THE STEREOSPECIFICITY OF THE GLUCOSE-6-PHOSPHATE BINDING SITE OF GLYCOGEN PHOSPHORYLASE b

M. K. BATTERSBY and G. K. RADDA
Department of Biochemistry, University of Oxford, Oxford, England

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1. Introduction

Kinetic studies under conditions simulating those in muscle [1] suggest that the activity of glycogen phosphorylase b is dependent on the intracellular concentrations of the activator, AMP and inhibitors, glucose-6-phosphate and ATP. More detailed studies indicate that the inhibition by glucose-6-phosphate is a competitive effect with respect to AMP, as a result of inhibitor induced conformational changes at the AMP binding site [2–4]. The interaction between phosphorylase b and glucose-6-phosphate is, therefore, of importance in the control of glycogenolysis and, with the sugar phosphate being located at the branch point of important metabolic pathways, is of particular interest since its effect on phosphorylase b controls glucose-6-phosphate production via phosphoglucomutase.

There have been several reports of anomeric specificity of enzymes acting on glucose-6-phosphate (5–8) and the question has been raised as to whether rate-limiting anomerizations occur, providing a role for mutarotases in carbohydrate metabolism [8].

In this paper we describe the use of ^{31}P NMR to study the binding of glucose-6-phosphate to phosphorylase b in order to determine the anomeric specificity of the binding site. This method can also be applied in a general way to follow specific binding of phosphate containing compounds where, in solution, mixtures of isomers or several derivatives are present.

2. Materials and methods

Phosphorylase b was prepared according to the method of Fischer and Krebs [9], substituting dithiothreitol for cysteine and was crystallised twice before use.

The preparation of spin-labelled phosphorylase b, the method of titration and the treatment of results have been described previously [10].

All experiments were carried out in a buffer consisting of 50 mM triethanolamine, 100 mM potassium chloride, 1 mM EDTA, at pH 7.0.

ESR measurements were made on a Varian E-line Century Spectrometer at ambient temperature (18°C).

^{31}P NMR spectra were taken on a Brucker WH-90 in the Fourier Transform mode at 36.43 MHz (2.1 Tesla) using an external D_2O lock. Broad band proton decoupling now used throughout (ambient temperature was 27°C).

Glucose-6-phosphate and fructose-6-phosphate were obtained from Sigma Chemical Co.

3. Results and discussion

At pH 7.0 the ^{31}P NMR spectrum of glucose-6-phosphate consists of two resonances (fig.1(a)). Integrating the areas under the two peaks shows them to be in the ratio of approximately 60% : 40%. The larger of the resonances is 3.297 ppm downfield from phosphoric acid and the smaller is separated from the larger by an upfield chemical shift of 2.45 Hz (0.066 ppm). The linewidth of both resonances is less than 1 Hz.

On the basis of the anomeric specificity of glucose-6-phosphate dehydrogenase for the β-anomer of glucose-6-phosphate [5], Bailey et al. [8] determined the anomeric composition of glucose-6-phosphate in

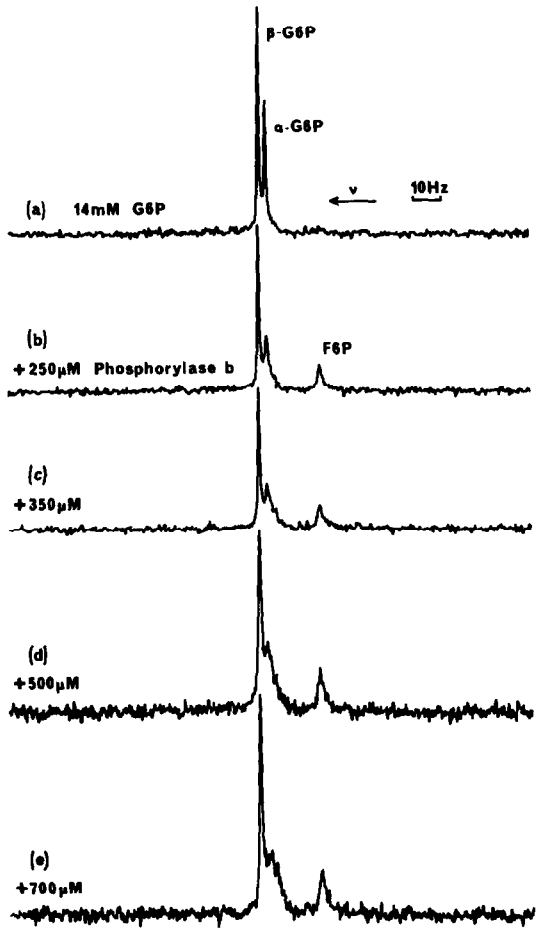

Fig.1. (a–e). The effect of increasing concentrations of phosphorylase b on the ^{31}P NMR spectrum of 14 mM glucose-6-phosphate in 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1 mM EDTA, pH 7.0, 2000 at 70° pulses were applied every 4.1 s for each spectrum. The arrow shows the direction of increasing frequency.

solution as 38.5% α-glucose-6-phosphate and 61.5% β-glucose-6-phosphate. Thus we assign the larger resonance to the β-anomer, and the smaller to the α-anomer [11].

Figure 1 (b–e) shows the effect of increasing concentrations of glycogen phosphorylase b on the ^{31}P NMR spectrum of glucose-6-phosphate. While the β-glucose-6-phosphate resonance is essentially unaffected, the α-glucose-6-phosphate resonance shows marked broadening as the concentration of the enzyme is increased.

This broadening is interpreted as the result of a reduction in the transverse relaxation time of the phosphorus nucleus of the α-anomer when bound to the enzyme molecule. The resonance of the β-anomer is only slightly broadened (a change of linewidth of 0.3 Hz is observed between the initial and final spectra). This change is small in comparison with the broadening of the α-anomer resonance, and is a result of increased viscosity and of the slight drift in field homogeneity during the course of the titration, which takes about 12 h in all.

The differential effect of increasing enzyme concentrations on the resonances of the two anomers shows that the enzyme interacts with α-glucose-6-phosphate only, the binding site being stereospecific so as to exclude interaction with the β-anomer.

Contamination by small amounts of other enzymes becomes significant in experiments at the high phosphorylase concentrations used here. The resonance at 0.59 ppm upfield from the β-glucose-6-phosphate signal coincides with that of fructose-6-phosphate. The presence of glucose-phosphate isomerase as a contaminant was established by its ability to form glucose-6-phosphate from fructose-6-phosphate, added as substrate, as observed by ^{31}P NMR. This side reaction, however, does not affect the conclusions and allowance can be made, by integration of the spectra, for the conversion of a small amount of glucose-6-phosphate to fructose-6-phosphate. It is of interest to note that five-fold recrystallisation from 30 mM dithiothreitol, 1 mM AMP, 10 mM magnesium acetate failed to remove the glucosephosphate isomerase contamination from phosphorylase b.

We have shown that the dissociation constants of ligands for phosphorylase can be measured using a spin-label specifically attached to a sulphydryl group on the enzyme [10,12]. This method allows the dissociation constant for the α-anomer of glucose-6-phosphate to be determined. Figure 2 shows four superimposed titrations of spin-labelled phosphorylase b with glucose-6-phosphate. The change in the ESR spectrum is shown as the ratio of the heights of the low-field to centre peaks (the ESR ratio, R) which has been shown to be an index of the mobility of the spin-label [13].

The curve is calculated by computer assuming that the following equilibria hold –

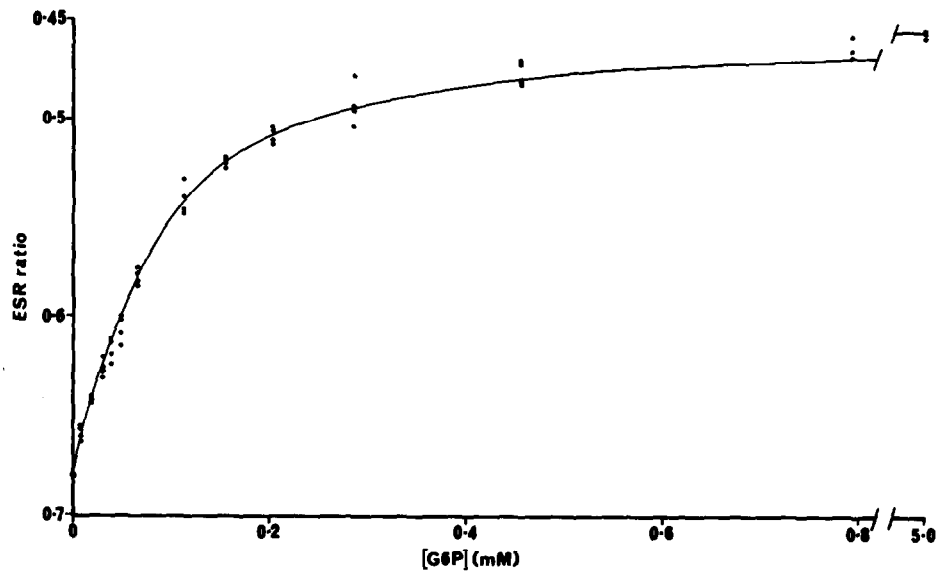

Fig.2. Four superimposed titrations of 59 μM spin-labelled phosphorylase b with glucose-6-phosphate in 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, 1 mM EDTA pH 7.0. The curve is a calculated binding curve based on the assumptions outlined in the text.

$$K = \frac{38.5}{61.5} \quad \begin{matrix} \beta G6P \\ \downarrow\uparrow \\ \alpha G6P \end{matrix} + Ppb \overset{K_d}{\rightleftharpoons} (Ppb{-}G6P)$$

The equilibrium constant for anomerization is that derived from the proportions of the anomers [8].

The ESR ratio at any point in the titration (R_t) may be predicted from the relationship –

$$\frac{R_t - R_o}{R_{oo} - R_o} = \frac{[EL]}{[E_o]}$$

where R_{oo} and R_o are the ESR ratios of spin-labelled enzyme in the absence and presence of saturating amounts of ligand respectively, $[EL]$ and $[E_o]$ are the concentrations of enzyme–ligand complex and total enzyme respectively. The computer uses K_d to determine a theoretical value for R_t at each glucose-6-phosphate concentration in the titration and then iterates about K_d to determine the best fit to the experimental data, as defined by the lowest root-mean-square difference between predicted and observed values of R_t. This method assumes that only the α-anomer binds, that there is one ligand site per monomer and ignores homotropic interactions.

The value for the dissociation constant for α-glucose-6-phosphate, determined in this way, is 19.5 ± 1 μM.

A control ^{31}P NMR experiment showed that at the concentrations of spin-labelled enzyme and glucose-6-phosphate used, the effect of glucose-phosphateisomerase contamination was negligible.

4. Conclusions

^{31}P NMR unequivocally established that the binding of glucose-6-phosphate to phosphorylase is stereospecific for the α-anomer to the exclusion of observable interaction with the β-anomer. Besides the obvious possibility of applying our method to other similar situations, the observation has metabolic implications. First, the dissociation constants previously derived for glucose-6-phosphate binding to phosphorylase are in error as they are based on total glucose-6-phosphate concentrations. The importance of glucose-6-phosphate in metabolic regulation has been discussed [1,14,15] and many considerations, are based on precise knowledge of ligand binding constants [15].

The second notable feature of the observed stereospecificity is that the anomer of glucose-6-phosphate

which inhibits phosphorylase, is the same as that produced by phosphoglucomutase [7]. Although the interconversion of the α- and β-forms of glucose-6-phosphate is relatively rapid, even in the absence of enzymatic catalysis [8], in a flux situation, like in glycogenolysis, such correspondence in stereospecificity of the two consecutive enzymes in the pathway bears on the efficiency and speed of feedback control.

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