Probing the ionization state of substrate α -D-glucopyranosyl phosphate bound to glycogen phosphorylase *b*

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The ionization state of the substrate α -D-glucopyranosyl phosphate bound at the active site of glycogen phosphorylase has been probed by a number of techniques. Values of K_i determined for a series of substrate analogue inhibitors in which the phosphate moiety bears differing charges suggest that the enzyme will bind both the monoanionic and dianionic substrates with approximately equal affinity. These results are strongly supported by ^{31}P - and ^{19}F -NMR studies of the bound substrate analogues α -D-glucopyranosyl 1-methylenephosphonate and 2-deoxy-2-fluoro- α -D-glucopyranosyl phosphate, which also suggest that

the substrate can be bound in either ionization state. The pH-dependences of the inhibition constants K_i for these two analogues, which have substantially different phosphate $\text{p}K_a$ values (7.3 and 5.9 respectively), are found to be essentially identical with the pH-dependence of K_m values for the substrate, inhibition decreasing according to an apparent $\text{p}K_a$ value of 7.2. This again indicates that there is no specificity for monoanion or dianion binding and also reveals that binding is associated with the uptake of a proton. As the bound substrate is not protonated, this proton must be taken up by the proton.

INTRODUCTION

Glycogen phosphorylase catalyses the reversible phosphorolysis of glycogen, generating glucose-1-*P* from glycogen plus phosphate. Despite considerable information on its three-dimensional structure in both the T- and R-states [1–4], as well as structures of various enzyme–substrate analogue complexes [5–8], along with decades of mechanistic studies (for reviews see refs. [9–11]), the catalytic mechanism is still unclear. Of particular interest has been the role of the coenzyme pyridoxal phosphate (PLP) in this mechanism. Numerous studies have been performed on the ionization state of the essential phosphate moiety of the cofactor at various stages of activation, most notably by the application of ^{31}P -NMR techniques. These have led to a number of proposals for its mode of action, including one in which it functions as a Bronsted acid/base catalyst, protonating the phosphate moiety of the substrate glucose-1-*P* on binding and thus assisting leaving-group departure [6,11]. This latter proposal was based primarily on results of ^{31}P -NMR studies of the enzyme in the presence of various activators and substrate analogues. However, other studies involving PLP analogues [12], as well as solid-state ^{31}P -NMR studies [13,14], have cast very serious doubt on the idea that the cofactor phosphate is involved in an essential proton transfer to the substrate phosphate moiety.

Relatively little attention has, however, been paid to the ionization state of the bound substrate, an equally important question as this is the group suggested to be protonated on binding. Previous attempts to determine ligand ionization states on binding to proteins have involved several different approaches. These have included binding studies using isosteric analogues of fixed charge [15,16], and also analogues of different $\text{p}K_a$ values in studies of the pH-dependence of binding [17,18]. They have also involved NMR studies of the binding of suitably NMR-active (and ionization-sensitive) nuclei to the proteins, in some cases

coupled with studies of the pH-dependence of the bound and free resonances [19]. Useful compounds for these kinds of study on glycogen phosphorylase would be analogues of glucose-1-*P* which are resistant to turnover or at least turn over only very slowly. These compounds should be as close in structure to glucose-1-*P* as possible, retaining a diabolic moiety, and bind with similar affinity. Ideally they should also contain at least one NMR nucleus which resonates in a region free of protein resonances. Two glucose-1-*P* analogues that have been used previously with glycogen phosphorylase are glucose-1,2-cyclic phosphate and 5-thioglucose-1-phosphate. The former compound, although it is a very close analogue of glucose-1-*P* in many ways and binds well to phosphorylase [20,21], nevertheless has only a monobasic phosphate moiety and thus cannot become dianionic. It is therefore useful for some of the experiments proposed, but not for others. It is also somewhat hydrolytically unstable, decomposing over long periods in the presence of the enzyme during ^{31}P -NMR experiments, and generating products that obscure enzymic resonances of interest (the PLP and, in the case of phosphorylase *a*, the serine phosphate) [21]. The latter compound is dibasic but has a ^{31}P -NMR spectrum that overlaps with protein resonances. It has, however, proved useful in X-ray-crystallographic studies of enzyme–substrate complexes [22].

Two analogues that would appear to be particularly suitable for such a study are 2-deoxy-2-fluoro- α -D-glucopyranosyl phosphate (2F α GlcP) and α -D-glucose-1-methylene phosphonate (1d α GlcCP), both of which are dibasic (Figure 1). The former compound is known to bind to phosphorylase with a similar affinity to that of glucose-1-*P* and to only turn over extremely slowly [23]; further, its mode of binding at the active site has been determined by X-ray crystallography [7]. In addition, it has two resolvable NMR-active nuclei, ^{31}P and ^{19}F , whose chemical shift could be expected to be sensitive to phosphate ionization state. The second analogue has been synthesized previously [24], but

Abbreviations used: glucose-1-*P*, α -D-glucopyranosyl phosphate; 1d α GlcCP, α -D-glucose-1-methylene phosphonate; 2F α GlcP, 2-deoxy-2-fluoro- α -D-glucopyranosyl phosphate; 2F α GlcP-F, 2-deoxy-2-fluoro- α -D-glucopyranosyl phosphorofluoridate; α ManP, α -D-mannopyranosyl phosphate; α ManP-F, α -D-mannopyranosyl phosphorofluoridate; DTT, dithiothreitol; AMPS, adenosine 5'-O-thiophosphate; PLP, pyridoxal phosphate; TEAD, buffer system comprising 50 mM triethanolamine, 100 mM KCl, 1 mM EDTA, 1 mM DTT.

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This paper is dedicated to the memory of Dr. Stephen Waley.

Figure 1 Structures of analogues useful for the study of the ionization states of enzyme–substrate complexes

tested only briefly as a competitive inhibitor of glycogen phosphorylase [7], although in the same study active-site binding was demonstrated by X-ray crystallography. The presence of the C–C bond at the anomeric centre renders it completely enzymically inert. Each compound individually should therefore be a useful probe of the substrate ionization state. In addition, as quite different pK_{a2} values can be expected for these two analogues, studies of the pH-dependence of their inhibition should provide further useful insights, especially when coupled with studies on other monoanionic sugar phosphates. Other compounds that should be useful are monobasic monoanionic analogues of glucose-1-*P*, such as the 1-phosphorofluoridate or 1,2-cyclic phosphate derivatives of glucose [8,16]. The present paper describes a detailed ^{31}P - and ^{19}F -NMR study of the interaction of these analogues with glycogen phosphorylase, the results of which, coupled with comparative studies of the pH-dependence of their inhibition and with studies of the binding of monoanionic substrate analogues, provide new insights into the ionization state of the substrate at the active site of the enzyme.

MATERIALS AND METHODS

Syntheses

The syntheses of the following compounds have been described previously: 2Fα-GlcP [25]; 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphorofluoridate (2FαGlcP-F) and α-D-mannopyranosyl phosphorofluoridate (αManP-F) [16]. Synthesis of 1dαGlcCP was achieved essentially by the method of Nicotra et al. [24], except that the bromodemercuriation step was replaced by an iododemercuriation step, with a very significant increase in yields resulting from the lack of unwanted removal of benzyl protecting groups, as follows.

(2,3,4,6-Tetra-*O*-benzyl-1-deoxy-α-D-glucopyranosyl) methyl iodide

To a solution of iodine (0.753 g) in 60 ml of dry methylene chloride, 2.1 g (2.7 mmol) of (2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl) methylmercuric chloride [26], was added and left stirring for 18 h at room temperature. Iodine was removed by washing with 10% Na_2SO_3 and 5% NaI followed by drying with Na_2SO_4 . After removal of the solvent, crystallization of the residue from methanol gave the product as a white solid (1.55 g, 91%); m.p. 87–88 °C; ^1H -NMR data ($[\text{H}]$ chloroform,

270 MHz): δ (p.p.m.) 7.34–7.10 (20 H, aromatic OBn), 4.89–4.47 (8 H, benzylic OBn), 4.15 (dt, 1 H, $J_{2,3}$ 4.3, $J_{2,1}$ 11.5, $J_{2,1'}$ 4.3 Hz, H-2), 3.80–3.49 (6 H, H-3, H-4, H-5, H-6, H-7, H-7'), 3.48 (AB multiplet, 2 H, $J_{1,1'}$ 11.5, $J_{1,2}$ 4.1, $J_{2,1'}$ 11.5 Hz, H-1, H-1').

Diethyl (2,3,4,6-tetra-*O*-benzyl-1-deoxy-α-D-glucopyranosyl) methylphosphonate

A solution of (2,3,4,6-tetra-*O*-benzyl-1-deoxy-α-D-glucopyranosyl) methyl iodide (1.5 g, 2.3 mmol) in triethylphosphite (17 ml) was heated at reflux for 2.5 h. The volatile products and excess reagent were removed by distillation under reduced pressure and the residue purified by flash chromatography (methylene chloride/ethyl acetate, 4:1, v/v) to give the product as a colourless oil (1.245 g, 80%). ^1H -NMR data ($[\text{H}]$ chloroform, 270 MHz): δ (p.p.m.) 7.31–7.12 (20 H, aromatic OBn), 4.90–4.45 (8 H, benzylic OBn), 4.09 (q, 2 H, J 8.0 Hz, $\text{O}-\text{CH}_2-\text{CH}_3$), 4.06 (q, 2 H, J 8.0 Hz, $\text{O}-\text{CH}_2-\text{CH}_3$), 4.01 (ddd, 1 H, $J_{2,3}$ 4.5, $J_{2,1}$ 7.6, $J_{2,1'}$ 3.2 Hz, H-2), 3.79–3.62 (6 H, H-3, H-4, H-5, H-6, H-7, H-7'), 2.20 (m, 2 H, $J_{2,1'}$ 7.6, $J_{2,1}$ 3.2, $J_{\text{H-P}}$ 18 Hz, H-1, H-1'), 1.29 (t, 3 H, J 8.2 Hz, $\text{O}-\text{CH}_2-\text{CH}_3$), 1.27 (t, 3 H, J 8.2 Hz, $\text{O}-\text{CH}_2-\text{CH}_3$).

Cyclohexylammonium (1-deoxy-α-D-glucopyranosyl) methylphosphonate

To a solution of diethyl (2,3,4,6-tetra-*O*-benzyl-1-deoxy-α-D-glucopyranosyl) methylphosphonate (0.982 g, 1.5 mmol) in dry carbon tetrachloride (0 °C) under dry nitrogen was added iodo-trimethylsilane (1.9 ml, 13.4 mmol) and stirring continued for 45 min. The reaction was quenched by the addition of 2 ml of water followed by removal of all solvents *in vacuo*. After repeated extraction with diethyl ether, the gum was redissolved in methanol and an excess of cyclohexylamine added. Addition of ether followed by cooling in a solid CO_2 /acetone bath precipitated the product which was isolated by filtration and quickly dried *in vacuo*. This procedure was repeated four times to give the product as a white amorphous solid (0.33 g, 62%). ^1H -NMR data ($^2\text{H}_2\text{O}$, 270 MHz): δ (p.p.m.) 4.30 (m, 1 H, $J_{2,3}$ 4.8, $J_{2,1}$ 4.0, $J_{2,1'}$ 10.1, $J_{2,P}$ 10.1 Hz, H-2), 3.76–3.26 (6 H, H-3, H-4, H-5, H-6, H-7, H-7'), 3.06 (m, 1 H, $\text{CH}-\text{NH}_3^+$), 1.89–1.07 (12 H, cyclohexyl, H-1, H-1'); ^{31}P -NMR data ($[\text{H}]_2\text{O}$ (20 mM), 109.29 MHz): δ (p.p.m.) 22.20 (m, $J_{\text{P,H-2}}$ 9.47, $J_{\text{P,H-1}}$ 27.55, $J_{\text{P,H-1'}}$ 27.19 Hz). Anal. Calc. for $\text{C}_{13}\text{H}_{28}\text{O}_8\text{PN}$: C, 43.70; H, 7.90; N, 3.92. Found: C, 43.28; H, 8.20; N, 3.70.

Enzymology

Rabbit muscle phosphorylase *b* was prepared by the method of Fischer and Krebs [27] using dithiothreitol (DTT) instead of cysteine and recrystallized at least three times before use. Protein concentration was determined from absorbance measurements at 280 nm by using the absorbance index $A_{1\%}^{1\text{cm}}$ of 13.2 [28]. Rabbit liver glycogen (type III) purchased from Sigma Chemical Co. was purified on a Dowex 1-Cl column and assayed by the method of Dishe [29]. All $^2\text{H}_2\text{O}$ used in NMR experiments was pretreated with Chelex 100 to remove bivalent metal ions. A Radiometer PHM 82 pH-meter was used for all pH measurements, and those measurements made in $^2\text{H}_2\text{O}$ are uncorrected.

NMR studies

^{31}P -NMR spectra were recorded at 109.29 MHz on a Bruker HX270 superconducting spectrometer operating in the Fourier-transform mode with quadrature phase detection, using a 10 mm ^{31}P probe at 28 °C. A spectral width of 6000 Hz was used with a 50–70° pulse angle (15–20 ms) and repetition times of 0.5–2 s depending on the experiment. Chemical shifts are quoted relative

to 85% orthophosphoric acid ($\delta = 0.00$ p.p.m.) and were measured with reference to a 100 mM sample of β -glycerophosphate ($\delta = 4.751$ p.p.m.) or orthophosphoric acid contained in a 1 mm capillary tube inserted into the sample during data acquisition.

^{19}F -NMR spectra were recorded at 254 MHz using a 5 mm ^{19}F -NMR probe. A spectral width of 10000 Hz was generally used and experiments were conducted using either broad-band proton decoupling or gated proton decoupling (decoupler on during acquisition only). A pulse angle of 50 – 70° (7–10 ms) with a repetition time of 100 ms was used during continuous decoupling, and a 90° pulse angle and a repetition delay of 3 s were used during gated decoupling. Chemical shifts are quoted relative to CFCl_3 ($\delta = 0.00$ p.p.m.) and were measured with reference to external hexafluorobenzene ($\delta = 162.90$ p.p.m.) contained in a concentric capillary.

The NMR titration of 'free' $1\alpha\text{GlcCP}$ was performed by dissolving a sample (20 mM) in 100 mM KCl (50% $^2\text{H}_2\text{O}$ /50% H_2O) then running ^{31}P -NMR spectra after adjusting the pH as desired over the range of pH values from 0.5 to 10.7. Probe temperature was maintained at 28°C .

Phosphorylase *b* was freed from AMP as described previously [30], and dialysed into TEAD buffer (50 mM triethanolamine, 100 mM KCl, 1 mM EDTA, 1 mM DTT, pH 6.8) containing 50% $^2\text{H}_2\text{O}$. For ^{31}P -NMR spectra, sample volumes were 1.4–1.6 ml, and solutions of effectors (pH adjusted to 6.8) were added directly to the enzyme solution giving the final concentrations shown in the Figure legends. For ^{19}F -NMR spectra, sample volume was 0.5 ml and enzyme concentrations between 0.6 and 0.68 mM were employed. Solutions of effectors were again added directly to the sample tube. Exponential line-broadening of 20 Hz was used before Fourier transformation, and all linewidth data quoted have been corrected for this

Kinetic studies

Initial reaction rates were determined by the Fiske–Subbarow phosphate analysis in the direction of saccharide synthesis as described by Engers [31]. The same TEAD buffer as was employed for the NMR experiments was used. Reaction mixtures were 0.2 or 0.5 ml containing 1 mM AMP and 1% glycogen, and studies were performed at pH 6.8 and 30°C , except in the case of pH variation. In these cases the same buffer system was used and the desired pH values were attained by addition of small volumes of 2 M HCl or 2 M NaOH. Final pH values of 5.40, 5.52, 5.73, 5.89, 6.09, 6.55, 6.83, 7.13 and 7.53 were recorded directly in mixtures containing all the reagents except glycogen phosphorylase. Equilibrium constants for the phosphorylase reaction at each pH value were kindly supplied by Dr. N. B. Madsen (University of Alberta), and all data have been corrected for this. Approximate values for inhibition constants (K_i) were determined by measuring initial reaction rates at a constant concentration of glucose-1-P (2.8 mM), AMP (1 mM) and glycogen (1%) in the presence of the following inhibitor concentrations: $2\text{F}\alpha\text{GlcP}$ 0, 1.1, 2.2, 3.3, 4.4, 5.5 mM; $1\alpha\text{GlcCP}$, 0, 0.50, 0.99, 1.98, 2.97, 4.95 mM. Accurate determinations of inhibition constants were made using the same range of inhibitor concentrations, but at a series of substrate concentrations. Data were analysed by use of the non-linear regression program GraFit [32].

RESULTS

Kinetic studies

Inhibition constants for a series of glucose-1-P and mannose-1-P analogues are shown in Table 1. As indicated, inhibition was

Table 1 Reversible inhibition of glycogen phosphorylase *b* by substrate analogues

Inhibitor	K_i (mM)	Inhibition type
$1\alpha\text{GlcCP}$	0.7	Competitive
$2\text{F}\alpha\text{GlcP}^*$	2.0	Competitive
$2\text{F}\alpha\text{GlcP-F}$	6	N.D.†
$\alpha\text{ManP-F}$	16	Competitive
$\alpha\text{ManP}^\ddagger$	35	Competitive

* Taken from Street et al. [23].

† Full kinetic study not performed.

‡ Taken from Sprang et al. [33].

Figure 2 pH-Dependence of K_i values for $2\text{F}\alpha\text{GlcP}$ and $1\alpha\text{GlcCP}$

○, $2\text{F}\alpha\text{GlcP}$; ●, $1\alpha\text{GlcCP}$. Approximate values for inhibition constants (K_i) were determined by measuring initial reaction rates at a constant concentration of glucose-1-P (2.8 mM), AMP (1 mM) and glycogen (1%) in the presence of various concentrations of the inhibitor. The buffer systems used for the different pH ranges are described in the Materials and methods section.

competitive with substrate glucose-1-P in each case studied, although unfortunately the small quantities of material available precluded such a study in the case of $2\text{F}\alpha\text{GlcP-F}$. Inhibition constants for two of these compounds, $2\text{F}\alpha\text{GlcP}$ and $1\alpha\text{GlcCP}$, were also determined over a range of pH values within the pH-stability range of the enzyme and these results are shown in the form of a plot of $-\log K_i$ against pH in Figure 2. Possible substrate activity of $2\text{F}\alpha\text{GlcP-F}$ was examined by incubating the analogue (10 mM) with a large quantity of glycogen phosphorylase (10.2 mg) in the presence of the activator AMP (1 mM) and second substrate glycogen (0.5%). After 48 h this sample was examined by ^{19}F -NMR for the appearance of a resonance due to 2-deoxy-2-fluoro-substituted glycogen, as has been seen previously on reaction with $2\text{F}\alpha\text{GlcP}$ under similar conditions [23]. No such resonance was observed, indicating that no significant reaction occurred and putting a limit on the rate of any reaction that may occur of at least 10 times slower than that with $2\text{F}\alpha\text{GlcP}$, which itself was very slow ($V_{\text{max}} = 2.8 \times 10^4 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$).

NMR studies

The pH-dependence of the ^{31}P -NMR chemical shift for the inhibitor $1\alpha\text{GlcCP}$ free in solution is shown in Figure 3. Fitting of these data to a titration curve using the program GraFit [32] yielded values of $\text{p}K_1 = 2.0$ and $\text{p}K_2 = 7.3$ for the two ionizations

Figure 3 pH-Dependence of ^{31}P -NMR chemical shift for $1\text{d}\alpha\text{GlcCP}$

The NMR titration of $1\text{d}\alpha\text{GlcCP}$ was performed by dissolving a sample (20 mM) in 100 mM KCl (50% $^2\text{H}_2\text{O}/50\%$ H_2O) and acquiring the ^{31}P -NMR spectra after adjusting the pH. Probe temperature was maintained at 28 °C.

Figure 4 ^{31}P -NMR spectra of phosphorylase *b* in the presence of $1\text{d}\alpha\text{GlcCP}$ and cofactors

(a) Phosphorylase *b* (0.99 mM) plus AMPS (1.84 mM) and $1\text{d}\alpha\text{GlcCP}$ (0.91 mM); (b) phosphorylase *b* (0.96 mM) plus AMPS (1.78 mM), $1\text{d}\alpha\text{GlcCP}$ (0.88 mM) and glycogen (0.5%); (c) phosphorylase *b* (0.89 mM) plus AMPS (1.71 mM) and $1\text{d}\alpha\text{GlcCP}$ (4.88 mM). All spectra represent the average of 10 000 transients.

of the phosphonate ester, with chemical shift values of about 28, 22 and 18 p.p.m. for the neutral, monoanionic and dianionic species respectively.

^{31}P -NMR spectra of the complex of this inhibitor with glycogen phosphorylase are shown in Figure 4. Figure 4(a) shows the

Figure 5 Binding of $1\text{d}\alpha\text{GlcCP}$ to phosphorylase *b*: a plot of $1/\Delta\delta$ versus total $1\text{d}\alpha\text{GlcCP}$ concentration

The sample contained 1 mM phosphorylase *b*, AMPS (2 mM) and 0.5% glycogen. The chemical-shift values for the exchange-averaged signal from the free and enzyme-bound $1\text{d}\alpha\text{GlcCP}$ were recorded at various concentrations of the inhibitor. The change in chemical shift ($\Delta\delta$) is expressed relative to the chemical shift of $1\text{d}\alpha\text{GlcCP}$ obtained in the absence of phosphorylase *b* under identical buffer conditions.

spectrum of phosphorylase *b* plus the nucleotide activator AMPS (2 equiv.) and the inhibitor $1\text{d}\alpha\text{GlcCP}$ (1 equiv.). Under these conditions AMPS is clearly quite tightly bound in the slow exchange regime, as evidenced by the separate signals for free and bound AMPS. This is consistent with the inhibitor stabilizing the activated R-state conformation of the enzyme. A single resonance only is seen for $1\text{d}\alpha\text{GlcCP}$, suggesting that binding is in the medium-to-fast exchange regime, as expected for an inhibitor of this affinity ($K_i = 0.7$ mM). The resonance for PLP comprises two components, one at $\delta = 3.9$ p.p.m. ($\Delta\nu_{1/2} = 120$ Hz) and a smaller component at $\delta = 0.5$ p.p.m. ($\Delta\nu_{1/2} = 290$ Hz). The consequences of adding glycogen to this sample are seen in Figure 4(b), when the resonances for AMPS now appear as a single broad exchange-averaged signal at $\delta = 41.0$ p.p.m., but with no apparent effect on the signal for $1\text{d}\alpha\text{GlcCP}$. The proportion of the upfield component of the PLP spectrum appears to increase slightly on glycogen addition. Addition of excess $1\text{d}\alpha\text{GlcCP}$ to this sample (Figure 4c) restored the AMPS signals to separate resonances and apparently further increased the proportion of the broader upfield component. The small sharp signal at 3.3 p.p.m. is due to AMP formed by the slow hydrolysis of AMPS after prolonged incubation. NMR spectra of this system were also accumulated at a series of concentrations of $1\text{d}\alpha\text{GlcCP}$ in the presence of an essentially constant concentration of enzyme (1 mM), AMPS (2 mM) and glycogen (0.5%) (spectra not shown). Within such a series a small progressive upfield shift of the exchange-averaged resonance due to free and bound $1\text{d}\alpha\text{GlcCP}$ is seen as the percentage of the bound ligand increases, and the results of this experiment are presented in Figure 5 as a plot of $1/\Delta\delta$ versus $[1\text{d}\alpha\text{GlcCP}]$. This plot yields a value (mean $\pm$ S.E.M.) for K_d of 0.6 ± 0.4 mM, which is consistent with the value of $K_i = 0.7$ mM obtained from the kinetic studies. It also gives a limiting chemical shift for the fully bound ligand of 21.30 p.p.m., representing a total upfield chemical-shift change on binding of 0.25 p.p.m.

The pH-dependence of the ^{19}F -NMR chemical shift for the inhibitor $2\text{F}\alpha\text{GlcP}$ free in solution has been determined previously by both potentiometric and NMR titrations [25], and yielded values of $\text{p}K_1 = 0.3$ and $\text{p}K_2 = 5.9$ for the two ionizations observed, which are assigned as those of the phosphate ester. The resonances from the monoanionic and dianionic species were shifted 1.3 and 2.5 p.p.m. downfield relative to the resonance

Figure 6 Proton-decoupled ^{19}F -NMR spectra of $2\text{F}\alpha\text{GlcP}$ binding to phosphorylase *b*

(a) Phosphorylase *b* (0.68 mM), $2\text{F}\alpha\text{GlcP}$ (0.8 mM), 45 000 transients; (b) phosphorylase *b* (0.68 mM), $2\text{F}\alpha\text{GlcP}$ (1.6 mM), 45 000 transients; (c) phosphorylase *b* (0.63 mM), $2\text{F}\alpha\text{GlcP}$ (2.2 mM), 15 000 transients.

position of the neutral species. There is therefore a substantial difference in chemical shifts between the monoanionic and dianionic species.

The ^{19}F -NMR spectra of the complexes of phosphorylase with $2\text{F}\alpha\text{GlcP}$ are shown in Figure 6. A small amount (20% in total) of the β -anomer of this compound, which is known not to bind to phosphorylase, was included in this sample in order to provide an internal chemical shift ($\delta = 198.7$ p.p.m.) and linewidth reference. A single resonance is observed for the free and bound $2\text{F}\alpha\text{GlcP}$ ($\delta = 199.2$ p.p.m.) in each case, indicating an intermediate-to-fast exchange regime. The linewidth of this resonance is observed to increase as the percentage of the ligand bound increases, but with no detectable change in chemical shift. Importantly, there is no such change in the signal from the β -anomer, indicating that the changes observed for the α -anomer are due to binding to the enzyme, and not the consequence of changes in viscosity. The signal at $\delta = 200.2$ p.p.m. in Figures 6(b) and 6(c) arises from 2-deoxy-2-fluoroglycogen formed due to the presence of small quantities of contaminating glycogen copurified with the enzyme [23].

DISCUSSION

Comparison of binding affinities of phosphate-containing ligands with those of ligands in which the phosphate moiety is forced to be monoanionic through the conservative substitution of an ionizable hydroxyl has previously provided clear answers to questions regarding the ionization states of bound ligands. For example, the phosphorofluoridate analogue of AMP was found to bind to glycogen phosphorylase *b* some 65-fold more weakly (corresponding to a $\Delta\Delta G^\circ$ value of $10.5 \text{ kJ}\cdot\text{mol}^{-1}$) than did the parent compound [34]. Similarly, phosphorofluoridate analogues of glucose-1-P and glucose 6-phosphate were found to bind 75–150 times more weakly to phosphoglucomutase (corresponding to an average $\Delta\Delta G^\circ$ value of approx. $11.3 \text{ kJ}\cdot\text{mol}^{-1}$) than

the corresponding substrates [16]. These results, coupled with the inactivity of the complexes so derived, clearly indicated that the dianionic form of the ligand was the active bound species in both these cases. Equivalent studies with glycogen phosphorylase did not provide such clear results. First, synthesis of the preferred monoanionic analogue, α -D-glucopyranosyl phosphorofluoridate, proved impossible because of the facile reaction of its 2-hydroxyl with the activated phosphate moiety yielding glucose-1,2-cyclic phosphate [16]. This necessitated the synthesis and use of phosphorofluoridate analogues that could not undergo this intramolecular cyclization reaction, namely the manno- and 2-deoxy-2-fluorogluco-derivatives. Studies with these analogues revealed similar affinities for the phosphorofluoridates and their corresponding dibasic parents, suggesting that either only the monoanionic species is bound or the enzyme binds monoanions and dianions with approximately equal affinity. However, should the former be the case, it might be expected that $2\text{F}\alpha\text{GlcP-F}$ would be bound considerably more tightly than $2\text{F}\alpha\text{GlcP}$ ($\text{p}K_a = 5.9$) rather than three times less tightly. The simplest conclusion is therefore that the enzyme can bind the monoanionic and dianionic forms with similar affinities.

These conclusions are supported by the results of the study of the pH-dependence of binding of two of these ligands. As seen in Figure 2, an essentially identical dependence of K_i on pH is seen above pH 6 for both $2\text{F}\alpha\text{GlcP}$ and $1\text{d}\alpha\text{GlcCP}$. Fitting of both sets of data to an equation for a single ionization [32] suggests a dependence on apparent $\text{p}K_a$ values of 7.1 and 7.2 respectively, essentially identical with that of $\text{p}K_a = 7.2$ found previously for the K_m value of glucose-1-P [12]. Previous work [31] has shown that the substrate K_m in this system is a true dissociation constant, thereby validating this comparison.

The increase in K_i value below pH 6 for $2\text{F}\alpha\text{GlcP}$ is of interest and probably arises from replacement of the substrate 2-hydroxyl by fluorine as no such effect is seen with $1\text{d}\alpha\text{GlcCP}$ or with substrate glucose-1-P. The X-ray-crystal structure of the $2\text{F}\alpha\text{GlcP}$ –phosphorylase *b* complex [7] provides a clue to the source of this induced pH-dependence. Interaction of Glu-672 with the 2-position was abolished on replacement of the 2-hydroxyl by fluorine because the side chain of Glu-672 moves significantly away from the sugar in the structure with $2\text{F}\alpha\text{GlcP}$. Previous work [23] has suggested that Glu-672 is deprotonated in this complex, the hydrogen bond to the 2-hydroxyl keeping it deprotonated across the pH range. On removal of this interaction it is feasible that the side chain might then be protonated at lower pH values, decreasing the affinity of the $2\text{F}\alpha\text{GlcP}$ as seen. This lower pH ionization ($\text{p}K_a = 5.5$) may therefore be that of Glu-672. The $\text{p}K_a$ value observed is certainly within the range expected and seen previously for glutamic and aspartic acid side chains in proteins.

The NMR studies performed also suggest that the substrate can be bound in either ionic form. The chemical shift of free $1\text{d}\alpha\text{GlcCP}$ was shown to be sensitive to the ionization state of the phosphonate moiety, yet binding of this ligand to the protein in the presence of activator AMP and substrate glycogen results in only a very small ($\Delta\delta = 0.25$ p.p.m.) upfield chemical shift of its resonance. Assuming that this shift indeed reflects a change in ionization state of the phosphonate moiety, this would imply a slight increase in amount of the dianionic form of the ligand. However, as there is a difference of approx. 4 p.p.m. between the chemical shifts of the mono- and di-anionic forms of the free ligand, this change in dianion concentration on binding is very small. Further, as in free solution at pH 6.8 some 71% of $1\text{d}\alpha\text{GlcCP}$ is present as the monoanionic species, this would reflect a small shift towards binding of the two species with roughly equivalent affinities. As has been noted previously,

however, [35] interpretation of ^{31}P -NMR shifts is a hazardous occupation, thus further confirmation of the above result was required.

Such confirmation was obtained through a ^{19}F -NMR study of the binding of $2\text{F}\alpha\text{GlcP}$ to phosphorylase, again in the presence of substrate glycogen and activator AMP. This kind of study should be quite informative because, although the ^{19}F -NMR chemical shift is probably sensitive to the ionization state of the adjacent phosphate, it should be relatively insensitive to factors such as distortion of P–O bond angle, factors that would severely affect the ^{31}P -NMR chemical shift and compromise the interpretation. The study requires that the ^{19}F -NMR chemical shift of $2\text{F}\alpha\text{GlcP}$ be sensitive to the ionization state of the phosphate. This indeed is known to be the case because a substantial difference in chemical shift ($\Delta\delta = 1.2$ p.p.m.) is seen on conversion from monoanion into dianion [25]. It further requires that changes in ^{19}F -NMR chemical shift on binding due to local environmental effects or substrate distortion, rather than changes in ionization state, be minimal. The three-dimensional structure of the phosphorylase b - $2\text{F}\alpha\text{GlcP}$ complex [7] revealed no such substrate distortion, and in order to provide some measure of whether local environmental effects might be important, the binding of 2-deoxy-2-fluoroglucose to phosphorylase was monitored by NMR (results not shown). No change in ^{19}F -NMR chemical shift was observed, although linewidth changes were observed that were consistent with the binding of the ligand. This implies that any changes in chemical shift observed for $2\text{F}\alpha\text{GlcP}$ probably reflect true differences in ionization state.

Titration of phosphorylase b with $2\text{F}\alpha\text{GlcP}$ resulted in linewidth changes for the ligand that were consistent with its binding to the protein. However, no significant change in chemical shift, which must be a weighted average of that for the free and bound forms, was observed, suggesting that no significant change in the ionization state of the ligand occurs on binding. As the titration was performed at pH 6.8 and the pK_a of $2\text{F}\alpha\text{GlcP}$ is 5.9 [25] (by way of comparison, the pK_a of glucose-1- P is 6.1 [36]), the ligand must exist in free solution primarily in its dianionic form and the data suggest that it is also bound to the enzyme primarily in that form under these conditions. It would therefore appear that glycogen phosphorylase can bind substrate glucose-1- P in either its monoanionic or dianionic forms, with no great discrimination between the two.

These results are incompatible with the hypothesis that PLP and the substrate phosphate are involved in a proton-transfer relay, at least in the ground-state enzyme–substrate complex, as no appreciable proton donation was observed by ^{19}F -NMR in the $2\text{F}\alpha\text{GlcP}$ -enzyme complex. However, it does not exclude the possibility that reaction occurs *via* a small percentage of the protonated substrate or that extensive proton donation occurs only at the transition state. Nonetheless, as the proton-shuttle hypothesis was based largely on results obtained from ^{31}P -NMR studies performed on ground-state enzyme–ligand complexes, although this study revealed no proton transfer under equivalent conditions, some doubt is cast on the interpretations of those experiments. A second line of evidence against the proton-shuttle hypothesis comes from the ^{31}P -NMR studies on the $1\alpha\text{GlcCP}$ -phosphorylase–glycogen complex. Formation of this complex is accompanied by changes in the PLP resonance that are essentially identical with those occasioned by the binding of glucose-1,2-cyclic phosphate [21] or pyridoxal 5'-diphosphate glucose [37] and were interpreted as reflecting either the formation of a protonated coenzyme phosphate in the intermediate exchange regime, or a constrained and distorted dianionic coenzyme phosphate moiety [21,37]. Klein et al. [38] argued that, as glucose-1,2-cyclic phosphate has a very low pK_a value and thus

could not be protonated at these pH values, the results obtained were not representative of the situation with normal substrate. However, observation of essentially identical results with the $1\alpha\text{GlcCP}$ (pK_a 7.3) indicates that this is not the case. Further, it is highly unlikely that the shifting and broadening of the coenzyme phosphate resonance on binding of substrate analogues observed here or elsewhere [38] is due to proton transfer between the two phosphates, given that extensive proton transfer could not occur with the compounds of lower pK_a . If the broad resonance observed for the coenzyme phosphate in the presence of substrate does reflect an exchangeable protonated phosphate, the exchange cannot be with the substrate, but must be with other residues in the active site.

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REFERENCES

- 1 Sprang, S. R. and Fletterick, R. J. (1979) *J. Mol. Biol.* **131**, 523–551
- 2 Weber, I. T., Johnson, L. N., Wilson, K. S., Yeates, D. G. R., Wild, D. L. and Jenkins, J. A. (1978) *Nature (London)* **274**, 433–437
- 3 Barford, D., Hu, S.-H. and Johnson, L. N. (1991) *J. Mol. Biol.* **218**, 233–260
- 4 Sprang, S. R., Withers, S. G., Goldsmith, E. J., Fletterick, R. J. and Madsen, N. B. (1991) *Science* **254**, 1367–1371
- 5 Goldsmith, E. J., Sprang, S. R., Hamlin, R., Xuong, N.-H. and Fletterick, R. J. (1989) *Science* **245**, 528–532
- 6 Johnson, L. N., Acharya, K., Jordan, M. D. and McLaughlin, P. J. (1990) *J. Mol. Biol.* **211**, 645–661
- 7 Martin, J. L., Johnson, L. N. and Withers, S. G. (1990) *Biochemistry* **29**, 10745–10757
- 8 Withers, S. G., Madsen, N. B., Sprang, S. R. and Fletterick, R. J. (1982) *Biochemistry* **21**, 5372–5382
- 9 Fletterick, R. J. and Sprang, S. R. (1982) *Acc. Chem. Res.* **15**, 361–369
- 10 Madsen, N. B. and Withers, S. G. (1986) in *Coenzymes and Cofactors: Pyridoxal Phosphate and Derivatives*, pp. 355–389, Wiley, New York
- 11 Palm, D., Klein, H. W., Schinzel, R., Buehner, M. and Helmreich, E. J. M. (1990) *Biochemistry* **29**, 1099–1107
- 12 Withers, S. G., Shechosky, S. and Madsen, N. B. (1982) *Biochem. Biophys. Res. Commun.* **108**, 322–328
- 13 Challoner, R., McDowell, C. A., Stirtan, W. and Withers, S. G. (1993) *Biophys. J.* **64**, 484–491
- 14 Taguchi, J. E., Heyes, S. J., Barford, D., Johnson, L. N. and Dobson, C. M. (1993) *Biophys. J.* **64**, 492–501
- 15 Robertson, H. E. and Boyer, P. D. (1956) *Arch. Biochem. Biophys.* **62**, 380–395
- 16 Percival, M. D. and Withers, S. G. (1992) *J. Org. Chem.* **57**, 811–817
- 17 Yount, R. G. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol.* **43**, 1–56
- 18 Blackburn, G. M., Kent, D. E. and Kolkmann, F. (1981) *J. Chem. Soc. Chem. Commun.*, 930–932
- 19 Campbell, I. D., Jones, R. B., Kiener, P. A. and Waley, S. G. (1979) *Biochem. J.* **179**, 607–621
- 20 Hu, H. Y. and Gold, A. M. (1978) *Biochim. Biophys. Acta* **525**, 55–60
- 21 Withers, S. G., Madsen, N. B. and Sykes, B. D. (1981) *Biochemistry* **20**, 1748–1756
- 22 Madsen, N. B., Kasvinsky, P. J. and Fletterick, R. J. (1978) *J. Biol. Chem.* **253**, 9097–9101
- 23 Street, I. P., Rupitz, K. and Withers, S. G. (1989) *Biochemistry* **28**, 1581–1587
- 24 Nicotra, F., Ronchetti, F. and Russo, G. (1982) *J. Org. Chem.* **47**, 4459–4462
- 25 Withers, S. G., MacLennan, D. J. and Street, I. P. (1986) *Carbohydr. Res.* **154**, 127–144
- 26 Pougny, J. R., Nassr, M. A. M. and Sinay, P. (1981) *J. Chem. Soc. Chem. Commun.*, 375–376
- 27 Fischer, E. H. and Krebs, E. G. (1962) *Methods Enzymol.* **5**, 369–373
- 28 Buc, M. H. and Buc, H. (1968) in *Symposium on Regulation of Enzyme Activity and Allosteric Interactions*, p. 109, Academic Press, New York
- 29 Ashwell, G. (1957) *Methods Enzymol.* **3**, 73–105
- 30 Withers, S. G., Sykes, B. D., Madsen, N. B. and Kasvinsky, P. J. (1979) *Biochemistry* **18**, 5342–5348
- 31 Engers, H. D., Shechosky, S. and Madsen, N. B. (1970) *Can. J. Biochem.* **48**, 746–754
- 32 Leatherbarrow, R. J. (1990) *GraFit*, Version 2.0, Erithacus Software Ltd., Staines
- 33 Sprang, S., Goldsmith, E. J., Fletterick, R. J., Withers, S. G. and Madsen, N. B. (1982) *Biochemistry* **21**, 5364–5371

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- 34 Withers, S. G. and Madsen, N. B. (1980) *Biochem. Biophys. Res. Commun.* **97**, 513–519
- 35 Gorenstein, D. (1984) in *Phosphorus-31 NMR*, p. 7, Academic Press, San Francisco
- 36 O'Connor, J. V. and Barker, R. (1979) *Carbohydr. Res.* **73**, 227–243
- 37 Withers, S. G. (1985) *J. Biol. Chem.* **260**, 841–845
- 38 Klein, H. W., Im, M. J., Palm, D. and Helmreich, E. J. M. (1984) *Biochemistry* **23**, 5853–5861
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