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# Proton magnetic resonance studies on SH groups in glycogen phosphorylase b

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SUMMARY

The reaction between 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and the SH groups of glycogen phosphorylase b (α-1,4-glucan:orthophosphate glycosyltransferase, EC 2.4.1.1) has been investigated. The interaction of glycogen phosphorylase b and its allosteric activator AMP was detected by proton magnetic resonance spectroscopy. Specific broadening of the $H_2$ and $H_8$ resonance lines of AMP, caused by the AMP–glycogen phosphorylase b interaction, decreased quantitatively upon the DTNB treatment.

$^{60}Co$ γ-irradiation of the enzyme also decreased the AMP–enzyme interaction.

It is assumed that SH groups of the glycogen phosphorylase b are involved in AMP binding.

High resolution nuclear magnetic resonance spectroscopy provides a good tool for making direct observations on the binding of small molecules to proteins. The glycogen phosphorylase b enzyme has an obligatory allosteric activator, AMP. The unexchangeable protons of AMP, having clearly observable resonance lines, make it suitable for studying interactions between the nucleotide and glycogen phosphorylase b[1]. Recently several authors have made attempts to carry out NMR work on glycogen phosphorylase b using the single peaks of the unexchangeable $H_2$ and $H_8$ protons of AMP as indicators[2,3]. None of them tried to make definite assumptions about the nature of the AMP binding site *i.e.* the allosteric center of the enzyme.

This paper presents some experimental data on the effect of specific SH blocking agents and of ionising radiation on the interaction between glycogen phosphorylase b and AMP, detected by proton magnetic resonance spectroscopy. Comparing our results with

Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

those gained earlier by different methods, it is assumed that SH groups are directly involved in binding the allosteric activators, AMP, to the glycogen phosphorylase b[4,5].

Glycogen phosphorylase b has been prepared from rabbit skeletal muscle and purified as reported earlier[5]. The purified enzyme has been recrystallized at least three times in a medium containing $^2H_2O$ instead of water. All of the reagents were dissolved either in the deuterated enzyme solution or in 99.8% $^2H_2O$. The $p^2H \approx 7$ was provided by 0.05 M Tris–$^2$HCl. Proton magnetic resonance spectra were observed in a JEOL 100 Mc NMR spectrometer. The position of the resonance lines were calibrated for those of sodium 2,2-dimethyl-silapentane-5-sulfonate. The half-widths of the $H_2$ and $H_8$ proton resonance lines of AMP were evaluated from the PMR spectra.

The incidental nonspecific interference of the specific SH reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with the resonance lines of AMP has been checked. DTNB and also cysteine caused a mild chemical shift of the $H_2$ and $H_8$ proton resonances, but neither of them produced broadening of the lines. The Tris–$^2$HCl had no effect upon the observed PMR spectral region of AMP.

The irradiation, when it is stated, was carried out by $^{60}$Co $\gamma$-rays at a dose rate of of 4500 R/min. The dose was determined with a Fricke dosimeter.

All other experimental details are indicated in the legends of the figures.

Fig. 1 shows the PMR spectra of $H_2$ and $H_8$ adenine protons of AMP. The broadening of the $H_2$ and $H_8$ resonance lines is assumed to be due to the specific interaction between AMP and glycogen phosphorylase b. The paramagnetic $Mn^{2+}$ amplifies this effect by making an enzyme–AMP–$Mn^{2+}$ complex. From the selective broadening of the AMP lines we calculated the $H_2$–$Mn^{2+}$ and the $H_8$–$Mn^{2+}$ distances in the AMP–glycogen phosphorylase b–$Mn^{2+}$ complex by the aid of the Solomon–Bloembergen theorem[6,7].

The calculated $H_2$–$Mn^{2+}$ and $H_8$–$Mn^{2+}$ distances were 7.08 Å and 4.57 Å, respectively. These values are in good agreement with data published by Bennick *et al.*[2]. The effect of the enzyme on the resonance lines of the AMP before and after different treatments is also demonstrated.

Fig. 2 demonstrates the effect of different DTNB concentrations on the half-widths of the $H_2$ and $H_8$ proton resonance lines of AMP in the presence of glycogen phosphorylase b and $Mn^{2+}$.

The width at half-height of the resonance line has a reciprocal relation to $T_2$, *i.e.* the transverse relaxation time ($T_2 = 1/\pi\Delta\nu$). The curves show that broadening of the proton resonance lines of the effector molecules decreases in non-linear fashion with increasing DTNB concentrations. DTNB is known to decrease the activity of the glycogen phosphorylase b by blocking specifically the free SH groups of the enzyme[8]. As a consequence of this blocking, the enzyme loses its ability to bind AMP. Upon the basis of the above results, it is assumed that the less the broadening of the resonance lines of the $H_2$ and $H_8$ protons, the less the AMP bound to the glycogen phosphorylase b. The half-width values of the $H_2$ and $H_8$ protons, extrapolated for infinite DTNB concentration, proved to be smaller than those of the AMP–$Mn^{2+}$ complex without enzyme and DTNB.

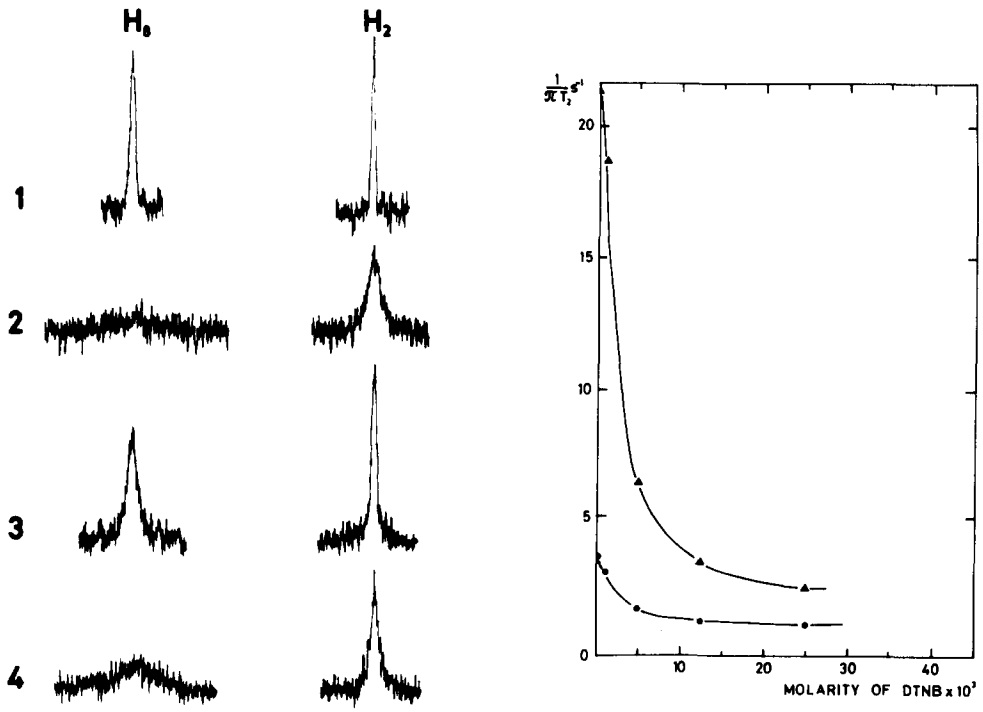



Fig.1. PMR spectra of the $H_8$ and $H_2$ protons of AMP. Curves 1, 20 mM AMP alone; Curves 2, 20 mM AMP + 240 μM phosphorylase b + 45 μM $MnCl_2$; Curves 3, 20 mM AMP + 240 μM phosphorylase b + 25 mM DTNB + 45 μM $MnCl_2$; Curves 4, 20 mM AMP + 240 μM phosphorylase b irradiated with 50 000 R + 45 μM $MnCl_2$. All the solutions were in 0.05 M Tris–$^2$HCl buffer at $p^2H) \approx 7$. PMR experiments were done at 30 °C and at 100 Mc.

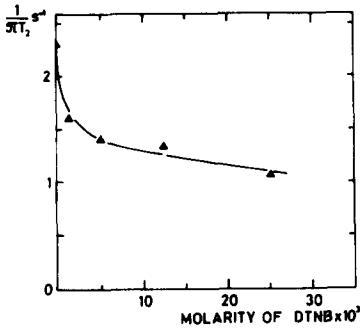
Fig.2. Effect of DTNB concentration on the half-width of AMP PMR peaks in the presence of 240 μM phosphorylase b and 45 μM $MnCl_2$. $H_8$ proton resonance peak half-width values, ▲——▲; $H_2$ proton resonance peak half-width values, ●——●. Conditions were as in Fig.1.

In the case of the AMP–$Mn^{2+}$ complex the half-width values were as follows: $\Delta\nu_{H_2} = 2.88\ s^{-1}$ and $\Delta\nu_{H_8} = 20.40\ s^{-1}$. This suggests that DTNB released the AMP from the enzyme–AMP–$Mn^{2+}$ complex and that $Mn^{2+}$ was retained by the protein.

Fig.3 shows the effect of DTNB on the half-width of the $H_8$ proton resonance line of AMP without the use of $Mn^{2+}$. The lines belonging to the $H_8$ protons showed a less extensive but still clearly observable effect than those demonstrated in Fig.2. However, the half-width of the $H_2$ resonance lines were too small without $Mn^{2+}$ to observe the broadening of them. Nevertheless the shape of the curve in Fig.3 obtained by the $H_8$ proton resonance lines of the enzyme–AMP complexes, was in good correlation with the curves demonstrated in Fig.2 for the enzyme–AMP–$Mn^{2+}$ complexes.

Fig.4 shows the effect of $^{60}$Co γ-irradiation of the enzyme on the half-width of the $H_2$ and $H_8$ proton resonance lines during the AMP–enzyme interaction. The SH groups of phosphorylase b are known to be very radiosensitive[5]. The irradiation had to be carried out in a medium containing cysteine and a little amount of AMP, which strongly

protected the enzyme[9]. For technical reasons, such as the high concentration of the enzyme necessary for the NMR experiment, it was easier to increase the irradiation dose



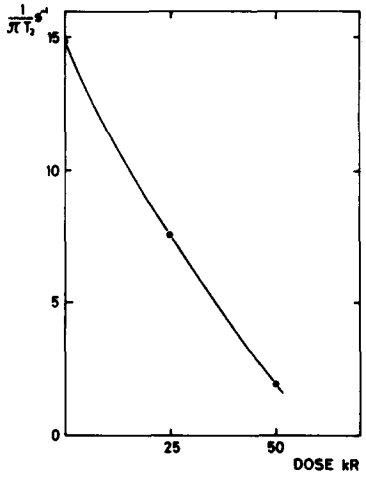




Fig. 3. Effect of DTNB concentration on the half-width value of $H_8$ PMR peak of 20 mM AMP without $Mn^{2+}$. Phosphorylase b concentration was 240 $\mu$M. Other experimental conditions were as in Fig. 1.

Fig. 4. Effect of irradiation of 240 $\mu$M phosphorylase b on the half-width value of the $H_2$ proton resonance peak of 20 mM AMP, in the presence of 45 $\mu$M $MnCl_2$. The irradiation was carried out by $^{60}Co$ $\gamma$-rays and the doses were determined by Fricke dosimeter. Other experimental conditions were as in Fig. 1.

instead of removing the protective agents. The curve shows a similar fashion to the case of the DTNB reactions.

We suggest, in accordance with our earlier opinion, that the AMP binding site, *i.e.* the allosteric site, of the glycogen phosphorylase b enzyme contains SH groups. Recently experiments were carried out with affinity labeling of the enzyme by activator analog, which rendered this assumption also very likely[10]. The exact number of SH groups involved in binding the AMP will be discussed in another paper.

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