PYRIDOXAL PHOSPHATE IN GLYCOGEN PHOSPHORYLASE: A PHOSPHORUS NMR STUDY

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1. Introduction

In spite of numerous investigations the role of pyridoxal phosphate in maintaining the activity of glycogen phosphorylase is still a mystery (for summary see [1]). Reconstitution experiments with chemically modified pyridoxal analogues have helped to define the functionally essential features of the coenzyme [2,3]. One observation of particular interest is that a subunit containing pyridoxal-5′-phosphate monomethyl ester confers activity on a neighbouring subunit whilst itself remaining inactive. This suggests that the phosphate may have a direct catalytic function in the enzyme. Spectroscopic observations using absorption [4,5] and fluorescence quenching techniques [6] have indicated ligand-induced changes in the environment of the heterocyclic chromophore. Neither the nature of these changes nor the exact form of the chromophore responsible for the observed electronic transitions is yet fully understood.

Nuclear magnetic resonance can provide detailed structural information about individual nuclei in a macromolecule. We have used high sensitivity phosphorus NMR spectroscopy to observe both the state of pyridoxal phosphate in phosphorylase *b* and *a* and the response of the cofactor to enzyme–ligand interactions.

2. Materials and methods

The preparation of phosphorylase *b* and *a* and the sources of chemicals have been described previously [7,8]. NMR spectra at 129 MHz (7.5 Tesla) were obtained on an instrument constructed by D.I. Hoult and R. E. Richards [9], using Quadrature Fourier Transformation with a Cyclically Ordered Phase Sequence (CYCLOPS) of transmitter pulses, and appropriate hardware routing in the computer store in order to orthonormalise the two free induction decays. A Bruker WH-90 spectrometer operating in the Fourier Transform mode was used to record spectra at 36.43 MHz (2.1 Tesla).

3. Results and discussion

^{31}P NMR spectra of phosphorylase *b*, recorded at two frequencies, are shown in fig.1. Two features of the spectra are apparent. (i) The signal arising from a single phosphate residue per subunit has more than one component. (ii) The total width of the resonance is larger at 129 MHz than 36.43 MHz (approximately 900 and 150 Hz respectively). The former observation implies that the phosphate residue of the coenzyme experiences at least two different environments, i.e. that the phosphorylase *b* subunit has a minimum of two forms (conformations), which on the NMR timescale are not rapidly interconvertible. At 36.43 MHz two peaks are resolved in the phosphorylase *b* spectrum. They correspond in frequency to the resonances of the dianion and monoanion forms of both pyridoxal-5′-phosphate and a pyridoxal-5′-phosphate-butylamine conjugate. Assuming that these signals correspond to two interconverting forms of the enzyme, a lower limit can be placed on the 'lifetime', τ, for the interconversion from the theory of chemical exchange. (τ is the

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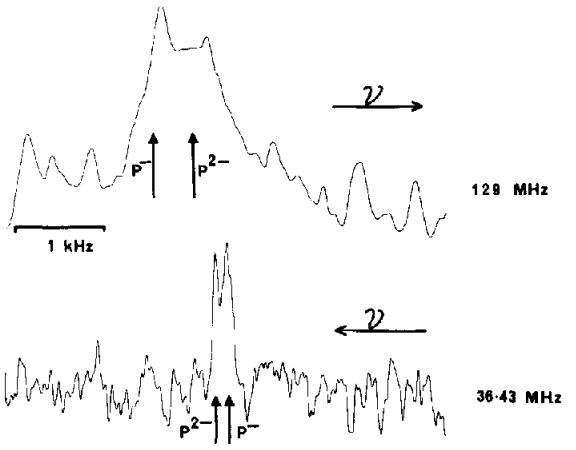

Fig.1. Phosphorus NMR spectra of phosphorylase *b* at 129 and 36.43 MHz. The spectra in fig.1-3 were recorded at 20°C without proton irradiation. External deuterium oxide was used for the field-frequency lock. P^- and P^{2-} indicate the resonance positions of the monoanionic and dianionic forms of the butylamine conjugate of pyridoxal phosphate (10 mM). ν indicates the direction of increasing frequency in each spectrum. The spectrum at 129 MHz is that of a sample of 2.0 mM phosphorylase *b* in 50 mM triethanolamine hydrochloride, 100 mM potassion chloride, 1 mM EDTA, pH 7.78. Radiofrequency radiation was applied as 18 102 45°C pulses at intervals of 2s. The 36.43 MHz spectrum was recorded from a sample of 1.0 mM phosphorylase *b* in the triethanolamine buffer system at pH 7.50. 6700 70° pulses were applied at 8 s intervals.

reciprocal of the sum of the forward and backward rate constants for interconversion.) At 36.43 MHz the two peaks are separated by approximately 150 Hz and hence τ ($> 1/(2\pi\Delta\nu)$, where $\Delta\nu$ is the frequency separation in Hertz) must exceed about 1 ms. As the pK_a of free pyridoxal phosphate is 6.2, a signal corresponding to the dianionic form of the cofactor would be expected at the pH of the above experiments (7.5). If the two components of the signal observed at 36.43 MHz do in fact correspond to the two states of ionisation of the phosphate group, it is likely that proton exchange requires a concomitant conformational change in the enzyme. Whilst throughout this communication we indicate that the two components of the signal could correspond to the two states of ionisation of the phosphate group, current data do not provide sufficient evidence to prove this hypothesis. Other explanations are equally possible.

The difference in the linewidths at the two frequencies cannot be accounted for by chemical exchange and must, at the high magnetic field strengths employed here, contain contributions from chemical shift anisotropy of the phosphorus resonance as observed in other systems [10]. Magnetic relaxation due solely to anisotropy of the phosphate group generates a signal linewidth which is proportional to the square of the applied magnetic field intensity and hence accounts for the major proportion of relaxation at 129 MHz, whilst being subordinate to dipolar relaxation at the lower frequency.

Addition of the activator AMP, or of the inhibitor glucose-6-phosphate, results in a dramatic change in the NMR signal of pyridoxal phosphate (fig.2). The assignments shown in fig.2 were deduced from spectra recorded at differing relative concentrations of ligand to enzyme. The pyridoxal phosphate resonance of phosphorylase *b* saturated with glucose 6-phosphate consists predominantly of one signal of frequency corresponding to that of the dianion form of pyridoxal phosphate. The cofactor signal has a minor component at the monoanion frequency. By contrast, in AMP-saturated phosphorylase *b* the major portion

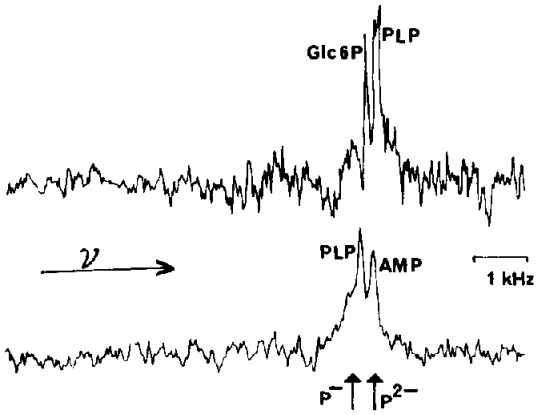

Fig.2. 129 MHz phosphorus NMR spectra of phosphorylase *b* in the presence of glucose 6-phosphate or AMP. The upper spectrum is that of 1.11 mM phosphorylase *b* and 1.14 mM glucose 6-phosphate in the triethanolamine buffer system (see fig.1) at pH 7.89. 10 722 45° pulses were applied at 6s intervals. The lower spectrum was recorded from a sample of 1.16 mM phosphorylase *b* and 1.7 mM AMP in triethanolamine buffer at pH 6.8, and is the accumulated magnetization from 14 400 45° pulses applied at intervals of 2 s. Other details are given in fig.1.

of pyridoxal phosphate magnetization is at the monoanion frequency though there may be some adsorption at the dianion position which is obscured by the AMP signal. Both effectors reduce the linewidth of the pyridoxal phosphate signal as compared to the unliganded enzyme. These results could be most simply explained by a reduction in the number of conformations accessible to the phosphorylase–ligand complex.

Phosphorylase *b* may also be activated to the *a* form by phosphorylation of a single serine residue. The phosphorus NMR spectrum of phosphorylase *a* is shown in fig.3. The composite signal from both the seryl and pyridoxal phosphates is narrower (approximately 120 Hz) than the signal from phosphorylase *b* alone and corresponds in frequency to the dianion form of pyridoxal phosphate (or the amine conjugate). Assignments of both phosphates to the single phosphorylase *a* resonance is based on an experiment in which the intensity of the enzyme signal was compared to that of a standard concentration of pyridoxal phosphate. The chemical shifts of the phosphates correspond to the value for the pyridoxal phosphate in phosphorylase *b* in the presence of the inhibitor glucose 6-phosphate.

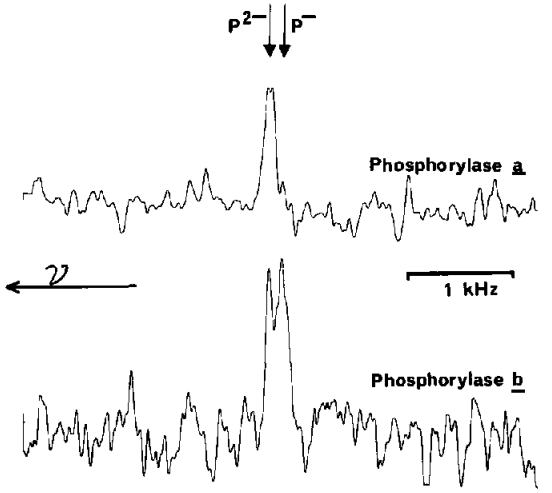

Fig.3. 36.43 MHz phosphorus NMR spectra of phosphorylase *a* and *b*. 6800 pulses were applied at 10 s intervals to a 0.723 mM sample of phosphorylase *a* in triethanolamine buffer at pH 7.5 (80° pulses were used). The phosphorylase *b* spectrum is the same as that shown in fig.1. Other details are given in the legend to that figure.

4. Conclusions

Pyridoxal phosphate in phosphorylase *b* undoubtedly experiences more than one 'chemical environment'. On the simplest assumption that the two component signal seen for phosphorylase *b* alone at 36.43 MHz represents a slow interchange between two enzymic conformations, an upper limit of 1 kHz can be given for the rate of exchange between these forms. Addition of ligands (AMP or glucose 6-phosphate) shifts the pyridoxal phosphate into one of the environments. There is apparently no direct relation between the characteristics of the pyridoxal phosphate resonances and enzymatic activity, in view of the fact that activation by AMP or by covalent phosphorylation produce opposite changes in the spectrum.

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