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^{19}F NMR Investigations of the Catalytic Mechanism of Phosphoglucomutase Using Fluorinated Substrates and Inhibitors†

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ABSTRACT: The complexes of phosphoglucomutase with a number of fluorinated substrate analogues have been investigated by ^{19}F NMR and the effects of the binding of Li^+ and Cd^{2+} to these complexes determined. Very large downfield chemical shift changes (−14 to −19 ppm) accompanied binding of the inhibitors 6-deoxy-6-fluoro-α-D-glucopyranosyl phosphate and α-glucosyl fluoride 6-phosphate to the phosphoenzyme. Smaller shift changes were observed for ligands substituted with fluorine at other positions. Addition of Li^+ to enzyme/fluorinated ligand complexes caused a 10^2- to 10^3-fold decrease in ligand dissociation constants as witnessed by the change from intermediate to slow-exchange conditions in the NMR spectra. Measurement of the ^{19}F NMR spectra of complexes of the Li^+–enzyme with each of the fluoroglucose 1-phosphates and 6-phosphates has provided some insight into the environment of each of these fluorines (thus also parent hydroxyls) in each of the complexes. Results obtained argue strongly against a single sugar binding mode for the glucose 1- and 6-phosphates. Two enzyme-bound species were detected in the ^{19}F NMR spectra of the complexes formed by reaction of the Cd^{2+}–phosphoenzyme complex with the 2- and 3-fluoroglucose phosphates. These are tentatively assigned as the fluoroglucose 1,6-bisphosphate species bound in two different modes to the dephosphoenzyme. Only one bound species was observed in the case of the 4-fluoroglucose phosphates. The results from this investigation, and those above, are consistent with an exchange type of mechanism [Ray, W. J., Mildvan, A. S., & Long, J. W. (1973) *Biochemistry 12*, 3724] for the enzyme in which there are two distinct glucose ring binding sites.

As described in greater detail in the preceding paper (Percival & Withers, 1992), the general mechanism of phosphoglucomutase is now largely established as involving an initial phosphoryl-transfer step from the serine phosphate of the phosphoenzyme to the bound sugar phosphate, yielding a dephosphoenzyme/glucose-1,6-diP[1] complex. This complex then rearranges in some way to place the nontransferred

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[1] Abbreviations: glucose-1-P, α-D-glucopyranosyl phosphate; deoxyglucose-1-P, deoxy-α-D-glucopyranosyl phosphate; fluoroglucose-1-P, deoxyfluoro-α-D-glucopyranosyl phosphate; glucose-6-P, D-glucose 6-phosphate; α-D-glucosyl fluoride-6-P, α-D-glucosyl fluoride 6-phosphate; glucose-1,6-diP, α-D-glucose 1,6-bisphosphate; PGM, phosphoglucomutase; 4FMeGlc, methyl 4-deoxy-4-fluoro-α-D-glucopyranoside; 6FMeGlc, methyl 6-deoxy-6-fluoro-α-D-glucopyranoside.

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phosphate adjacent to the serine hydroxyl group after which a second phosphoryl transfer occurs, giving the sugar phosphate product and regenerating the phosphoenzyme. Less clear, however, is the mode of reorganization of the dephosphoenzyme/glucose-1,6-diP complexes. The two mechanisms proposed, the minimal motion and exchange mechanisms (Ray et al., 1973), were described in some detail in the preceding paper (Percival & Withers, 1992). The principal focus of this paper is upon our attempts to distinguish between these two possible mechanisms by means of ^{19}F NMR studies. A considerable amount of work has already been done by others in studying various phosphoglucomutase/substrate complexes by NMR techniques including ^{1}H, ^{31}P, ^{113}Cd, and ^{7}Li NMR (Rhyu et al., 1984, 1985a,b; Post et al., 1989; Ray et al., 1990).

One of the principal distinguishing features of these two mechanisms is the fact that the minimal motion mechanism requires only a single sugar binding mode, whereas the exchange mechanism requires two. The relative movement required for the minimal motion mechanism is provided by a protein conformational change. Therefore, any technique which could determine whether or not the sugar moiety in the glucose-1-P complex binds in the same protein site as that in the glucose-6-P complex would allow distinction of these two mechanisms. The first approach described in this paper involves the ^{19}F NMR study of nonproductive complexes of the phosphoenzyme with each of the fluorinated glucose-1-P analogues and each of the fluorinated glucose-6-P analogues. Since ^{19}F NMR chemical shifts are highly sensitive to local environment, they may be used as probes of the specific protein binding site of each fluorine, and therefore also of the corresponding hydroxyl. If the minimal motion mechanism is correct, there should only be a single sugar binding mode and the environment of each fluorine should be the same in the sugar 1-phosphate and 6-phosphate complexes. In that case similar chemical shift *changes* should be observed upon binding for each of the pairs of fluorosugar phosphates. If the exchange mechanism is correct, quite different chemical shift changes might be expected.

Such an approach requires that stable nonproductive complexes of the enzyme with each fluorosugar phosphate be formed to allow the necessary ^{19}F NMR measurements to be made. This is simple for those analogues in which the attacking hydroxyl has been replaced by a fluorine (6-fluoroglucose-1-P and α-glucosyl fluoride-6-P) since these are catalytically incompetent and thus function as competitive inhibitors. For those analogues which are substrates it was necessary to study their complexes with the Li^+–enzyme, whose activity has been shown (Ray et al., 1989) to be some 10^8 times lower than the native enzyme, allowing ^{19}F NMR studies to be performed. This approach has been used previously (Ma & Ray, 1980; Rhyu et al., 1984, 1985a,b) to stabilize phosphoglucomutase/substrate complexes for several hours, allowing their study by physical techniques.

A second approach to this problem involved the use of a series of three difluorinated sugar 1-phosphate analogues in which *both* the 6-hydroxyl and *one* of the other hydroxyls, in each case, had been replaced by fluorine. These compounds have been shown previously (Percival & Withers, 1992) to act as competitive inhibitors of the enzyme, and to bind in the same mode as the parent glucose-1-P. ^{19}F NMR studies of complexes of the enzyme with each of these inhibitors should in principle provide insight into the binding environment of each of the ring fluorines without the need for addition of Li^+ to stop reaction.

A final approach that has been employed in this study is to study the complexes formed by these fluorinated substrates with the Cd^{2+}–phosphoenzyme. It has been shown previously (Ray & Long, 1976a) that this weakly activating metal ion causes a redistribution among the three enzyme-bound species (the central complex) such that the dephosphoenzyme/glucose-1,6-diP complex dominates, allowing ^{31}P NMR spectra of the bound glucose-1,6-diP to be measured. A similar approach is used in this study in which ^{19}F NMR is used to investigate the various fluoroglucose-1,6-diP complexes. If the minimal motion mechanism is correct, there should only be one binding mode for the sugar moiety of glucose-1,6-diP, whereas two different binding modes would be expected for the exchange mechanism. Therefore, if two different ^{19}F NMR signals are observed in such spectra for each of the fluorosugar complexes, this would provide strong support for the exchange mechanism, whereas observation of only a single resonance in each case might suggest that the minimal motion mechanism is the more likely.

Experimental Procedures

The syntheses of the 2-, 3-, and 4-fluoro-D-glucose 6-phosphates were achieved by the enzymatic phosphorylation of the appropriate nonphosphorylated sugar (Withers et al., 1986) as detailed in the preceding paper (Percival & Withers, 1992). The synthesis of α-D-glucosyl fluoride-6-P is also described therein. The syntheses of the 2-, 3-, 4-, and 6-fluoro-α-D-glucopyranosyl phosphates and their difluorinated analogues have been described previously (Withers et al., 1986, 1989).

The procedures for the isolation of rabbit muscle phosphoglucomutase (phospho form) and the demetalation of enzyme, ligands, buffers, and other additives were kindly provided by Prof. W. J. Ray, Purdue University. All glassware, NMR tubes, and pipets were soaked in a solution of 0.1 mM EDTA overnight and were rinsed with distilled, double-deionized water from a Millipore water purification system prior to use. Enzyme concentrations were determined, using a molecular weight of 61 600, by absorbance measurement at 278 nm, based upon a 1% w/v solution having an absorbance of 7.0 (Ray et al., 1983).

Lithium chloride was obtained from Johnson Matthey Chemicals and was Puratronic grade. Cadmium acetate was from BDH and was Analar grade.

^{19}F NMR spectra (254 MHz) were obtained in the Fourier transform mode on a Bruker HXS-270 spectrometer equipped with a 5-mm ^{19}F high-resolution probe. Sweep widths of 20 or 34 kHz, 8K data sets, 90° pulse angles (14 μs), and 0.5-s pulse delay times were used for all spectra, except when gated ^{1}H decoupling was used, for which 2.0-s delays were employed. In most cases 20 000 scans were used to acquire spectra. The line broadening factor resulting from exponential apodizations was 20 Hz and has been subtracted from all the line widths quoted. Chemical shifts are reported relative to trichlorofluoromethane (δ = 0.0 ppm) and were determined relative to one of the internal references 4FMeGlc (δ = 199.2 ppm) or 6FMeGlc (δ = 236.2 ppm). NMR measurements were made at 20 °C with a sample volume of 0.4 mL containing approximately 1.0 mM phosphoglucomutase (phospho form) in a buffer of 20 mM Tris·HCl, pH 7.5, and 50% D_2O (unless stated otherwise). No attempt was made to correct for the isotope effect on pH. Other experimental details are given in the figure legends. Analysis of the composition of the NMR samples containing 3-fluoroglucose phosphates was performed according to a modification of the procedure of Ray and Long (1976b) in which the rapid quenching by perchloric acid was

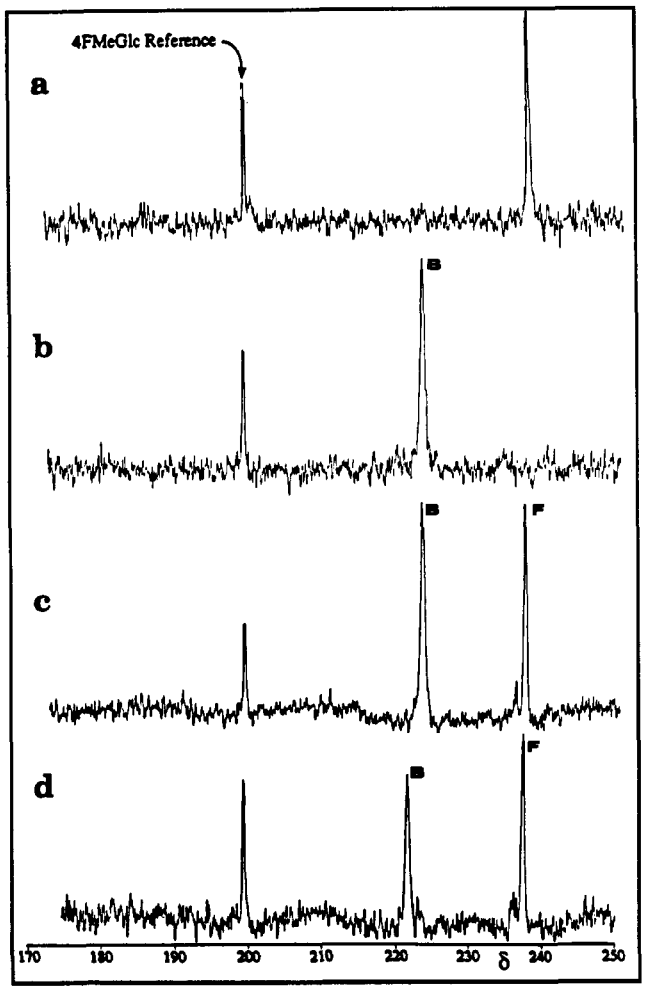

FIGURE 1: ^{19}F NMR titration of phosphoglucomutase with 6-fluoroglucose-1-P and Li^+. The reference employed in each sample was 4FMeGlc. (a) Spectrum of 6-fluoroglucose-1-P; (b) phosphoglucomutase (1.4 mM) plus 6-fluoroglucose-1-P (1.4 mM); (c) phosphoglucomutase (1.4 mM) plus 6-fluoroglucose-1-P (2.8 mM); (d) phosphoglucomutase (1.0 mM) plus 6-fluoroglucose-1-P (2.0 mM) and Li^+ (10 mM). B = bound 6-fluoroglucose-1-P; F = free 6-fluoroglucose-1-P.

replaced by heating the enzyme to 100 °C for 10 min.

Results

"Incompetent" Fluoroglucose Phosphate Inhibitors. The ^{19}F NMR spectrum illustrating the titration of a solution of phosphoenzyme with 1.0 molar equivalent of 6-fluoroglucose-1-P (Figure 1b) shows that a new resonance appears at 223.2 ppm, shifted 14.2 ppm downfield from that of the free ligand (Figure 1a) in the absence of phosphoglucomutase (237.4 ppm). The absence of any resonance due to free ligand indicates that binding is relatively tight, as found in the previous kinetic study (Percival & Withers, 1992). The addition of a second equivalent of ligand (Figure 1c) results in the appearance of a second resonance at the same chemical shift as that of the free ligand, showing that binding is in the slow-exchange regime. Addition of 10 mM Li^+ to this complex (Figure 1d) causes a further 1.7 ppm downfield shift of the bound ligand (221.5 ppm). These complexes are very stable since essentially identical spectra could be obtained after storage of the sample for 2 weeks at 4 °C.

Broad-band proton decoupling causes the complete disappearance of the signal from the bound ligand and a reduction in the intensity and line width of the signal due to free ligand (spectrum not shown). The disappearance of the signal from the bound ligand is caused by the negative nuclear Overhauser effect (NOE) obtained in this case due to the relatively long correlation time of the bound ligand and the dominance of the dipolar spin–lattice relaxation mechanism. The reduction in intensity of the signal from free ligand is due to magnetization transfer from the bound species as a consequence of chemical exchange. These results are in accord with those of Sykes et al. (1974), who observed the same phenomenon for signals from fluorotyrosine-labeled alkaline phosphatase. Fluorine–proton spin–spin splitting could also be removed by the use of a pulse sequence in which the ^{1}H decoupler is gated on during acquisition of the free induction decay and gated off during the delay between pulses. In this way any multiplets are collapsed but no NOE is induced. Gated decoupling of the sample consisting of a 2:1 molar ratio of ligand to enzyme resulted in a collapse of the multiplets of the signals from the reference ($\Delta\nu_{1/2}$ = 80–5 Hz) and free ligand ($\Delta\nu_{1/2}$ = 105–40 Hz), but only a relatively small reduction in line width of the bound ligand ($\Delta\nu_{1/2}$ = 130–100 Hz). The narrow line width of the reference peak ($\Delta\nu_{1/2}$ = 5 Hz) confirms that the broadening of the free ligand is not due to a viscosity effect, but rather to exchange with the bound species. It also confirms that the internal references have little affinity for the enzyme as anticipated since the K_m of glucose itself is 0.7 M (Layne & Najjar, 1978).

Table I: ^{19}F NMR Chemical Shifts for Fluorinated Sugar Phosphates Free or Complexed with Li^+–Phosphoglucomutase

ligand (L)	free δ (ppm)	bound δ (ppm)	difference Δδ (ppm)
α-2F-Glc-6-P	200.3	197.9	−2.4
β-2F-Glc-6-P	200.1	197.9	−2.2
α-3F-Glc-1-P	201.2	197.4	−3.8
α-3F-Glc-6-P	200.6	201.1	+0.5
β-3F-Glc-6-P	195.7	197.0	+1.3
α-4F-Glc-1-P	199.0	192.9	−6.1
α-4F-Glc-6-P	198.3	195.6	−2.7
β-4F-Glc-6-P	200.2	198.8	−1.4
α-6F-Glc-1-P	237.4	221.5	−15.9
α-6P-Glc-1-F	151.3	133.1	−18.2

Titration of the phosphoenzyme with the ligands α-glucosyl fluoride-6-P and Li^+ resulted in a similar series of ^{19}F NMR spectra (not shown) to that obtained with 6-fluoroglucose-1-P, a large downfield shift (−19.7 ppm) being observed once again upon binding to the enzyme. The chemical shifts of the various free and bound species and the changes in chemical shift which accompany complex formation are summarized in Table I.

The spin–lattice relaxation times of the internal reference and 6-fluoroglucose-1-P, both free and enzyme bound, were measured by the progressive saturation method both in the presence and in the absence of protein. Values for T_1 of around 1.0 and 0.6 s were found for the reference 4FMeGlc and ligand 6-fluoroglucose-1-P, respectively, in the absence of enzyme at 20 °C in 50% D_2O. Increasing the D_2O/H_2O ratio to 99:1 raised the T_1 of 4FMeGlc by 0.15 s, showing that ^{1}H–^{19}F dipolar interactions contributed significantly to longitudinal relaxation. In the presence of phosphoglucomutase the T_1 value for "free" 6-fluoroglucose-1-P dropped to 0.4 s while that for the reference 4FMeGlc was unaffected. Binding of 6-fluoroglucose-1-P to phosphoglucomutase decreased its T_1 value to 0.21 s while changing the buffer composition from 50% D_2O to 99% D_2O had no effect on T_1 values for the bound ligand, indicating that the fluorine, once bound, was no longer solvent-accessible.

Fluoroglucose Phosphate Substrates. The ^{19}F NMR spectra of the ternary complexes of phosphoenzyme, Li^+, and each fluoroglucose phosphate substrate were measured, and

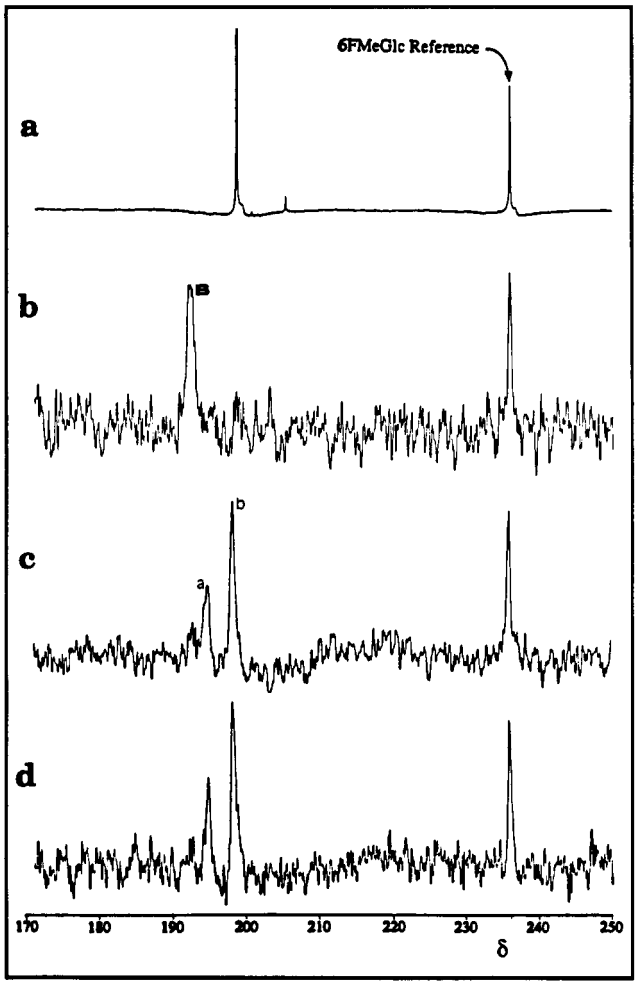

FIGURE 2: ^{19}F NMR titration of Li^+–phosphoglucomutase with 4-fluoroglucose-1-P. The reference employed in each sample was 6FMeGlc. (a) ^1H-decoupled spectrum of 4-fluoroglucose-1-P; (b) phosphoglucomutase (1.0 mM) plus LiCl (10 mM), EDTA (1.0 mM), and 4-fluoroglucose-1-P (1.0 mM); (c) same as (b) but 10 h later; (d) phosphoglucomutase (1.0 mM) plus LiCl (10 mM), EDTA (1.0 mM), and 4-fluoroglucose-6-P (1.0 mM). B = bound 4-fluoroglucose-1-P; a = bound α-4-fluoroglucose-6-P; b = bound β-4-fluoroglucose-6-P.

the chemical shifts measured for each free and bound sugar are presented in Table I. As an example of the spectra obtained, the results for the 4-fluoroglucose phosphates are presented in Figure 2. The addition of 1 equiv of 4-fluoroglucose-1-P to the Li^+–phosphoenzyme produces a complex with a chemical shift of 192.9 ppm (Figure 2b), shifted 6.1 ppm downfield from that of the free ligand in the absence of phosphoglucomutase (199.0 ppm) (Figure 2a). Over a period of 10 h the signal from this species disappeared and two new resonances at 195.6 and 198.8 ppm appeared (Figure 2c). This final spectrum is indistinguishable from that produced by a 1:1 molar ratio of α/β-4-fluoroglucose-6-P and Li^+–phosphoenzyme (Figure 2d); thus turnover has occurred. Addition of a second equivalent of α/β-4-fluoroglucose-6-P to this latter sample resulted in the appearance of additional resonances at the expected chemical shifts for free α- and β-4-fluoroglucose-6-P (not shown), confirming that binding is tight and in the slow-exchange regime.

Such enzyme-catalyzed turnover was not seen for 3-fluoroglucose-1-P as was evidenced by the absence of any change in the spectrum as a function of time. In addition, enzymatic analysis of the NMR samples following measurement of the spectra showed that none of the 3-fluoroglucose-6-P had been formed. This is presumably a consequence of the lower V_{max} for the 3-fluoro substrate than the 4-fluoro (Percival & Withers, 1992) and probably a more complete demetalation of this particular sample. Unfortunately, this enzymatic assay could not be used to analyze the 2-fluoroglucose-1-P sample because of the low activity of the coupling enzyme, glucose-6-phosphate dehydrogenase, with 2-fluoroglucose-6-P. This rendered the spectra obtained with 2-fluoroglucose-1-P uninterpretable as the same chemical shift was measured as was found for bound 2-fluoroglucose-6-P, indicating that turnover may have occurred. Fortunately, other data were available from the difluorinated inhibitor study to give a value for the chemical shift change upon binding and thereby to complete the study.

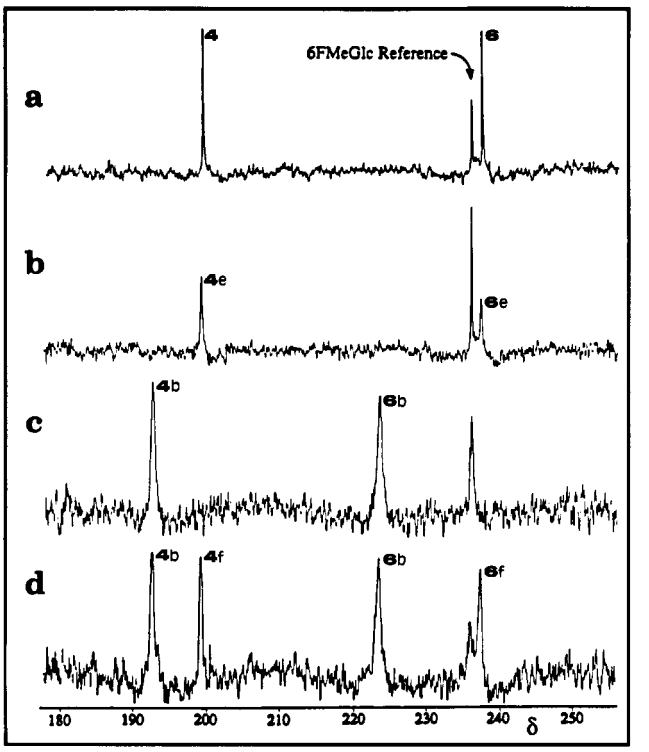

FIGURE 3: ^{19}F NMR titration of phosphoglucomutase with 4,6-difluoroglucose-1-P and Li^+. The reference employed in each sample was 6FMeGlc. (a) ^1H-decoupled spectrum of 4,6-difluoroglucose-1-P; (b) ^1H-decoupled spectrum of phosphoglucomutase (1.0 mM) plus 4,6-difluoroglucose-1-P (1.0 mM); (c) phosphoglucomutase (1.0 mM) plus LiCl (10 mM), EDTA (1.0 mM), and 4,6-difluoroglucose-1-P (1.0 mM) (^1H coupled); (d) phosphoglucomutase (1.0 mM) plus LiCl (10 mM), EDTA (1.0 mM), and 4,6-difluoroglucose-1-P (2.0 mM) (^1H coupled). 4 = resonance from F-4; 6 = resonance from F-6; e = exchange-averaged resonance; b = bound resonance; f = free resonance.

Difluorinated Inhibitors. Figure 3 shows the ^{19}F NMR spectra resulting from a titration of phosphoenzyme with the inhibitor 4,6-difluoroglucose-1-P. The ^1H-decoupled spectrum of the free inhibitor plus reference is shown in Figure 3a. When demetalated phosphoglucomutase is added in a 1:1 molar ratio with the inhibitor, the gated proton-decoupled signal from F-6 is broadened ($\Delta\nu_{1/2}$ = 70 Hz) and shifted slightly downfield by 0.2 ppm to 237.4 ppm. Similarly, F-4 is broadened ($\Delta\nu_{1/2}$ = 65 Hz) and is also shifted downfield by approximately 0.2 ppm (Figure 3b). Addition of 10 mM Li^+ to the binary enzyme–inhibitor complex caused the ligand peaks to broaden considerably more ($\Delta\nu_{1/2}$ = 110 Hz) and shift further downfield by 7.0 and 14.2 ppm for F-4 and F-6, respectively, relative to the free ligand shift (Figure 3c). On addition of a second equivalent of 4,6-difluoroglucose-1-P two new resonances appeared at the same chemical shifts as that of the ligand in the absence of protein (Figure 3d), confirming

Table II: ^{19}F NMR Chemical Shifts for Difluorinated Sugar Phosphates Free and Bound to Li^+–Phosphoglucomutase

ligand (L)	chemical shift, δ (ppm)					
	free ligand		ligand + E_p·Li^+		difference ($\Delta\delta$)[a]	
	F-6	F-X[b]	F-6	F-X	F-6	F-X
4,6-difluoroglucose-1-P	237.7	199.5	223.5	192.5	−14.2	−7.0
3,6-difluoroglucose-1-P	237.7	201.5	222.9	197.7	−14.8	−3.8
2,6-difluoroglucose-1-P	237.4	200.3	222.2	194.2	−15.2	−6.1

[a] Chemical shift change from free ligand upon binding. [b] F-X = F-4 for 4,6-difluoroglucose-1-P, F-3 for 3,6-difluoroglucose-1-P, and F-2 for 2,6-difluoroglucose-1-P.

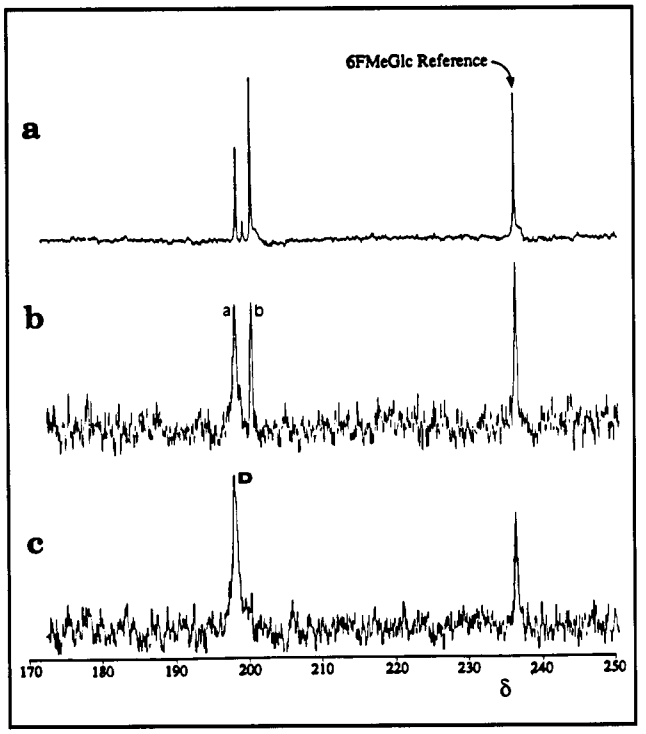

FIGURE 4: ^{19}F NMR titration of phosphoglucomutase with 4-fluoroglucose-6-P and Cd^{2+}. The reference employed in each sample was 6FMeGlc. (a) ^{1}H-decoupled spectrum of 4-fluoroglucose-6-P; (b) phosphoglucomutase (1.0 mM) plus 4-fluoroglucose-6-P (1.0 mM) (^{1}H coupled); (c) phosphoglucomutase (1.0 mM) plus $Cd(OAc)_2$ (1.1 mM) and 4-fluoroglucose-6-P (1.0 mM) (^{1}H coupled). a = α-4-fluoroglucose-6-P; b = β-4-fluoroglucose-6-P; D = bound diphosphate resonance.

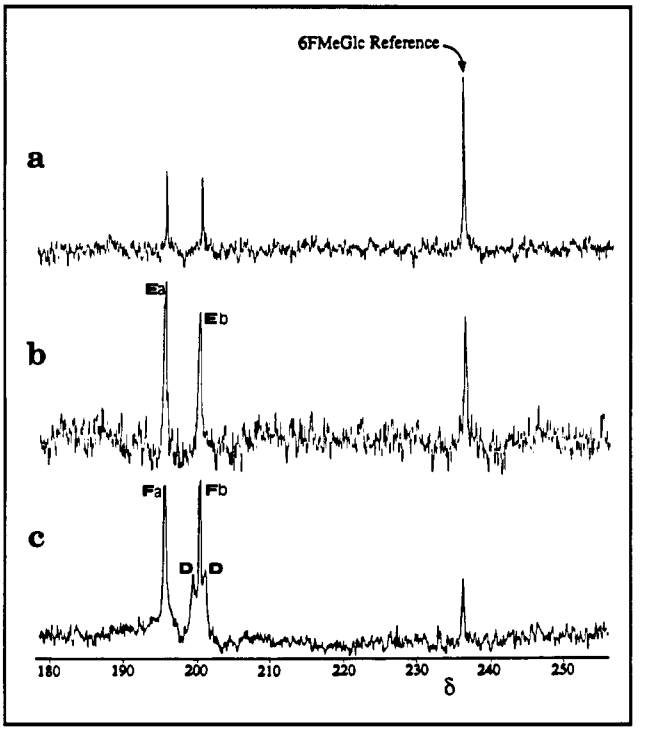

FIGURE 5: ^{19}F NMR titration of phosphoglucomutase with 3-fluoroglucose-1-P and Cd^{2+}. The reference employed in each sample was 6FMeGlc. (a) ^{1}H-decoupled spectrum of α/β-3-fluoroglucose-6-P; (b) phosphoglucomutase (1.0 mM) plus 3-fluoroglucose-1-P (1.0 mM) (^{1}H coupled); (c) phosphoglucomutase (1.0 mM) plus $Cd(OAc)_2$ (1.1 mM) and 3-fluoroglucose-1-P (2.0 mM) (^{1}H coupled). E = exchange averaged resonance; F = free resonance; D = bound diphosphate resonance; a = α-3-fluoroglucose-6-P; b = β-3-fluoroglucose-6-P.

that binding is tight and in the slow-exchange regime. Similar series of spectra were obtained with 2,6- and 3,6-difluoroglucose-1-P, the results being summarized in Table II.

Fluoroglucose Phosphate Substrates plus Cd^{2+}. The complexes of each of the fluoroglucose phosphate substrates with the Cd^{2+}–phosphoenzyme were studied by ^{19}F NMR, and spectra obtained are shown in Figures 4–6. Figure 4a shows the ^{1}H-decoupled ^{19}F NMR spectrum of α/β-4-fluoroglucose-6-P and reference 6FMeGlc (δ = 236.2 ppm). The small peak at 199.1 ppm is a contaminant of 4-fluoroglucose-1-P presumably caused by a contaminant of phosphoglucomutase in the hexokinase used in the synthesis of 4-fluoroglucose-6-P. Figure 4b shows the proton-coupled spectrum of a solution containing a 1:1 molar ratio of phosphoenzyme and 4-fluoroglucose-6-P. An essentially identical spectrum was produced by an equimolar ratio of phosphoenzyme and 4-fluoroglucose-1-P, showing that, in spite of the demetalation procedure, sufficient activating metal ion was present to allow the isomerization of 4-fluoroglucose-1-P to the thermodynamically more stable α/β-4-fluoroglucose-6-P. The line widths of the ligand and internal reference peaks were determined by gated proton decoupling as 50, 40, and 5 Hz, respectively. The addition of 1.1 equiv of Cd^{2+} to the above mixture resulted in the appearance of a single resonance at 198.2 ppm (Figure 4c). The proton-decoupled line width of this peak was subsequently shown to be 130 Hz.

The corresponding spectra for complexes of the Cd^{2+}–enzyme with the 2- and 3-fluoroglucose phosphates are shown in Figures 5 and 6. Figure 5a shows the spectrum of α/β-3-fluoroglucose-6-P plus reference 6FMeGlc. The spectrum of the demetalated phosphoenzyme plus equimolar 3-fluoroglucose-1-P is shown in Figure 5b. The two resonances at 200.1 and 195.2 ppm arise from the α- and β-anomers of 3-fluoroglucose-6-P produced by enzymic turnover, presumably due to a trace metal ion contamination. Addition of a second equivalent of 3-fluoroglucose-6-P caused the appearance of no additional peaks, just the intensification of the peaks at 200.1 and 195.2 ppm (spectrum not shown). Addition of Cd^{2+} to this sample (Figure 5c) resulted in the appearance of two new broad resonances at 199.2 and 200.9 ppm which are assigned to the glucose-1,6-diP complexes. Similar results were obtained with 2-fluoroglucose-1-P, and these are shown in Figure 6. Figure 6, panels a and b, shows the spectra of free α/β-2-fluoroglucose-6-P and 2-fluoroglucose-1-P, respectively, plus reference 6FMeGlc. The small peak at δ = 199.7 ppm in Figure 6b is due to contaminating β-2-fluoroglucose-1-P.

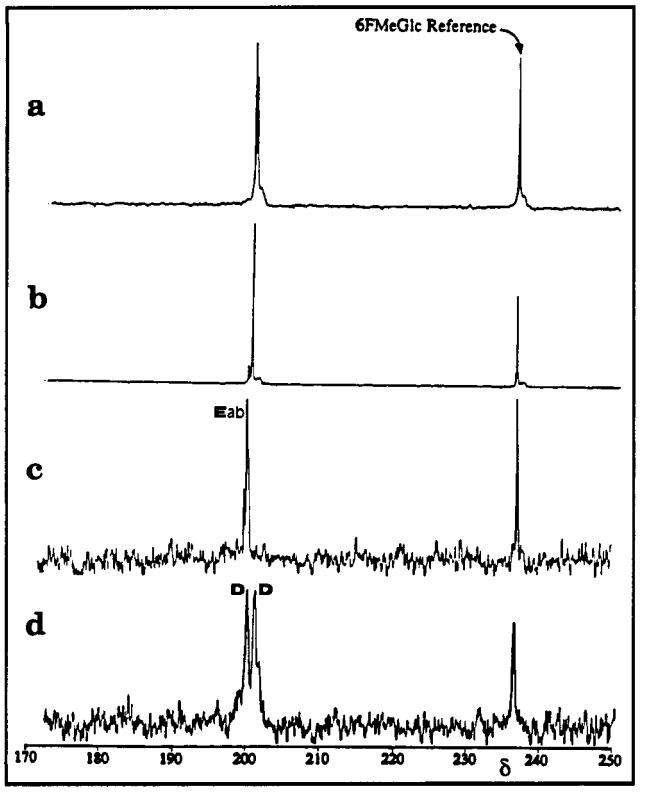

FIGURE 6: ^{19}F NMR titration of phosphoglucomutase with 2-fluoroglucose-1-P and Cd^{2+}. The reference employed in each sample was 6FMeGlc. (a) ^1H-decoupled spectrum of α/β-2-fluoroglucose-6-P; (b) ^1H-decoupled spectrum of 2-fluoroglucose-1-P; (c) phosphoglucomutase (1.0 mM) plus 2-fluoroglucose-1-P (1.0 mM) (^1H coupled); (d) phosphoglucomutase (1.0 mM) plus $Cd(OAc)_2$ (1.1 mM) and 2-fluoroglucose-1-P (1.0 mM) (^1H coupled). E = exchange-averaged resonance; a = α-2-fluoroglucose-6-P; b = β-2-fluoroglucose-6-P; D = bound diphosphate resonance.

The spectrum resulting from the addition of 1 equiv of phosphoenzyme to 2-fluoroglucose-1-P is shown in Figure 6c and comprises two resonances at 199.9 and 200.0 ppm which are assigned to free and bound α/β-2-fluoroglucose-6-P in fast exchange. Addition of Cd^{2+} causes the appearance of two resonances at 199.9 and 200.9 ppm (Figure 6d) which again are assigned to the two fluoroglucose-1,6-diP complexes. The whole experiment was repeated with 2-fluoroglucose-6-P in place of 2-fluoroglucose-1-P, and identical spectra (not shown) were obtained.

Discussion

The downfield chemical shift changes which accompany binding of 6-fluoroglucose-1-P and α-glucosyl fluoride-6-P are extremely large. Indeed, these are considerably larger than any previously reported for a fluorinated ligand binding to a macromolecule without a change in covalency, presumably reflecting the unusual, solvent-inaccessible environment in which the fluorine substituent is placed, directly adjacent to the enzymic phosphate. The large size of the chemical shift change rules out anisotropic ring currents as the major cause, although a fraction of the observed shift could result from this type of effect (Webb, 1986; Campbell et al., 1985). Likewise, hydrogen bonding is unlikely to be the cause of the large shift since inhibition studies (Percival & Withers, 1992) showed that at most only very minor hydrogen-bonding interactions occur between the 1- and 6-hydroxyls and the protein. The downfield shifts on binding of both ligands to the enzyme are consistent with shifts observed on other systems (Gerig, 1978, 1982) which have been construed to reflect a change to a hydrophobic (buried) environment (Sykes & Weiner, 1980). However, this may not be consistent with the structure of the active site of phosphoglucomutase since the fluorine would be expected to be very close to the enzymic phosphate groups as well as the activating metal ion (when present). The remainder of the active site probably consists of polar side chains which form the hydrogen bonds to the sugar ring hydroxyl groups (Percival & Withers, 1992); thus the environment into which the fluorine has been introduced is probably hydrophilic rather than hydrophobic. In fact, the previous suggestion that downfield shifts upon binding are due to hydrophobic effects may simply reflect the nature of the previously employed fluorinated probes (e.g., fluorotyrosine, fluorotryptophan, fluorouracil) which were primarily aromatic and would necessarily have bound in a hydrophobic region. The downfield shift may be a more general phenomenon associated with desolvation or closer van der Waals contacts. The most likely cause of the enormous shift observed in this case is the large electric field gradient (Kimber et al., 1977) which would be experienced by this fluorine located in *very* close proximity to the enzymic phosphate. The similarity of these two chemical shift changes indicates that the two fluorine atoms are bound in very similar environments, presumably directly adjacent to the enzymic phosphate. However, these shifts are not useful in distinguishing between the minimal motion and exchange mechanisms since similar environments for the fluorine of the two inhibitors would be expected in both cases.

Interpretation of the chemical shifts measured for the ligands bound to the Li^+–phosphoenzyme complex in terms of the mechanism of normal catalysis hinges somewhat upon the assumption that the Li^+–phosphoenzyme binds its two substrates (glucose-1-P and glucose-6-P) in modes that are closely similar to those of the competent Mg^{2+}–enzyme. Fortunately, there is good evidence for the similarity of these two complexes from UV/visible spectroscopy studies (Ma & Ray, 1980; Ray et al., 1990); thus the assumption is reasonable. One attempt to obviate this problem involved the use of difluorinated inhibitors which are incapable of turnover. Unfortunately, these ligands were not bound sufficiently tightly to the enzyme in the absence of Li^+ to provide useful chemical shift information on bound species, thus making it impossible to determine bound chemical shifts with the demetalated enzyme. However, the addition of an excess of Li^+ once again tightened the binding and caused large downfield shifts of both fluorine nuclei. In all cases the chemical shift changes upon binding for the difluorinated inhibitors are very similar to those measured for the corresponding monofluorinated species. This provides good evidence that these difluorinated inhibitors do, at least in the presence of Li^+, bind to the phosphoenzyme in similar modes to those of the monofluorinated glucose phosphates and concurs with the previous demonstration by UV/visible difference spectroscopy that these difluorinated inhibitors bind to the Mg^{2+}–enzyme in the same mode as glucose-1-P (Percival & Withers, 1992).

This change in binding behavior upon addition of Li^+ is consistent with a decrease in ligand dissociation constant of at least 2 orders of magnitude and a concomitant decrease in exchange rate. This is very much in agreement with earlier estimates of the effects of Li^+ upon the binding of substrate monophosphates (Ray et al., 1978) which showed that enzyme–substrate dissociation constants are decreased by almost 3 orders of magnitude in the presence of Li^+. It contrasts however with more recent ^7Li NMR studies (Rhyu et al., 1985a), which have indicated that the decreases in enzyme–substrate dissociation constants caused by the binding of Li^+

are only on the order of 10-fold.

Inspection of the data in Tables I and II reveals that the chemical shift changes measured for pairs of fluorinated sugar phosphates are quite different. Thus, the 3-fluoro substituent of 3-fluoroglucose-1-P undergoes a 3.8 ppm shift downfield while the same substituent on the 6-phosphate shifts 0.5 ppm upfield upon binding. Similarly, the 4-fluoro substituent of 4-fluoroglucose-1-P shifts 6.1 ppm downfield upon binding whereas the equivalent resonance for the 6-phosphate shifts only 2.7 ppm downfield. The data for the 2-position rest upon a comparison of shifts for 2,6-difluoroglucose-1-P and 2-fluoroglucose-6-P. Once again there is no correspondence since downfield shifts of 6.1 and 2.4 ppm, respectively, are observed. The fact that there does not appear to be a satisfactory correspondence of the two sets of shifts therefore argues strongly against identical sugar ring binding modes for the two sets of substrates, thus strongly against the minimal motion mechanism.

Complexes of Fluoroglucose Phosphates and Cd^{2+}. Previous work has shown that the replacement of Mg^{2+} by Cd^{2+} in phosphoglucomutase results in an enzyme with 1% of the activity of the native form (Ray, 1969). In addition, the presence of the Cd^{2+} changes the distribution of the species in the central complex (Ray & Long, 1976a). For the Mg^{2+}–enzyme, the dephosphoenzyme–Mg^{2+}–glucose-1,6-diP complex accounts for about half of the total enzyme with the monophosphate complexes accounting for the remainder. However, with Cd^{2+} present approximately 90% of the equilibrium mixture is the dephosphoenzyme–Cd^{2+}–glucose-1,6-diP complex. Rate constants for the interconversions of these species in the central complex have been determined recently by ^{31}P NMR saturation-transfer and isotope-transfer experiments (Post et al., 1989; Ray et al., 1989).

The addition of Cd^{2+} to complexes of phosphoglucomutase and the fluoroglucose phosphates is found to have profound effects upon the appearance of the spectra, resulting in the appearance of new resonances whose line widths ($\Delta\nu_{1/2} \approx 130$ Hz) are suggestive of tightly bound species which are assigned to the fluoroglucose-1,6-diP complexes. In the cases of the 2- and 3-fluoroglucose phosphates (Figures 5 and 6), addition of Cd^{2+} causes the appearance of two broad resonances, whereas in the case of the 4-fluoroglucose phosphates (Figure 4) a single broad resonance is observed. The pair of resonances observed for each of the 2- and 3-fluoroglucose phosphates is proposed to arise from the two forms of the dephosphoenzyme–Cd^{2+}–fluoroglucose-1,6-diP complexes required in the exchange mechanism. This assignment is based both upon the fact that such complexes have been shown to predominate with the Cd^{2+}–enzyme, and upon the line widths of these resonances which are consistent with the submicromolar dissociation constants inferred for such diphosphates (Percival & Withers, 1992). Furthermore, it is thought to be unlikely that these resonances arise from phosphoenzyme–Cd^{2+}–α,β-fluoroglucose-6-P complexes (of which there would be two) since Cd^{2+} is not known to cause a dramatic decrease in enzyme–*substrate* dissociation constants in the way that Li^+ does. Thus, observation of two such species for each of the 2- and 3-fluoroglucose diphosphates provides substantial further evidence in support of the exchange mechanism. It is not immediately obvious, however, why only a single enzyme-bound 4-fluoroglucose-1,6-diP species should be observed whereas two such species were observed for the 2- and 3-fluoroglucose diphosphates. This could be due to coincidence of resonances, although no hint of that is obtained upon inspection of chemical shifts for the bound (Li^+–phosphoenzyme) fluoroglucose 1- and 6-phosphates since there is an approximately 3–4 ppm difference between the shifts of *each* of these bound substrate pairs, making it less likely that two such resonances would be superimposed. Alternatively, it is possible that, for some reason, one of these complexes is much more stable than the other in the case of 4-fluoroglucose-1,6-diP such that only a single complex is observed. This would be well precedented since only a single dephosphoenzyme–glucose-1,6-diP complex was seen in previous ^{31}P NMR studies (Rhyu et al., 1984; Post et al., 1989), whether in the absence of metal ions or in the presence of Li^+ or Cd^{2+}. In that case it was suggested that the observed glucose-1,6-diP complex is that which is bound in the glucose-1-P mode, in accord with UV spectral studies (Ma & Ray, 1980) in which the spectrum of the dephosphoenzyme–Li^+–glucose-1,6-diP complex was found to be similar to that of phosphoenzyme–Li^+–glucose-1-P complex and based upon the fact that glucose-1-P binds the enzyme substantially better than glucose-6-P (K_d = 8.4 and 57 μM, respectively; Ray & Long, 1976b).

The shifts observed for the Cd^{2+}–fluoroglucose-1,6-diP complexes, and changes therein (relative to either the free diphosphate species or free fluoroglucose monophosphates) upon binding bear no obvious resemblance to changes in chemical shifts observed for the corresponding monophosphates upon binding to the Li^+–phosphoenzyme. Reasons for this are not obvious, but they may relate to different direct effects of the metal itself upon chemical shift, or to differences in the mode of binding of the sugar moieties in the two cases. The fact that such differences might exist would not be unreasonable given the difference in affinities of the monophosphate and diphosphate complexes and therefore the tightness of interaction of each hydroxyl or fluorine with its hydrogen-bonding partners or other neighbors.

Consequences for the Mechanism. The data obtained in this study are inconsistent with the minimal motion mechanism. The alternative, which is consistent with the data, is an exchange mechanism in which each glucose ring hydroxyl has its own enzymic binding subsite. This mechanism is supported by the NMR data since the fluorine substituents on pairs of fluoroglucose phosphates underwent different chemical shift changes upon binding, indicating different binding environments. In addition, the observation of what are likely two distinct Cd^{2+}–diphosphate complexes is heavily supportive of some type of exchange mechanism. Several different versions of the exchange mechanism have been forwarded which differ in the proposed modes of reorganization of the complexes. One (I. A. Rose, 1987, personal communication) involves the sugar diphosphate essentially pivoting about its 3-hydroxyl along a C-3 to O-5 axis such that the two phosphate moieties exchange places and the 2- and 4-hydroxyl groups interchange binding locations. Another version (W. J. Ray, 1986, personal communication) involves a similar rotation, but this time along an axis linking O-5 and the midpoint of the C-3 to C-4 bond such that the 3- and 4-hydroxyl groups interchange locations. It is tempting to attempt to test these hypotheses by looking for similar chemical shift changes for the corresponding fluoroglucose phosphates as a consequence of binding to the same locales; e.g., according to this approach the Rose mechanism would predict similar chemical shift changes upon binding for 2-fluoroglucose-1-P and 4-fluoroglucose-6-P since the two fluorines should bind to the same site. However, such a simplistic analysis is unjustified since the fluorine chemical shifts are undoubtedly dependent upon the orientation of the fluorine substituent with respect to its immediate environment, and the two fluorines

in this case would be oriented quite differently within the site. Thus, while the data are strongly supportive of an exchange mechanism, the exact nature of this exchange cannot be deduced from these data. It could be either of the proposed versions, or a different one again in which there are no common hydroxyl binding sites.

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Rotational Diffusion of the Erythrocyte Integral Membrane Protein Band 3: Effect of Hemichrome Binding†

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ABSTRACT: Human erythrocyte band 3 was covalently labeled within the integral membrane domain by incubating intact erythrocytes with the phosphorescent probe eosinyl-5-maleimide. The rotational diffusion of band 3 in membranes prepared from these labeled cells was measured using the technique of time-resolved phosphorescence anisotropy. Three rotational correlation times ranging from 16 to 3800 μs were observed, suggesting that band 3 exists in different aggregate states within the plane of the membrane. The oxidizing agent phenylhydrazine was used to induce hemichrome formation within intact erythrocytes. The immobilization of band 3 in membranes prepared from these erythrocytes suggests that the binding of hemichromes induces clustering of band 3. The addition of purified hemichromes to erythrocyte ghosts leads to a similar effect. We have also examined the mobility of the cytoplasmic domain of band 3. This region was labeled indirectly using a phosphorescently labeled antibody which binds to an epitope within the cytoplasmic domain. We observed very rapid motion of the cytoplasmic region of band 3, which was only partially restricted upon hemichrome binding. This suggests that the integral and cytoplasmic domains of band 3 may be independently mobile.

Aging of erythrocytes in vivo is accompanied by the gradual oxidation of hemoglobin and the formation of hemichrome-containing Heinz bodies below the membrane surface (Peisach et al., 1975; Jacob & Winterhalter, 1970). The hemichromes have a high affinity for the cytoplasmic domain of band 3, the major integral protein of the red cell membrane. It has been suggested that hemichromes cross-link band 3 molecules into clusters that constitute the so-called "senescence antigen"

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