PROTON MAGNETIC RESONANCE STUDIES ON 5'- AMP SITE IN GLYCOGEN PHOSPHORYLASE b

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1. Introduction

Rabbit muscle phosphorylase b exhibits quaternary interactions which are strongly dependent on the presence of 5'-AMP, the activator of the enzymatic reaction [1]. It is, therefore, of interest to know how strongly this ligand is immobilized in its site. We present here an estimation of the mean residence time of 5'-AMP bound to the enzyme obtained by nuclear magnetic resonance (NMR) studies of the non-deuterated 5'-AMP protons in heavy water solutions. The experiments reported show that the binding site is rather highly immobilized. The rotational relaxation time for the 5'-AMP site is the order of 150 nsec at 300° K. This value is much larger than that reported by Bennick et al. for the 5'-AMP-Mn^{2+}–protein complex [2] and seems to question their assumptions. We estimate that H_2, in the bound 5'-AMP, lies 2.7 Å from a non-exchangeable proton of the protein and H_8 at Van der Waals distance from another such proton. A temperature dependent conformational change, noted by Birkett et al. [3] with other spectroscopic techniques, influences the $H_1{}'$ line width. Our estimated range for the mean residence time of 5'-AMP in its site (30–150 μsec) agrees with the inverse of the dissociation rate constant of the 5'-AMP-phosphorylase a complex as measured by fast relaxation techniques [4].

2. Material and methods

Broadening of the proton resonance lines of an effector molecule in the presence of an enzyme is detectable when the rotational motion of the binding site is slow enough. Moreover, measurements are possible only in a narrow range of dissociation constants such that the effector exchange is rapid enough to average the broadening on all the effector molecules and such that all binding sites are occupied at the concentrations used [5].

Among the different effectors of rabbit muscle glycogen phosphorylase b, we have therefore selected 5'-AMP, which has a dissociation constant of about 4×10^{-5} M at 22°, a value 5–10 times higher than that of most other activators or inhibitors of this enzyme [6].

Phosphorylase b, prepared according to Krebs et al. [7], was recrystallized in a solution of 10^{-3} M Tris acetate, 10^{-3} M β-mercaptoethanol in D_2O. The crystals were then redissolved at a final concentration of 10 mg per ml in a 10^{-1} M solution of 5'-AMP in D_2O. In most experiments, no buffer was added, since 5'-AMP per se at a concentration between 10^{-2} M and 10^{-1} M is a good enough buffer to maintain the pD between 6.9 and 7.0. Lyophilized 5'-AMP was dissolved in D_2O.

NMR experiments were performed on a Varian HA 60 spectrometer. The HDO resonance line was used as a lock for the field. For temperature calibration of the chemical shifts, a 0.05 M solution of tetramethyl ammonium chloride was used as a reference.

Transverse relaxation times, T_2, were evaluated from the width at half height of the resonance line of the non-exchangeable protons of 5'-AMP, either in the absence ($T_{2,0}$) or in the presence ($T_{2,obs}$) of enzyme. Each resonance line was scanned at a rate of 100 sec per hertz, using a time constant of 0.02 sec^{-1}. Under these conditions, the signal to noise ratio for the H_2 line of free 5'-AMP is equal to 20.

Without enzyme, 5′-AMP gives a resonance spectrum for each of its unexchangeable protons. Those which are not scalar coupled to other protons (H_8 and H_2) give a single peak. The others, like H_1' and H_5' give multiplets. The width of a peak is due mostly to proton–proton dipolar coupling which are partially but not totally averaged out by rotation of the 5′-AMP molecule. The faster the rotation, the narrower the peak. The width of the line, $\Delta\nu_{obs}$ is given by:

$$(1) \quad \frac{1}{T_{2,0}} = \Pi\Delta\nu_{obs} = \frac{10\,\mathrm{K}\,\tau_{ro}}{a^6}$$

τ_{ro} is the rotational correlation time of 5′-AMP. a is the center-to-center distance between a given non-exchangeable proton and its nearest neighbor. The factor 10 K, which is known, expresses the magnetic dipolar interaction between unlike spins at the Larmor frequency ω, in the limiting case:

$(\omega\tau_{ro})^2 \ll 1$ (or $\tau_{ro} < 2.6$ nsec in our measurements).

If phosphorylase b is added, a fraction of 5′-AMP molecules is immobilized on binding and, in the case of rapid exchange between bound and free molecules, this gives rise to a broadening of the observed lines. An extra-broadening may appear when strong dipole–dipole interactions are taking place between a given 5′-AMP proton and non-exchangeable protein protons close to it.

Quantitatively one can write:

$$(2) \quad \Pi\Delta\nu_{obs} = \frac{1}{T_{2,obs}} = \frac{1}{T_{2,0}} + \frac{C}{N_0}\,\frac{1}{T_{2p} + \tau_h}$$

T_{2p} is the transverse relaxation time of the observed proton when 5′-AMP is bound to the enzyme.

C is the 5′-AMP site concentration (all sites are occupied under our conditions).

N_0 is the free 5′-AMP concentration.

τ_h is the mean residence time of 5′-AMP on the enzyme.

$\dfrac{1}{T_{2p}}$ is expressed by an equation similar to (1):

$$(3) \quad \frac{1}{T_{2p}} = f(\omega,\tau_c)\left(\frac{\tau_c}{a^6} + \frac{\tau_c}{x^6}\right)$$

In our case (unlike spins) $f(\omega,\tau_c)$ reduces to 2.5 K for $\tau_c > 2.6$ nsec and to 10 K, as above, for $\tau_c < 2.6$ nsec [8]. τ_c is a correlation time of the dipolar relaxation; its value is given by

$$(4) \quad \tau_c^{-1} = \tau_r^{-1} + \tau_h^{-1}$$

where τ_r is the mean rotation time of bound 5′-AMP.

x is the distance between a given proton and its nearest neighbor at the AMP site of phosphorylase b. Several protons may contribute to the broadening; however, since their contributions go as the inverse of the sixth power of the distance, one is allowed to consider only the nearest neighbor. In the following section, we are going to use this approximation.

3. Results and discussion

3.1. *Free 5′-AMP*

We looked only at peaks which are well resolved and well separated from the HDO band (H_8, H_1', H_2) and measured the corresponding $T_{2,0}^{-1}$ values. However, the width of the H_2 proton is very narrow and appears to be limited only by field inhomogeneity. The absence of dipolar broadening shows that H_2 proton is more than 4 Å away from any other proton in the same 5′-AMP molecule.

The rotational correlation time τ_{ro} of 5′-AMP has been obtained directly by measuring the water proton relaxation rates in the presence of manganese ions at several Larmor frequencies (3.25, 7.00, 15.48, 28.52, 40.52 MHZ) and by calculating the rotational time of the 5′-AMP-Mn^{2+}–$(H_2O)_5$ complex as previously described [9]. τ_{ro} has been found equal to 58 psec at 300° K. In D_2O this value has to be multiplied by a factor 1.23 which takes into account the relative viscosities of the two solvents. Then, formula (1) can be simplified by inserting the correct value of τ_{ro}. At 300° K one gets:

$$(5) \quad \frac{1}{T_{2,0}} = \frac{53}{a^6}$$

a being expressed in angstroms and $T_{2,0}$ in seconds. The first two columns in table 1 show the internal consistency of data, since values of a computed according to [5] are close to measurements made directly on Leybold molecular models.

Table 1

	a (Å)	a *model* (Å)	$\frac{1}{T_{2,0}}$(300° K) (sec^{-1})	λ_{min} (Å)	λ_{max} (Å)	τ_c min (nsec)	τ_c max (nsec)
H_8	2.3	2.3–3.4	0.35	2.2	3.4	150	2800
H_2	> 3	3.4–6.4	0.1	2.2	6.4	55	3.5×10^4
H_1'	2.6	2.5–2.7	0.09	2.2	2.7	86	180

a is the distance of the nearest 5'-AMP non-exchangeable proton to the considered proton obtained from eq. (1).

a model is the distance of the nearest 5'-AMP non-exchangeable proton measured on a Leybold model.

λ_{min} and λ_{max} are extreme values of $(1/a^6 + 1/x^6)^{-1/6}$ (see text).

$\tau_{c\ min}$ and $\tau_{c\ max}$ are the corresponding correlation times in eq. (5). From the three proton resonance broadenings one finds 150 nsec < τ_c < 180 nsec.

3.2. *5'-AMP–phosphorylase b complex*

The figure shows the temperature dependence from 5° C to 34° C of $\frac{1}{T_2^*} = \frac{1}{T_{2,obs}} - \frac{1}{T_{2,0}}$, the broadening of the H_2, H_8 and H_1, resonance lines. At the concentration used, C/N_0 is slightly larger than 10^{-3} and $T_{2p} + \tau_h$ must therefore lie between 1.5 msec and 10 msec.

As it appears that $1/T_2$ increases as η/T, at least for H_8 and H_2, we shall first interpret our results assuming that τ_h is negligible with respect to T_{2p}. Then, according to equation (3), one finds that τ_c must be larger than 10 nsec for T_{2p} to lie within the necessary limits. In formula (3), $f(\omega,\tau_c)$ equal to 2.5 K, and $1/T_2$ can be rewritten as:

$$(6)\quad \frac{1}{T_2} = 2.5\, K \frac{C}{N_0} \tau_c \left(\frac{1}{a^6} + \frac{1}{x^6}\right)$$

We can now have a better estimate of τ_c. For a given observed value of $1/T_2$ the upper limit of τ_c is determined by the largest possible value of a when a is known from model examination and x is assumed to be larger than a. The lower limit of τ_c results from the assumption that x is as small as possible, the contact distance between two hydrogen atoms, and that a is equal to its smallest evaluation in the table. As these two limits depend on the observed proton and as τ_c is a characteristic of the whole site, one is led to take for τ_c the intersect of the three estimates, i.e. a value close to 160 nsec (±10 nsec) at 300° K.

Once τ_c is known, one can get for each resonance line the value of $\frac{1}{\lambda^6} = \frac{1}{a^6} + \frac{1}{x^6}$ through equation (6). At 300° K, H_8 must be in close contact with a proton of the protein while H_1' is not influenced by the protein. For H_2, λ is of the order of 2.7 Å, a value which indicates that this proton resonance is broadened by dipolar interaction with at least one proton of the site.

It appears in fig. 1 that there is a break in the variation of the width of the H_1' resonance.

This is interpreted as a local conformational change occurring around 13° C; below that temperature H_1' starts to "see" another non-exchangeable proton. Using again equation (6), one can deduce that the distance between H_1' and this proton moves from

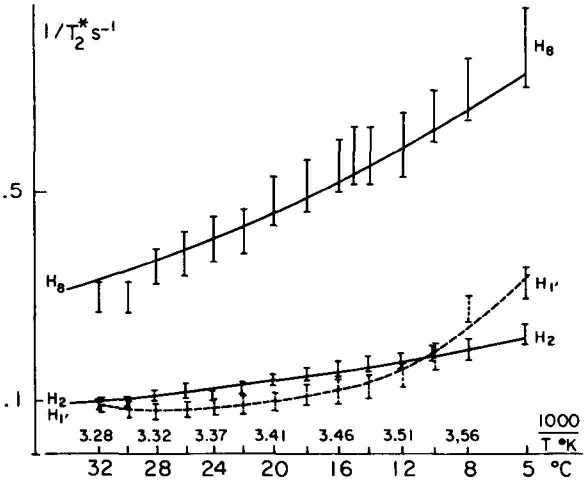

Fig. 1. Temperature variation of $\frac{1}{T_2^*} = \frac{1}{T_{2,obs}} - \frac{1}{T_{2,0}}$ for the protons $H_{8,2,1}'$ of 10^{-1} M 5'-AMP in a solution of 10 mg/ml Ph b in D_2O, pD 7.0. (——): Shows theoretical η/T variations. (- - - -) represents the temperature variation of H_1

more than 4 Å above 18° C to around 2.8 Å at 5° C.

According to equation (4), τ_c^{-1} is dominated either by τ_h^{-1} or by τ_r^{-1}. The largest value of τ_h^{-1}, the dissociation rate constant of the 5′-AMP–phosphorylase b complex, is obtained when the corresponding association rate constant is diffusion controlled. Under this assumption τ_h is equal to 30 μsec, a value which is very small with respect to τ_c. Therefore τ_c measures essentially the rotational relaxation time of the 5′-AMP site which is only 2.2 times smaller than the overall rotational relaxation time of a phosphorylase b dimer [10]. $1/T_2^*$ is, therefore, proportional to τ_r, and it is normal that it follows an η/T variation.

On the other hand, if τ_h were becoming significant with respect to T_{2p}, its contribution to the different proton resonances would give a different temperature variation for each of them, a result which is not observed. Therefore τ_h is smaller than $T_{2p}/5$ or 150 μsec.

The rough estimate of τ_h (30 μsec $< \tau_h <$ 150 μsec) is consistent with the value found for the residence time of 5′-AMP on phosphorylase a, 100 μsec at 25° C [4].

We must say, however, that all these results are only semi-quantitative. Several protons could have contributed to the broadening of H_8, though steric hindrance and the fast decrease in $1/x^6$ of the interaction severely restrict the influence of more distal protons. In this case τ_r should be smaller, but certainly not smaller than the lowest value given in the sixth column of the table (55 nsec) because it is quite unlikely that all the three 5′-AMP resonance lines under study could have been broadened by more than one proton. Furthermore, extra broadening can be due to causes other than proximity effects between protein protons and effectors since it is not certain that the relative geometrical positions of the protons of a given AMP molecule are the same in the presence and in the absence of enzyme[†].

4. Conclusion

Despite these restrictions, one can reliably measure the average rotational relaxation time of the site, because of favorable circumstances ($\tau_r < \tau_h < T_{2p}$), and because the protein protons interact strongly with some non-exchangeable protons and very weakly with others. It follows that mean distances between protein protons and ligand protons can be estimated; in particular, a conformational transition which is known to take place around 14° C [3, 12] is reflected by movement of a non-exchangeable proton of the site with respect to $H_{1'}$.

In a recent study [2] Bennick et al. have proposed a very attractive model according to which 5′-AMP and glucose-1-phosphate sites are very close. They suggest also that the 5′-AMP site is very mobile. These deductions rely primarily on proton magnetic resonance measurements on 5′-AMP in the presence of Mn^{2+}. These authors have subtracted the broadening of the resonances due to immobilization of 5′-AMP by the enzyme in the absence of Mn^{2+}. We present here, on the opposite, what can be derived from the measurement of this broadening. The effect of Mn^{2+} on the NMR spectrum of solutions of phosphorylase b and 5′-AMP has not been further investigated by us for two reasons. First the broadening of 5′-AMP lines due to Mn^{2+} is so important that the measure of T_2 which is the difference between two large numbers would have been very inaccurate. Second, the correlation time for the relaxation has in this case good chances to be limited by the electronic relaxation time of Mn^{2+} [9].

We conclude that the effector site is strongly immobilized once AMP is bound; this probably favors long distance interactions through an efficient positioning of distal groups of the protein, but it does not, of course, give any information about the flexibility of the corresponding empty site.

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[†] One could also object that changes in chemical shifts between protons in the free and bound AMP molecules might have increased T_{2p} [11]. But even in the unlikely case where all the proton resonances had been shifted by 1 ppm, the corresponding contribution to T_{2p} would have been only of 10%, which corresponds to the accuracy of the line width measurements.

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