Vol. 104, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1982 Pages 720-726

^{25}Mg NMR STUDY OF Mg^{2+}-ATP, ADP-CREATINE KINASE COMPLEXES

Toru Shimizu and Masahiro Hatano

Chemical Research Institute of Non-Aqueous Solutions
Tohoku University, Katahira, Sendai 980 JAPAN

Received December 3, 1981

^{25}Mg NMR spectroscopy was first applied to the ternary complexes consisting of Mg^{2+}, ATP, ADP and creatine kinase. The ^{25}Mg NMR spectra of the Mg^{2+}-ATP (or ADP) complex are remarkably broadened in the ternary Mg^{2+}-ATP(or ADP)-creatine kinase complex in contrast with previous prediction. From temperature dependence of the spectra of the protein-bound ion, it is suggested that Mg^{2+} of the protein-bound Mg^{2+}-ATP(or ADP) complex is not in the fast exchange regime. The ^{25}Mg NMR signal of the transition state analogue complex is narrower and less temperature-dependent than those of the ternary complex, suggesting that Mg^{2+} in the transition state analogue complex is in a more symmetrical environment or exchanges slower than that of the ternary complex.

^{25}Mg NMR has recently been applied to some biological substances(1-8). These studies were done mainly for determining the binding constants or for investigating the exchange behavior of Mg^{2+} to small molecules, DNA and proteins by using the half-band-widths($\Delta\nu_{1/2}$) of the NMR signals. On the basis of NMR data of highly concentrated Mg^{2+}(1.5 M) and calculations assuming the slow exchange limit involved, it was predicted(2) that the broadened ^{25}Mg NMR signal of the binary Mg^{2+}-ATP complex will be scarcely broadened furthermore and will show little temperature-dependence when the ternary Mg^{2+}-ATP-protein complex is formed.

We wish to describe in this paper that the ternary Mg^{2+}-ATP-creatine kinase complex, in fact, provides quite broad ^{25}Mg NMR signals compared with those of the binary Mg^{2+}-ATP complex and that the NMR signal of the ternary complex becomes narrower when the temperature is decreased. The temperature effect of the NMR spectra for the ternary complex is the reverse of that for the binary complex. The same results are obtained for the binary and ternary ADP complexes. The NMR of the transition state analogue complex is narrower and less temperature-dependent than that of the ternary complex. The reasons for the above-mentioned NMR findings of the ternary and the transition state analogue complexes will be discussed.

MATERIALS AND METHODS

^{25}Mg obtained as 95.66 % MgO from Prochem was dissolved in metal-free 1 M nitric acid and was neutralized to a final pH of 7.0. ATP, ADP and creatine of guaranteed grade were purchased from Sigma. The molecular activity of creatine kinase purchased from Sigma(Type I purified from rabbit muscle) was

0006-291X/82/020720-07$01.00/0

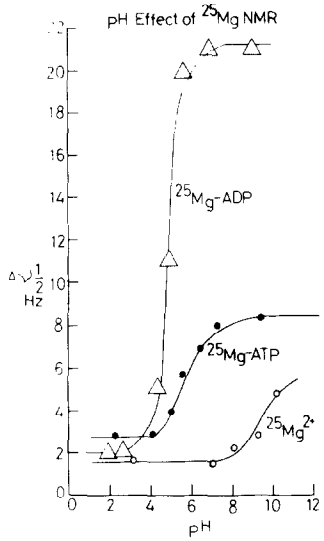


Figure 1. The pH effect on ^{25}Mg NMR of free $^{25}Mg^{2+}$(o)(3 mM), the binary $^{25}Mg^{2+}$-ATP(●)(Mg^{2+} 3 mM;ATP 0.1 mM), $^{25}Mg^{2+}$-ADP(Δ)(Mg^{2+} 3 mM;ADP 0.4 mM) complexes.

140 μmoles per mg protein. ATPase and other impure enzymatic activities in the creatine kinase were below 0.02 %. Doubly distilled water was used for the NMR measurements. Other chemicals were of guaranteed grade and used without further purification.

^{25}Mg NMR spectra were accumulated on a Bruker CXP-300 FT NMR spectrometer at 18.36 MHz in a spinning 10 mm sample tube with external D_2O for lock. An equipped transmitter provided 90-degree pulse width of 80 μs at a peak-to-peak voltage of 300 V. Typical spectra consisted of collecting 10000 transients for signal/noise > 10 using 2K or 4K data points over 5000 Hz spectral window. The signal/noise ratio was improved by exponential multiplication which introduced a few Hz line broadening. The acquisition time was changed depending upon the line widths of the samples to fit sufficient delay time. The sample temperature was maintained at 293 ± 0.5 K unless otherwise noted.

RESULTS

In order to investigate the chemical behavior of dilute Mg^{2+}(3 mM) complexes under physiological conditions, pH value was changed in the absence and presence of ATP and ADP. NMR results of the Mg^{2+} solutions are shown in Fig. 1. Increase of the spectral half-band-widths($\Delta\nu_{1/2}$) of the free Mg^{2+} around pH 9 indicates that the hydroxide complex is formed above this pH region for 3 mM Mg^{2+}. The formations of the Mg^{2+}-ATP(Mg^{2+} 3 mM;ATP 0.1 mM) and the Mg^{2+}-ADP (Mg^{2+} 3 mM;ADP 0.4 mM) complexes under these conditions are also clearly shown in Fig. 1. From these pH titration experiments, it is shown that ^{25}Mg NMR of dilute Mg^{2+}(3 mM) can be properly applied to protein system of our interest at pH 6.5 ∿ 8.0, ruling out any undesirable pH effect under those conditions.

^{25}Mg NMR($\Delta\nu_{1/2}$ = 8 Hz) of the Mg^{2+}-ATP complex(Mg^{2+} 3 mM;ATP 0.1 mM) is remarkably broadened by adding creatine kinase(0.1 mM per active site) to $\Delta\nu_{1/2}$ = 34 Hz(Table I). The spectrum of the Mg^{2+}-ATP complex becomes broad even when the protein concentration is 0.01 mM. Higher concentration of the

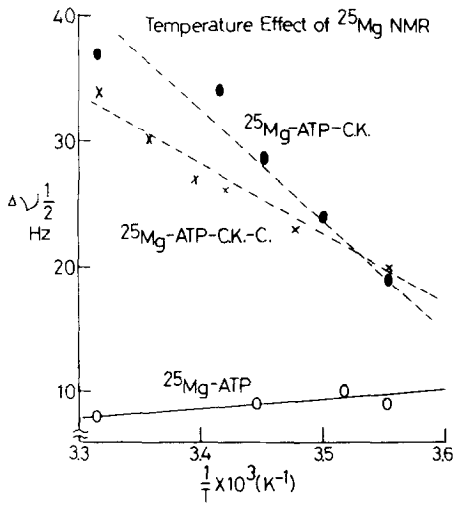
Table I. Half-band-widths($\Delta\nu_{1/2}$ in Hz) of ^{25}Mg NMR of the Mg^{2+} complexes at pH 8.0.

$MgSO_4$ 2M(pH 2.1)	4.5
$^{25}MgNO_3$ 3 mM	2
^{25}Mg(3 mM)-ATP(0.1 mM)	8
^{25}Mg(3 mM)-ATP(0.1 mM)-creatine kinase(0.1 mM)	34
^{25}Mg(3 mM)-ATP(0.1 mM)-creatine kinase(0.1 mM)-creatine(60 mM)	28
^{25}Mg(3 mM)-ADP(0.4 mM)	20
^{25}Mg(3 mM)-ADP(0.4 mM)-creatine kinase(0.1 mM)	52
^{25}Mg(3 mM)-ADP(0.4 mM)-creatine kinase(0.1 mM)-creatine(60 mM)	30
^{25}Mg(3 mM)-ADP(0.4 mM)-creatine kinase(0.1 mM)-creatine(60 mM)-NO_3^-(10 mM)	17
^{25}Mg(3 mM)-creatine kinase(0.1 mM)	7
^{25}Mg(3 mM)-ADP(0.4 mM)-bovine serum albumin(0.14 mM)	27

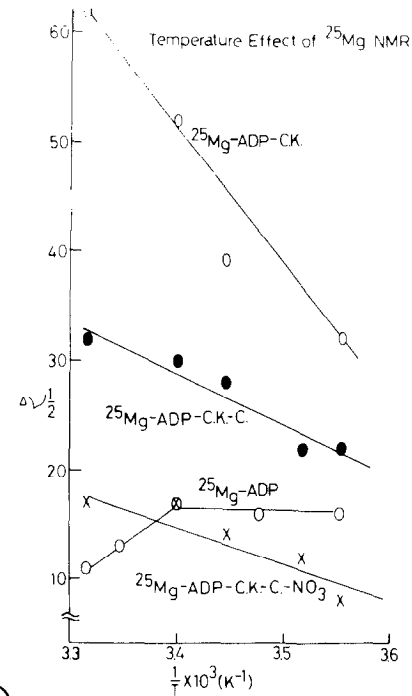
protein makes the NMR signal broad to more than 500 Hz. The half-band-width, $\Delta\nu_{1/2}$, of the ternary complex decreases to 28 Hz by adding saturated amount of creatine. The contraction of $\Delta\nu_{1/2}$ caused by addition of creatine is less pronounced at pH 6.5 than that at pH 8.0. The $\Delta\nu_{1/2}$ of the Mg^{2+}-ADP complex (Mg^{3+} 3 mM;ADP 0.4 mM) is also greatly broadened in the ternary Mg^{2+}-ADP-creatine kinase complex. Addition of creatine to the ternary ADP complex decreases the NMR band width to a certain extent. Nitrate nion, which is believed to form a transition state analogue complex(9,10), decreases the ^{25}Mg NMR line width furthermore.

To investigate whether those line broadening phenomena are exclusively observed for the Mg^{2+}-ATP(ADP)-creatine kinase system, creatine kinase was added to free Mg^{2+}(Table I). ^{25}Mg NMR of the free Mg^{2+} is broadened to a certain extent by adding creatine kinase, but the extent is much smaller than those for the nucleotide complexes. Effect of non-kinase protein on the ^{25}Mg NMR of the Mg^{2+}-ADP complex was also studied. NMR of the Mg^{2+}-ADP complex is broadened by adding bovine serum albumin, the broadeness being much smaller than those caused by creatine kinase.

Effect of temperature on the ^{25}Mg NMR was studied to investigate the exchange mechanism of Mg^{2+}. The ^{25}Mg NMR spectra of the Mg^{2+}-ATP and Mg^{2+}-ADP complexes are broader at low temperature than those at high temperature as shown in Figs. 2 and 3 where the ordinates express the $\Delta\nu_{1/2}$ in Hz plotted against reciprocal of temperature(K) as abscissa. These findings indicate that Mg^{2+} of these complexes is in the fast exchange regime like free(or hydrated) Mg^{2+}, which has the same temperature effect(not shown). In contrast with the findings for those simple molecules, the NMR line widths of the protein-bound Mg^{2+} decrease with decreasing temperature(Figs. 2 and 3), indicating that Mg^{2+} on the protein is not in the fast exchange regime. The



(2)



(3)

Figure 2. Temperature effect on ^{25}Mg NMR of the binary $^{25}Mg^{2+}$-ATP(o)(Mg^{2+} 3 mM; ATP 0.1 mM), the ternary $^{25}Mg^{2+}$-ATP-creatine kinase(●)(Mg^{2+} 3 mM;ATP 0.1 mM; creatine kinase 0.1 mM per site) and the quaternary $^{25}Mg^{2+}$-ATP-creatine kinase-creatine(x)(Mg^{2+} 3 mM;ATP 0.1 mM;creatine kinase 0.1 mM per site;creatine 60 mM) complexes.

Figure 3. Temperature effect on ^{25}Mg NMR of the binary $^{25}Mg^{2+}$-ADP(lower o) (Mg^{2+} 3 mM;ADP 0.4 mM), the ternary $^{25}Mg^{2+}$-ADP-creatine kinase(upper o)(Mg^{2+} 3 mM;ADP 0.4 mM;creatine kinase 0.1 mM per site), the quaternary $^{25}Mg^{2+}$-ADP-creatine kinase-creatine(●)(Mg^{2+} 3 mM;ADP 0.4 mM;creatine kinase 0.1 mM per site;creatine 60 mM) and the transition state analogue $^{25}Mg^{2+}$-ADP-creatine kinase-creatine-NO_3^-(x)(Mg^{2+} 3 mM;ADP 0.4 mM;creatine kinase 0.1 mM per site; creatine 60 mM;sodium nitrate 10 mM) complexes.

temperature effect on the NMR line width is pronounced for the protein-bound Mg^{2+} than for the free or the binary complexes, although the slope(minus against K^{-1}) for the protein-bound Mg^{2+} is the reverse(plus against K^{-1}) of that for the free or the binary Mg^{2+} complexes.

DISCUSSION

$^{25}Mg(I = -\frac{5}{2})$ has a large quadrupole moment and thus the relaxation rate of Mg^{2+} will be usually dominated by the quadrupole relaxation mechanism. The following equation is proposed for the quadrupole nuclei in the extreme narrowing limit(11,12)

$$\pi\Delta\nu_{1/2} = \frac{1}{T_1} = \frac{1}{T_2} = C(1 + \frac{\eta^2}{3})(\frac{e^2qQ}{\hbar})^2\tau_c \qquad (1)$$

where C is a constant value characteristic for each quadrupole nucleus, η is an asymmetry parameter that reflects the departure of the nuclear environment from the axial symmetry(η = 0 for C_{3v};η = 1 for C_{2v}), Q is the quadrupolar moment

for the nucleus(2.2×10^{-29} m^2 for Mg), q is the electrostatic field gradient at the nucleus and τ_c is a correlation time describing fluctuations in the magnitude and/or direction of the electrostatic field gradient. For small dilute molecules it may be possible to assume that the direction of the electrostatic field gradient is set up and also to normalize the line width to unit viscosity by measuring the viscosity of the sample solution, leading to semi-quantitative estimation of the quadrupolar coupling constant. However, actually it is impossible to measure the micro- or partial-viscosity around the metal ion bound to the large molecule or the protein. In addition, the binding process of Mg^{2+}-nucleotide complexes to the kinase is not describable in terms of a single two-site exchange model(12,13). In fact, temperature dependences of the NMR for the protein-bound Mg^{2+} suggest that the slow chemical exchange controls these systems and thus fast exchange limit cannot be applied to those systems, because semi-quantitative Debye-Stokes-Einstein model for reorientational correlation time τ_c indicates that the correlation time increases linearly with the ratio of the shear viscosity to temperature. Therefore, it seems like that the angle between the external magnetic field and the direction of the electric field gradient q is fixed for Mg^{2+} bound to the protein to a certain extent and the asymmetric parameter for the electric field gradient will be fairly large because of anisotropic rotational motion for the protein-bound Mg^{2+}. Since decreasing the temperature of the sample solution must make the anisotropic fluctuation of the magnetic and/or electric field gradient around the cation decrease due to lower reorientational motions, the asymmetrical contribution for the ^{25}Mg NMR will be reduced for the protein-bound Mg^{2+} at lower temperature. In addition, since the slow chemical exchange mechanism dominates the protein-bound Mg^{2+}, chemical exchange of Mg^{2+} bound to the protein may be reduced at lower temperature. Those may be part of reasons for the spectral narrowness at lower temperature. Mn^{2+} EPR results suggested that the symmetry and the mobility of binary Mn^{2+}-nucleotide complexes are essentially the same as those of the ternary complex(14). However, possible differences of the active-site environment between the Mn^{2+}- and Mg^{2+}-nucleotide-creatine kinase complexes have been suggested from ^{31}P NMR(10).

Since slow chemical exchange mechanism still dominates the ^{25}Mg NMR of the quaternary and the transition state analogue complexes(Figs. 2 and 3), the decrease of the anisotropic rotational and/or reorientational motions in those complexes can reasonably explain for the spectral narrowness of those complexes. Thus, the spectral narrowness found for the quaternary Mg^{2+}-ADP-creatine kinase-creatine complex suggests that the symmetry around the Mg^{2+} in this complex may be higher than that of the ternary complex and that the exchange rate of Mg^{2+} in this complex may be slower than that of the ternary complex. Similarly, Mg^{2+} in the transition state analogue complex may be in a more

symmetrical environment or exchange slower than those of the ternary and quaternary complexes. Or, in another words, life time of Mg^{2+} in the transition state analogue complex seems to be longer than those in the ternary and quaternary complexes. Infrared absorption results on anions(NO_3^- etc.) in the transition state analogue complexes provided convincing evidence for ligating of the anions to the activating divalent cations(Mg^{2+} etc.) and suggested that the divalent metal ion would be attached to one of the in-plane oxygens of the trigonal bipyramid in the transition state(9). Mn^{2+} EPR spectra on the quaternary and the transition state analogue Mn^{2+} complexes suggested that creatine or nitrate induces a change in structure around the active site which imposes a distortion or an asymmetry upon the Mn^{2+} environment or reduces the mobility of the Mn^{2+} in the coordination sphere(14). ^{25}Mg NMR results are in accordance with the latter suggestion. Possible difference of active-site structure between the Mn^{2+} and Mg^{2+} complexes should be reminded here(10). The equilibrium unbalance of the complex caused by those effectors should also be carefully considered for the ^{25}Mg NMR analyses.

^{25}Mg NMR of the free Mg^{2+} is broadened by adding creatine kinase, but the broadened effect observed for the free Mg^{2+} is much smaller than that observed for the nucleotide complexes. This will reflect that the binding constant of the free Mg^{2+} to the protein is much smaller(by fivefold) than that of the Mg^{2+}-ADP complex to the protein. This suggestion, of course, can be drawn from the assumption that the symmetry of the Mg^{2+} and/or the exchange rate of the Mg^{2+} in the non-nucleotide protein complex are exactly the same as those of the nucleotide protein complex. Actually the Mg^{2+} binding-site of the protein may be different from the Mg^{2+}-ADP binding-site of the protein. Similarly, it seems to be much easier for the Mg^{2+}-ADP complex to bind to creatine kinase(by threefold) than to bovine serum albumin on the assumption that the symmetry and/or the exchange rate of the Mg^{2+}-ADP complex bound to bovine serum albumin is not different from those of the complex bound to creatine kinase.

We know that these NMR data are not sufficient to solve the binding character of Mg^{2+} to the nucleotides and the protein, since the NMR spectra shown in this paper are ascribed to Mg^{2+} partly bound to the protein and most of Mg^{2+} in the solution is in a free or hydrated state. In addition, exact equilibrium study is practically impossible because the line broadening effect by adding the protein is quite large and we observe just small part of the effect. However, it should be emphasized that qualitative estimation of the symmetry, exchange or binding behavior of Mg^{2+} on the protein will be certainly feasible even for the ternary and quaternary complexes in terms of the ^{25}Mg NMR spectra.

REFERENCES

1. Magnusson, J. A. and Bother-By, A. A. (1971) in Magnetic Resonances in Biological Research(Franconi, C. ed.), pp.365-376, Gordon and Breach, New York.
2. Bryant, R. G. (1972) J. Magn. Res. 6, 159-166.
3. Robertson, Jr., P., Hiskey, R. G. and Koehler, K. A. (1978) J. Biol. Chem. 253, 5880-5883.
4. Robertson, Jr., P., Koehler, K. A. and Hiskey, R. G. (1979) Biochem. Biophys. Res. Commun. 86, 265-270.
5. Marsh, H. C., Robertson, Jr., P., Scott, M. E., Koehler, K. A. and Hiskey, R. G. (1979) J. Biol. Chem. 254, 10268-10275.
6. Reimarsson, P., Parello, J., Drankenberg, T., Gustavsson, H. and Lindman, B. (1979) FEBS Lett. 108, 439-442.
7. Rose, D. M., Bleam, M. L., Record, Jr., M. T. and Bryant, R. G. (1980) Proc. Natl. Acad. Sci. USA 77, 6289-6292.
8. Andersson, T., Drankenberg, T., Forsén, S. and Thulin, E. (1981) FEBS Lett. 125, 39-43.
9. Reed, G. H., Barlow, C. H. and Burns, Jr., R. A. (1978) J. Biol. Chem. 253, 4153-4158.
10. Nageswara Rao, B. D. and Cohn, M. (1981) J. Biol. Chem. 256, 1716-1721.
11. Harris, R. K. (1978) in NMR and the Periodic Table (Harris, R. K. and Mann, B. E. eds.) pp.1-19, Academic Press, London.
12. Forsén, S. and Lindman, B. (1981) Ann. Rep. NMR Spectr. 11A, 183-226.
13. Swift, T. J. and Connick, R. E. (1962) J. Chem. Phys. 37, 307-320.
14. Reed, G. H. and Cohn, M. (1972) J. Biol. Chem. 247, 3073-3081.