^{25}Mg NMR LINEWIDTH AS A PROBE IN PROTEIN BINDING STUDIES

Application to bovine serum albumin and nitrogenase Fe protein

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1. Introduction

Despite the importance of Mg^{2+}–protein interactions in many enzyme systems, no case of monitoring such an interaction by its effect upon the ^{25}Mg NMR spectrum has hitherto been reported [1,2]. The most accessible ^{25}Mg parameter is the linewidth, and a theoretical and experimental study [3] concluded that this was unlikely to be of use in measuring ternary complex formation between Mg^{2+}, ATP and a protein. This may have discouraged study of Mg^{2+}, protein binary systems, although the conclusions in [3] do not necessarily apply to the latter. Mg^{2+}–tripeptide interactions by this method have been studied [2] reiterating that Mg^{2+} binding to a macromolecule 'probably cannot be directly measured'.

We now report a preliminary linewidth study of two test proteins, bovine serum albumin (BSA) and the iron protein of nitrogenase from *Klebsiella pneumoniae* (Kp2), both having mol. wt ~67 000. The usefulness of the method is apparent with the natural abundance (10.05%) of ^{25}Mg used in this study and could be greatly enhanced by isotope enrichment.

2. Methods and materials

2.1. *Bovine serum albumin*

BSA was used as fraction V from Sigma Chemical Co. The albumin was deionized as in [4]. Two concentrations of albumin were used: 0.15 mM and the approximately physiological value of 0.6 mM. The solutions also contained 150 mM NaCl (BDH Analar grade) and were buffered with 0.67% (v/v) triethanolamine (obtained as triethanolamine hydrochloride, Sigma Chemical Co. 99% pure) at pH 7.4 measured with an EIL 7050 pH meter. The titrants contained either 0.15 mM or 0.6 mM albumin together with the above and 400 mM $MgCl_2$ (BDH Analar grade). All solutions were deoxygenated by bubbling with nitrogen. An EDTA–Eriochrome black T titration was used to measure the concentration of a stock solution of $MgCl_2$. The titrant was added through the vortex plug into the observed solution using a long-needled Hamilton syringe and thorough mixing was achieved by drawing the solution up into a syringe several times.

2.2. *Fe protein of nitrogenase*

Kp2 was prepared as in [5]. Because nitrogenase is irreversibly damaged by oxygen, air was rigorously excluded. The NMR tubes were evacuated on a vacuum line and the protein solution and titrant injected through a Subaseal closure with a long-needled Hamilton syringe, mixing as before. Two protein concentrations were used again. The solutions were buffered with 25 mM Tris at pH 7.4 and contained 1 mM sodium dithionite, which was added after bubbling with nitrogen for 15 min. The titrant did not contain Kp2 and again had a $MgCl_2$ at 400 mM. Assay before and after the experiment showed retention of enzyme activity.

2.3. *^{25}Mg NMR spectra*

^{25}Mg NMR spectra were recorded on a JEOL FX100

Fourier transform spectrometer, with multinuclear observation accessory, at 6.10 MHz, in spinning 10 mM tubes. An internal deuterium lock was provided by 10% D_2O (it has been shown that addition of D_2O does not affect the ^{25}Mg linewidth [3]). The probe temperature was 25°C. The spectra with the broadest lines were displayed with several values of a decaying exponential weighting function and corrected for the resultant artificial broadening. The practical lower limit, under these conditions, was ~15 mM $MgCl_2$ with an overnight run (~120 000 transients).

3. Results and discussion

Experiments were carried out by titrating the protein solutions with small aliquots of 400 mM $MgCl_2$ solution. This contained sufficient protein (in the case of BSA) to maintain constant concentration of the latter in the NMR sample. With Kp2 this was not practicable due to handling difficulties, and a correction was made for the slight dilution of protein during the course of a titration.

All ^{25}Mg spectra consisted of a single line, the width of which, measured at half-height, increased with increasing protein : Mg^{2+} ratio. $\Delta\nu$, the line broadening induced by binding to protein, was found by subtracting the limiting value at high Mg^{2+} concentration from the observed linewidth. The limiting widths (~2.5 Hz in the case of Kp2 samples; ~5 Hz for BSA) were found by extrapolation to high metal concentration, and were used in preference to the protein-free value (which we observed as ~1 Hz rather than 2–3 Hz [6]). The broadening at high metal was probably due to viscosity effects, the paramagnetism of Kp2, and possibly to traces of paramagnetic impurities. Plots of the reciprocal line broadening ($1/\Delta\nu$) against Mg^{2+} concentration are shown in fig.1.

Three features of these plots are apparent: (1) their linearity; (2) their convergence to a common intercept on the Mg^{2+} concentration axis for a given protein; (3) inverse proportionality of their slopes to the

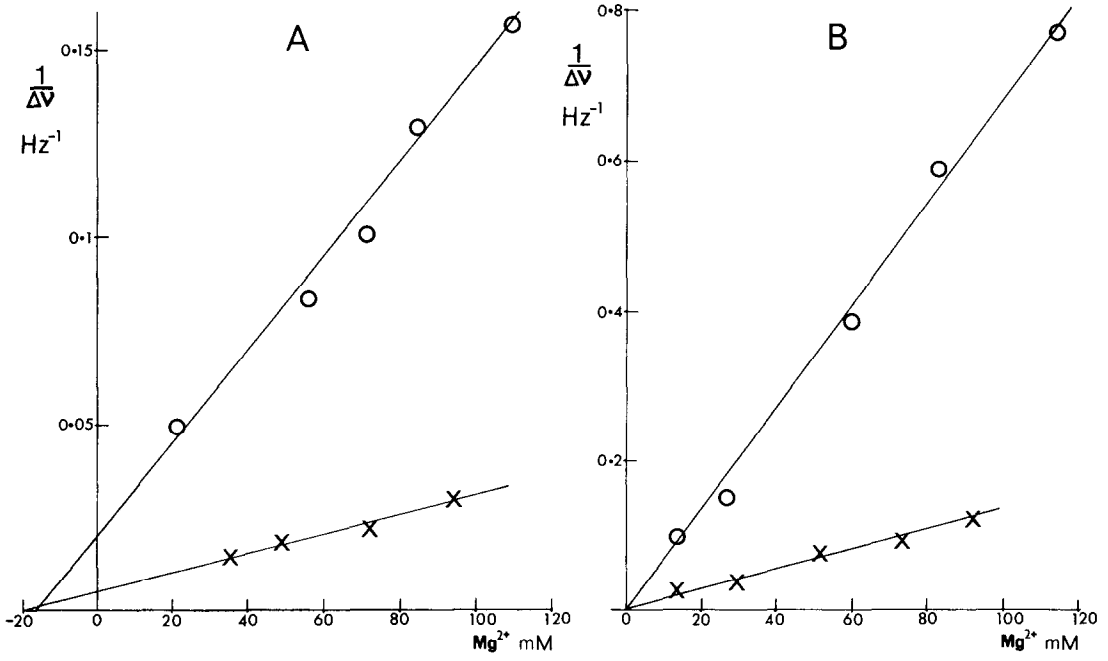

Fig.1. Plots of reciprocal line broadening ($1/\Delta\nu$) against total [Mg^{2+}]. (A) Titration of bovine serum albumin: (○——○) 0.6 mM; (×——×) 0.15 mM. (B) Titration of nitrogenase Fe protein, Kp2: (○——○) 0.068 mM; (×——×) 0.013 mM. The solid lines are linear least-squares fitted to experimental data.

protein concentration, again for a given protein. In view of the fairly large molar excess of Mg^{2+} over protein, all three features strongly suggest that the observed single-line spectra are caused by exchange of Mg^{2+} between free and bound conditions, and are either at or close to the fast exchange limit [7,8]. This was also supported by one preliminary experiment (with BSA) which indicated that the width of a well-broadened line was not further increased by lowering the temperature 15°C. Under fast-exchange conditions the Swift-Connick treatment [5] predicts an observed line width which is the weighted average of those for free and bound metal ions, and with a large excess of free over bound sites this leads to the following equation for line broadening [8]:

$$\frac{1}{\Delta\nu} = \frac{\pi T_2 m}{n[E_o]}(K_d + [S])$$

This assumes n equivalent sites for substrate (S) per protein molecule. If n is known, the bound linewidth ($1/\pi T_2 m$ in Hz) can be found from the slope of a $1/\Delta\nu$ versus [S] plot corresponding to protein concentration $[E_o]$, and the dissociation constant K_d of the protein–substrate complex is given by the intercept on the negative [S] axis. Previous studies of protein–Mg^{2+} binding indicate equivalent sites with values of $n = 6$, $K_d = 15.7$ mM for BSA [4], and $n = 4$, $K_d = 1.7$ mM for Kp2 [9], under conditions similar to those of the present study. Although K_d for the nitrogenase protein is too small to be found from the data of fig.1 (the observed intercept is not significantly different from zero) K_d for BSA is clearly accessible by the present study even without recourse to ^{25}Mg enrichment. The limited data of fig.1 indicates K_d ~15–20 mM, in agreement with the literature value. We have therefore no evidence for nonspecific binding of additional Mg^{2+}. With the published values of n, the data of fig.1 indicate linewidths of ~2750 ± 250 Hz for Mg^{2+} bound to Kp2, and 1000 ± 100 Hz for Mg^{2+} bound to BSA.

^{25}Mg is a spin 5/2 nucleus with a significant quadrupole moment, so that the dominant spin relaxation mechanism is almost certainly quadrupolar [1–3]. Enhanced transverse relaxation and line broadening on binding to a macromolecule is therefore expected on the basis of: (a) a higher electric field gradient due to the lower symmetry; (b) a longer correlation time for the quadrupolar interaction, due to reduced mobility of Mg^{2+} at the bound site. The bound linewidths that we find are however considerably less than in [3] guessed value of 'three orders of magnitude larger than that for Mg–ATP complex' (the latter reported as ~915 Hz), and this fact (with the added advantage of Fourier transform NMR) is largely responsible for our ability to follow line broadening under the present experimental conditions. A further requirement is approach to the fast exchange condition, and this probably indicates a rather small chemical shift displacement on binding. Certainly we could detect no displacement of the averaged peak. No displacement was found [2] on binding to a tripeptide (with a calculated bound line width of ~180 Hz), although a large displacement was inferred [3] on binding to ATP (the precise value in [3] is open to doubt since the calculations were based on a 1:1 complex rather than Mg_2ATP [10]).

Given fast exchange, line broadening could be observed for any system provided that a sufficiently large ratio of metal ion to macromolecule is taken. This can be of use in competition studies, but further information is available if the ratio can be reduced, without loss of the signal, to a point where dissociation of the complex becomes measurable. With limited availability or solubility of many macromolecules, it becomes important to take measurements to as low a concentration of metal ion as possible. We have been able to take measurements down to $[Mg^{2+}]$ ~15 mM in natural abundance, and enrichment in ^{25}Mg (preferably with the use of larger samples and/or higher fields) would allow this to be reduced further by an order of magnitude. This should yield accurate K_d values for systems resembling those of the present study, and in favourable cases the number and type(s) of binding site could also be investigated.

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