows bell-shaped changes, shown in Figure 3. These line-width changes may be explained by considering that, in the presence of subsaturating concentration of ligands, only a fraction of total protein binds ligand while the rest remains free. These two species undergo an exchange process, which can result in the broadening of the NMR signal. A two-site exchange simulation routine of Nicolet 1280 software was used to analyzed the data in Figure 3. For glucose-1-P binding, the line width and chemical shift of free enzyme, 490 Hz and −11.0 ppm, and those of the enzyme with 25 mM glucose-1-P, 470 Hz and −13.1 ppm, were used to represent the parameters of simple ligand-free and -bound species in the mixture. The fraction of protein containing bound ligand at different glucose-1-P concentrations was estimated from the curve in Figure 2. By use of different rates, ranging from 25 to 10 000 s^{-1}, 3300 ± 700 s^{-1} yielded the best fitting curve, shown as the solid

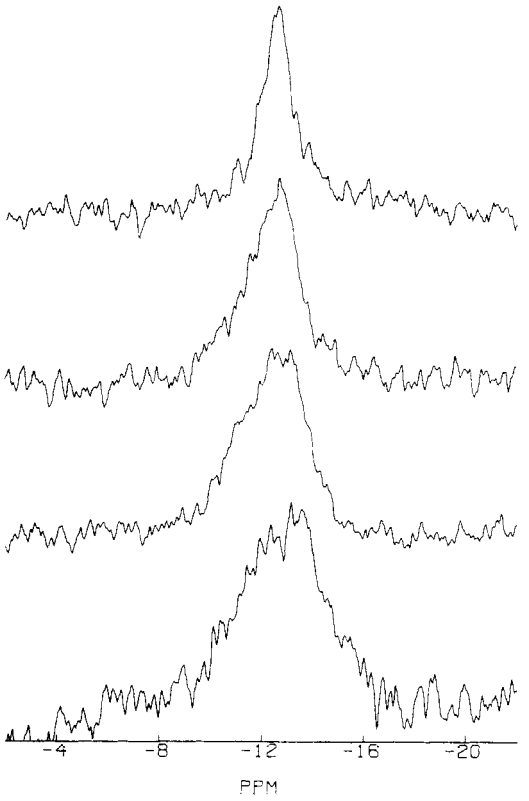

FIGURE 4: ^{19}F NMR spectra of phosphorylase reconstituted with 6-FPLP (30 mg/mL) in the presence of maltopentaose (1%), glucose-1-P (25 mM), inorganic phosphate (90 mM), and AMP (1 mM), recorded at 40, 30, 20, and 10 °C (from top to bottom).

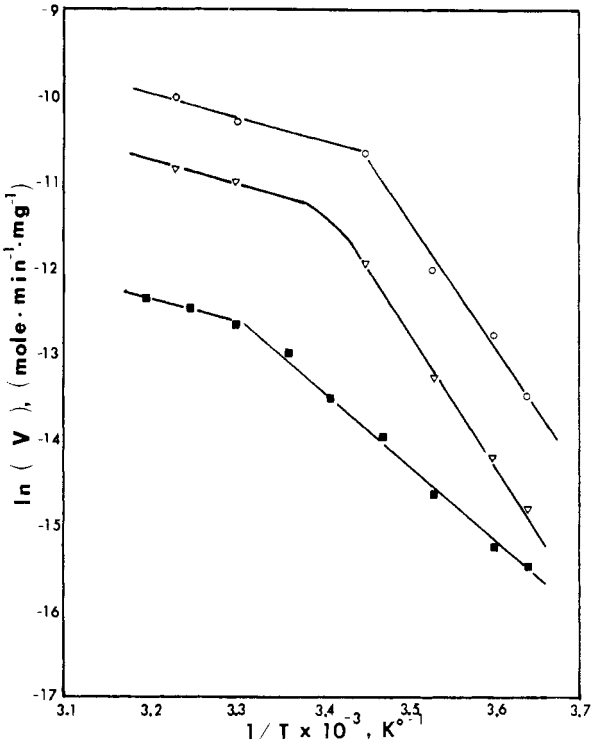

FIGURE 5: Arrhenius plots of enzymatic activity of the native phosphorylase (○) and phosphorylases reconstituted with 6-FPLP (▽) and 6-FPAL (■). The assay mixture for the native and 6-FPLP enzymes contained glucose-1-P (20 mM), AMP (1 mM), and glycogen (1%). The assay mixture for 6-FPAL–enzyme contained glucose-1-P (45 mM), AMP (1 mM), phosphite (5 mM), and glycogen (1%).

line in Figure 3. The (±) figure represents an estimate of the uncertainty due to the deviation of line-width values used in this analysis. The same analysis was also done to fit the line-width change due to the phosphate binding, and the exchange rate of 500 ± 100 s^{-1} yielded the best-fitting curve, also shown as a solid line in Figure 3. This analysis shows that the exchange rates between different protein–ligand complexes of phosphorylase at 30 °C are at least 5 times faster than the catalytic rate, 100 s^{-1} (Brown & Cori, 1961).

When the 6-FPLP–enzyme was mixed with AMP (1 mM) and a saturating amount of glucose-1-P, the line width of signals obtained at 40, 30, and 20 °C are respectively 410, 470, and 560 Hz. This gradual broadening effect can be explained by the slowed movement of the enzyme–AMP–glucose-1-P species at lower temperatures because of the increasing viscosity of the solution and by the change of the dimer–tetramer equilibrium of phosphorylase. However, a more pronounced temperature effect was found when the 6-FPLP–enzyme was actively catalyzing the reversible phosphorolysis of maltopentaose at equilibrium. In the presence of AMP (1 mM), maltopentaose (1%), glucose-1-P (25 mM), and phosphate (90 mM), the ^{19}F NMR spectra recorded at 40, 30, 20, and 10 °C show line widths of 360, 540, 850, and 970 Hz, respectively (Figure 4). No significant change of chemical shift is observed among the top two spectra, whereas the bottom two spectra seem to be composed of two broad bands. This observation can be explained by assuming the protein in this mixture forms two complexes and that these complexes undergo an exchange process. The pronounced temperature effect on the line shape is due to the increasing exchange rate between these complexes as the temperature rises and, hence, narrows the signal. This explanation is also consistent with result of the analysis of the rather broad signal of 6-FPLP–enzyme in the presence of substrates and AMP [(△) in Figure 1]. As the concentrations of ligands are much higher than their K_m values, the predominant species in the mixture are protein–glucose-1-P–AMP–maltopentaose and protein–phosphate–AMP–maltopentaose. The other protein–ligand complexes may only exist transiently in negligible amounts. Although the ^{19}F signals of these two main complexes are not available at this stage, it is likely that they have different chemical shifts because the binding of glucose-1-P and inorganic phosphate can strongly influence the chemical shift of 6-FPLP–enzyme to opposite directions.

The enzymatic activities of the native, 6-FPLP- and 6-FPAL-reconstituted phosphorylase were assayed at varied temperatures. For the first two enzymes, the resulting Arrhenius plots, shown in Figure 5, show a biphasic pattern, with a transition at around 17–19 °C. Segmental Arrhenius plots of the native phosphorylase have also been reported by Graves et al. (1965) and Kastenschmidt et al. (1968) at two different pH. Below these transition temperatures, these enzymes show higher activation energies than those at temperatures above these temperatures. Coincidentally, between 20 and 10 °C, the interconversion rate between the 6-FPLP–protein–AMP complex and the same complex with bound glucose-1-P also shows a pronounced change, shown in Figure 6. The experimental mixture includes 0.3 mM protein, 5 mM glucose-1-P, and 1 mM AMP. At 30 °C, the sample shows a rather symmetrical signal. A shoulder downfield from the main peak appears when the temperature is lowered to 20 °C. At 10 °C, the spectrum shows two bands at around −11.2 and −13.0 ppm, respectively, representing glucose-1-P-free and -bound species. The exchange rate between them was estimated to be around 250 s^{-1}. A similar change caused by lowering the temperature is also seen in a protein solution containing substrates and effector (Figure 4). Therefore, the higher activation energy of both native enzyme and 6-FPLP–

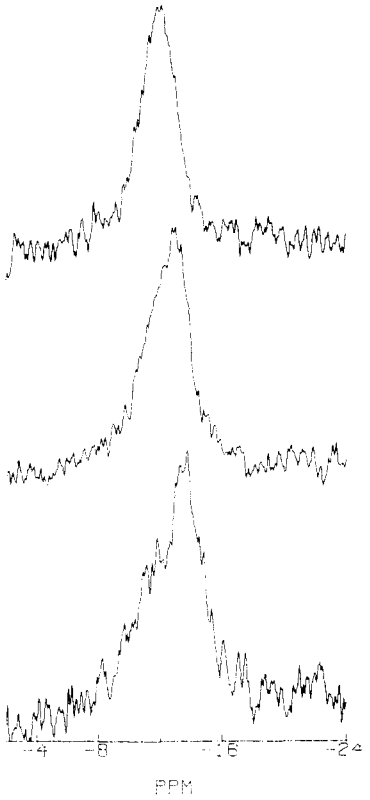

FIGURE 6: ^{19}F NMR spectra of phosphorylase reconstituted with 6-FPLP (30 mg/mL) in the presence of AMP (1 mM) and glucose-1-P (5 mM), recorded at 30 (top), 20 (middle), and 10 °C (bottom).

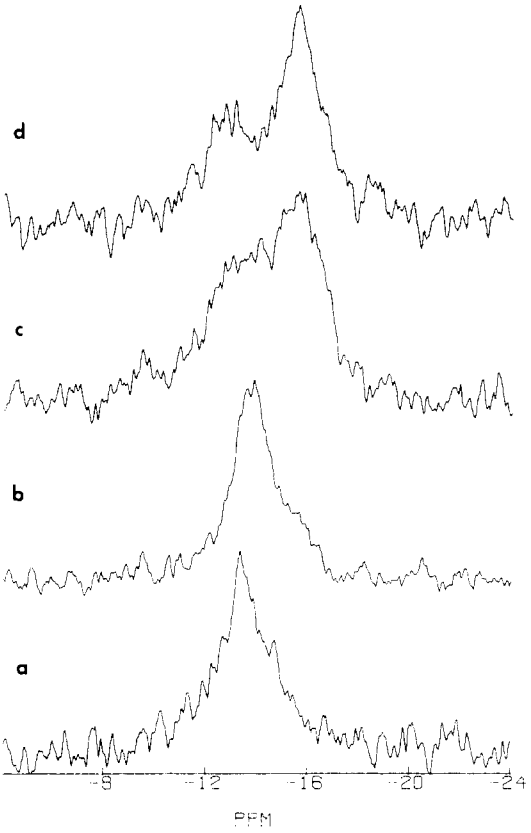

FIGURE 7: ^{19}F NMR spectra of phosphorylase reconstituted with 6-FPAL under different conditions. Spectrum a shows free 6-FPAL–enzyme (30 mg/mL) in buffer solution (40 mM β-glycerophosphate, 5 mM dithiothreitol, and 2 mM EDTA, at pH 6.8). Spectrum b shows 6-FPAL–enzyme (30 mg/ml) with AMP (1 mM) and phosphite (5 mM). Spectrum c shows 6-FPAL–enzyme (30 mg/mL) mixed with AMP (1 mM), phosphite (5 mM), and glucose-1-P (15 mM). Spectrum d shows 6-FPAL–enzyme (25 mg/mL) mixed with AMP (1 mM), phosphite (5 mM), and glucose-1-P (75 mM). Spectra were recorded at 30 °C.

enzyme below the transition temperature may be explained by the fact that the extra energy required for the interconversion between different protein–ligand complexes is added to the activation energy of catalysis.

That the interconversion process between different protein complexes is important for the catalysis is also implied by the ^{19}F NMR study and the temperature dependence of the activity of phosphorylase reconstituted with 6-FPAL. This enzyme [V_{max} = 4.2 μmol/(min·mg)] requires phosphite or other phosphate analogues for its enzymatic activity. The activity is lower than that of 6-FPLP-reconstituted enzyme [V_{max} = 17.8 μmol/(min·mg)] (Chang & Graves, 1985). The Arrhenius plot of its activity at various temperatures (in Figure 5) also shows a biphasic pattern. However, the transition temperature of this curve occurs at a much higher temperature than those of the other enzymes. ^{19}F NMR spectra of 6-FPAL–protein saturated with AMP and phosphite, in the presence of different glucose-1-P concentrations, are shown in Figure 7. Unlike the 6-FPLP–enzyme–AMP complexed with 5 mM glucose-1-P, the 6-FPAL–protein shows two signals at −12.5 and −14.5 ppm, representing the glucose-1-P-free and glucose-1-P-bound species, respectively, in the presence of 15 mM glucose-1-P (the K_m of glucose-1-P to 6-FPAL–enzyme is 14.5 mM). The peak at −12.5 ppm does not disappear even in the presence of 75 mM of glucose-1-P, which is well above the K_m value (14.5 mM). The interconversion rate between these two species is estimated by the computer simulation of line shape to be 250 s^{-1}, which is much slower than that found in the 6-FPLP–enzyme. Nevertheless, the binding of glucose-1-P shifts the ^{19}F signal of 6-FPAL–enzyme 2 ppm upfield. This change is virtually identical with that induced by the binding of the same ligand to 6-FPLP–enzyme. Therefore, the change of 6-FPAL– and 6-FPLP–proteins caused by the binding of glucose-1-P probably is similar, although the rates differ remarkably. The effect of temperature on the interconversion of glucose-1-P bound and free 6-FPAL–enzyme–AMP–phosphite complexes were also studied. The ^{19}F NMR spectra of 6-FPAL–enzyme with 15 mM glucose-1-P, 1 mM AMP, and 5 mM phosphite were recorded at 40, 30, 20, and 10 °C (Figure 8). Two signals centered at −12.5 and −14.5 ppm, representing the glucose-1-P-free and -bound protein complexes, respectively, are found in each spectrum. When the temperature is lowered from 40 °C, the peak corresponding to the glucose-1-P-bound species, at −14.5 ppm, decreases and the peak of glucose-1-P-free signal increases, as shown in Figure 8. Assuming that the ligand-bound and -free proteins are in an equilibrium and that the glucose-1-P concentration in the bulk solution is not significantly affected by binding to the protein (total protein concentration is 0.3 mM and glucose-1-P concentration is 15 mM), this change shows that the conversion of the 6-FPAL–enzyme–AMP–phosphite complex to a glucose-1-P–enzyme–AMP–phosphite complex is an endothermic reaction; i.e., the latter complex has a higher enthalpy than the former one.

Effects of other ligands, glucose and glucose cyclic 1,2-phosphate, on the ^{19}F NMR spectrum of 6-FPLP–enzyme were also studied. Glucose sharpened the 6-FPLP–enzyme–AMP complex signal to 360 Hz but did not change its resonating position. The change of line width can be correlated with the change in the dimer–tetramer equilibrium induced by the presence of glucose, which is known to favor the formation of the dimeric species (Withers et al., 1982a). Therefore, glucose leads to a sharpening of enzyme signals

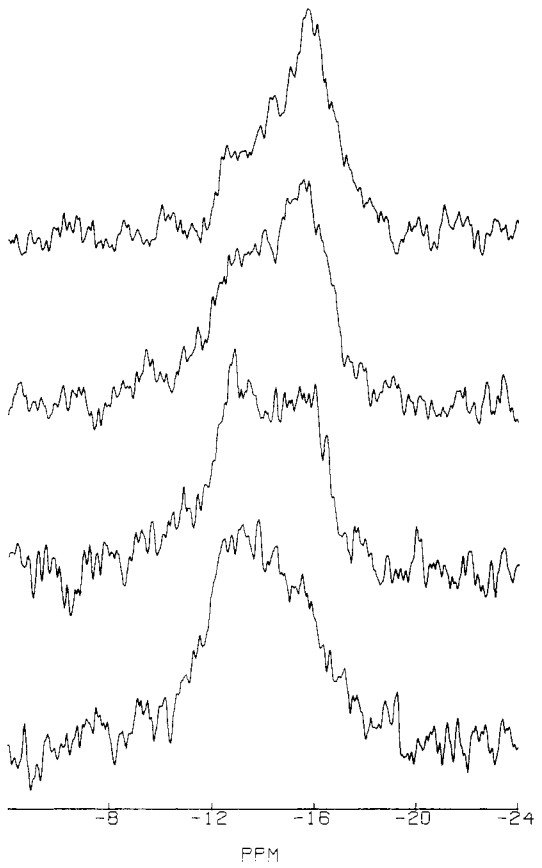

FIGURE 8: ^{19}F NMR spectra of 6-FPAL-reconstituted phosphorylase in the presence of AMP (1 mM), phosphite (5 mM), and glucose-1-P (15 mM), recorded at 40, 30, 20, and 10 °C, from top to bottom.

because of the smaller molecular size of the protein and shorter correlation time. Glucose cyclic 1,2-phosphate has been suggested to interact with the enzyme similarly to glucose-1-P (Withers et al., 1982b). When this cyclic compound replaces the glucose-1-P in a reaction mixture, in the presence of AMP and maltopentaose, the resulting ^{19}F signal is virtually identical with that of the enzyme–AMP complex. Therefore, it is doubtful whether this cyclic compound when enzyme bound can completely simulate an enzyme-bound glucose-1-P complex.

^{19}F NMR studies of 6-FPLP and Schiff's bases of 6-FPLP with amino acids showed that the chemical shift of the 6-fluorine nucleus is sensitive to the ionization state change of other groups, the 3-phenolic group, the 1-nitrogen, and the 4′-Schiff's base, of the coenzyme molecule (Chang & Graves, 1985; Scott et al., 1983). To determine whether the ionization states of these functional groups of an enzyme-bound 6-FPLP are affected by the pH change of the solution, the ^{19}F NMR spectra of 6-FPLP-reconstituted phosphorylase were recorded at different pH values. No significant change of the signal position is found between pH 6 and pH 9.1 (results not shown). This result agrees with the previous study showing that the pyridine ring of enzyme-bound PLP is deeply buried in a hydrophobic pocket in the enzyme and has no access to the solvent (Kupfer et al., 1977).

Discussion

The chemical shift of a fluorine nucleus embedded in a bulky protein molecule may be affected by interactions through chemical bonds or through space [see review of Gerig (1978)]. A previous study of phosphorylase reconstituted with 6-FPLP and 6-FPAL (Chang & Graves, 1985) has demonstrated that the ionization state of the 3-phenolic or of the pyridine nitrogen group of the coenzyme was unlikely to be affected by the binding of ligands. This finding rules out the possibility that the chemical shift change of 6-FPLP– and 6-FPAL–enzymes induced by ligands binding is due to interaction through bonds with the change of other functional groups of the coenzyme. Spatial interactions that affect the chemical shift of a protein-bound fluorine nucleus may include the van der Waals interactions and/or the second-order electric field effect between fluorine nucleus and neighboring protons (Hull & Sykes, 1976), or the ring-current-shielding effects caused by nearby aromatic rings (Haigh & Mallion, 1972), or electronic anisotropy of nearby carbonyl groups. The X-ray map of glycogen phosphorylase *b* shows that the pyridine ring of the coenzyme fits in a hydrophobic pocket made of residues of the C- and N-terminal domains (Jenkins et al., 1981, McLaughlin et al., 1984; Weber et al., 1978). The pyridine ring may interact with residues Lys-567 and Tyr-647 on one side, whereas the other side is in van der Waals contact with Tyr-90, Gly-134, Gly-135, and Val-649. In phosphorylase containing 6-FPAL or 6-FPLP, these amino acid residues may form interactions with the fluorine nucleus of the coenzyme and influence its chemical shift. In this study, we found that the ^{19}F signal of 6-FPLP– and 6-FPAL–enzymes were shifted when these enzymes formed complexes with ligands. This observation may be explained by certain conformational changes near the coenzyme binding site caused by ligand binding. These structural changes may alter the relative position of the protons or aromatic rings or carbonyl groups with respect to fluorine and, hence, affect its resonating position. This explanation is also supported by X-ray crystallographic studies of crystalline phosphorylase *b*. Comparisons of the X-ray map of phosphorylase complexed by glucose-1-P with those complexed with heptenitol, heptulose 2-phosphate–AMP–maltoheptaose (McLaughlin et al., 1984), and glucose cyclic 1,2-phosphate (Withers et al., 1982b) showed that substitutions of the substrate at the catalytic side with different ligands caused movements of glycine loop residues (133–136) and other disturbances around the pyridine ring. Therefore, each protein–ligand complex with a distinct ^{19}F signal may represent a unique conformation, and the interconversion between protein complexes found in this study may be viewed as certain transformations of protein structure.

On the basis of the study of isotopic exchange at equilibrium, Enger et al. (1969) found the rate-limiting step of phosphorylase catalysis is the chemical interconversion of the ternary complexes, i.e., the interconversion between protein–AMP–glucose-1-P–glycogen and protein–AMP–phosphate–glycogen complexes. The analysis of the field dependence of the line width and of the temperature effect of the ^{19}F NMR spectra of 6-FPLP phosphorylase (Figures 1 and 4) indicates that these two complexes have different chemical shifts, or conformations. Therefore, two changes, a conformational interconversion of protein structure and chemical steps of bond making and breaking, must occur during an individual turnover of the catalysis of phosphorylase. It is likely that the activation energy of catalysis is influenced by the energy barrier of the conformational interconversion. The segmental Arrhenius plot of phosphorylase catalysis has been interpreted by Graves et al. (1965) and Kastenschmidt et al. (1968) to arise from conformational changes of the enzyme which occur at lower temperature. In this study, we found that, similar to the native phosphorylase, phosphorylase reconstituted with 6-FPLP and 6-FPAL also shows a biphasic pattern. We also observed that the exchange rate of protein–ligand complexes of 6-FPAL–enzyme is much slower than that of the corresponding process

of 6-FPLP–enzyme. This difference is likely related to the much higher transition temperature found in the Arrhenius plot of the former enzyme than that found in the 6-FPLP–enzyme. When the ^{19}F NMR spectrum of 6-FPLP–enzymes was taken at varied temperatures (Figures 4 and 6), we observed a significant temperature effect on the interconversion rate between different protein–ligand complexes. For instance, the exchange rate of the interconversion between the 6-FPLP–protein–AMP complex and the same complex with bound glucose-1-P is 3300 s^{-1} at 30 °C, whereas it is 250 s^{-1} at 10 °C (Figure 6). A temperature difference of 20 °C changes the exchange rate by a factor greater than 10. This change is much larger than the temperature effect usually found in enzyme catalysis. The Q_{10} value, the ratio of enzymatic activities measured at two temperatures with a difference of 10 °C, usually is between 1 and 2 (Dixon & Webb, 1964). Therefore, we propose that the biphasic character of the phosphorylase activity at different temperature, or the cold inactivation found by Graves et al. (1965), is a result of the rate-limiting step of phosphorylase catalysis being comprised of two successive reactions, a pure chemical reaction of the bond breaking and forming and a conformational interconversion between ternary complexes.

According to the Arrhenius plots of the native enzyme and 6-FPLP-enzyme above 30 °C, these enzymes show virtually identical activation energies. Because these enzymes have almost identical K_m values for substrates and AMP at the same temperature, the different catalytic rates are likely due to different probability constants of the Arrhenius equation, A, which is directly related to the entropy of activation (Jencks, 1969). Various studies have shown that the entropy of activation is a measure of how many substrates bound to the active sites of the protein actually react (Jencks, 1969; Bender et al., 1964). In other words, this parameter shows how well the substrate is fitted to a specific structure of the protein required for catalysis. On the basis of model-building studies, Johnson et al. (1980) have shown that the active site of phosphorylase *b* can accommodate glucose-1-P with at least two different orientations. Therefore, the lower activity of 6-FPLP–enzyme may be due to the poor orientation of bound substrate in the active site, and as a result, the probability of reaction is low. The activation energy of the 6-FPAL–enzyme above its transition temperature is also similar to those of the native enzyme and 6-FPLP–enzyme. Its low activity may be explained by the even poorer orientation of the bound substrate at its active site compared to the other two enzymes in the same figure.

X-ray crystallographic studies of phosphorylase show that the pyridine nitrogen of the coenzyme does not form any strong interactions with nearby amino acid residues (McLaughlin et al., 1984). The possibility that this functional group, with a pK_a value of 8.7 (Perrin, 1965), may interact with the protein when the enzyme structure undergoes changes, however, cannot be ruled out. When the coenzyme PLP in phosphorylase is replaced by 6-FPLP, the potential interactions between the pyridine nitrogen of the coenzyme ($pK_a < 1$) with the protein become unlikely. The absence of these interactions may account for the lower activation entropy of the 6-FPLP–enzyme. In the native phosphorylase, the 5′-phosphate group of the coenzyme is tightly bound to the protein by forming interactions with surrounding basic groups (Johnson et al., 1980; McLaughlin et al., 1984). When the covalent linkage between the 5′-phosphate and pyridine ring of the coenzyme in 6-FPLP–enzyme was cleaved, such as in the 6-FPAL-enzyme, the energy barrier of the interconversion between different conformers increased and the activation entropy decreased. These observations indicate that the interactions between the pyridine ring of the coenzyme with the protein are important for the functions of phosphorylase. Comparative studies of phosphorylase isolated from different sources, ranging from *Escherichia coli* to rabbit, by X-ray crystallography showed that the amino acid residues surrounding the coenzyme molecule are very much conserved (Palm et al., 1985). These observations may also be interpreted as that the interactions between the coenzyme with the protein are important for the functions of the enzyme.

Because the active site region of phosphorylase lies where three major domains come together and PLP is in contact with residues from two domains, bound PLP may provide a proper arrangement of domains needed for aligning the catalytic groups for binding substrates and effective catalysis. However, when the contacts between coenzyme ring and protein domains are severed by different modifications of the coenzyme such as in the 6-FPLP– or 6-FPAL–enzyme, part of the active site structure becomes disoriented. As a result, the structural changes around the active site during the course of catalysis become less coordinated, the probability of substrates being bound to the active site in an unproductive mode becomes higher, and the catalytic rates of these enzymes become lower.

In conclusion, results of this study provide us with information about dynamic properties of glycogen phosphorylase in solution. First, the transformation of protein structure can be induced by the binding of ligands, and the energy of each of the resulting complexes may be different from that of a free enzyme. Second, the conversion from one complex to another has to pass through an energy barrier posing between them. Third, the integrity of the protein structure, the well-oriented domains and the coenzyme, may be a prerequisite for minimizing the energy barriers. Lastly, the coenzyme molecule, sitting in the heart of the bulky protein, plays an important role in coordinating the proper orientation of the domains, which in turn form the well-constructed active site region. This may be a major role played by PLP in glycogen phosphorylase.

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SUPPLEMENTARY MATERIAL AVAILABLE

Calculations of the overall rotational correlation times of native phosphorylase and the fluorine nucleus in 6-FPLP-reconstituted phosphorylase (4 pages). Ordering information is given on any current masthead page.

Registry No. 6-FPAL, 42242-42-2; 6-FPLP, 90932-80-2; PLP, 54-47-7; P_i, 14265-44-2; glucose-1-P, 59-56-3; glycogen phosphorylase, 9035-74-9; D-glucose, 50-99-7.

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