^{25}Mg and ^{31}P NMR Studies of Acetate Kinase from *Bacillus stearothermophilus*

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Received June 1, 1983

Acetate kinase is widely distributed in microorganisms and is involved in the phosphorization of acetate anion [1–3]. Like other kinases, Mg^{2+} is essential for the phosphorization to proceed. Acetate kinase purified from *Bacillus stearothermophilus* is a tetrameric enzyme consisting of identical subunits, each of which has a molecular weight of about 40,000, and is unique in that the enzymatic activity is maintained even at 65 °C over one hour [1–3].

We have previously shown that ^{25}Mg NMR can be applicable to biological systems such as ternary Mg^{2+}–ATP(and ADP)–creatine kinase solutions [4]. We present here ^{25}Mg NMR studies extended to acetate kinase system, showing that ^{25}Mg NMR spectra of some kinase solutions are different from each other and thus can be very useful for studying the active-site structure of kinases. Furthermore, ^{31}P NMR studices confirmed the suggestion deduced from ^{25}Mg NMR findings on the active-site structure of the kinase.

Experimental

^{25}Mg was purchased from Prochem Co. as 95.66% MgO. Acetate kinase purified from *Bacillus stearothermophilus* was obtained from Unitika Co., Osaka. The enzyme was homogeneous upon polyacrylamide

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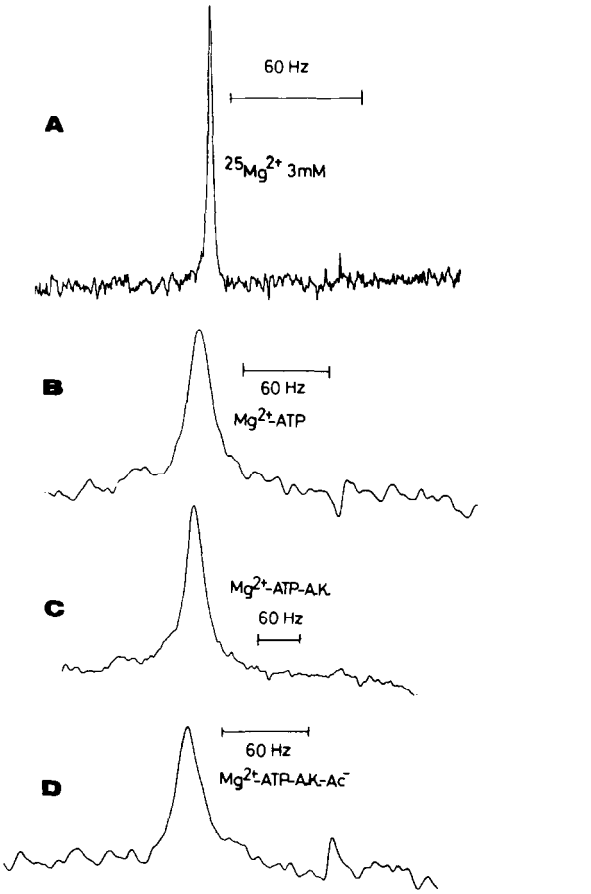

Fig. 1. Typical ^{25}Mg NMR of (A) enriched $MgCl_2$ (3 m*M*); (B) enriched Mg^{2+}(3 m*M*)–ATP(0.2 m*M*); (C) enriched Mg^{2+}(3 m*M*)–ATP(0.2 m*M*)–acetate kinase (0.018 m*M*); (D) enriched Mg^{2+}(3 m*M*)–ATP(0.2 m*M*)–acetate kinase (0.018 m*M*)–acetate anion (100 m*M*) in 50 m*M* HEPES-10% glycerol buffer (pH 7.2). They consisted of collecting 800 transients (A) or 15,000 transients (B)–(D) using 90° pulse (80 μs), 4k data points and an acquisition time of 4 s(A) or 0.25 s(B)–(D) over 5,000 Hz spectral window in quadrature detection mode. Signal/noise was improved by using exponential line-broadenings of 0.4 Hz(A) or 5.0 Hz (B)–(D).

TABLE I. Half-band Widths ($\Delta\nu_{1/2}$) of Selected ^{25}Mg NMR of the Acetate Kinase Solutions.

Solutions	$\Delta\nu_{1/2}$ (Hz)
$^{25}MgCl_2$ (3 m*M*)	4.2
$^{25}Mg^{2+}$ (3 m*M*)–ATP(0.2 m*M*)	14.5
$^{25}Mg^{2+}$ (3 m*M*)–ATP(0.2 m*M*)–enzyme(0.0287 m*M*)	29.5
$^{25}Mg^{2+}$ (3 m*M*)–ATP(0.2 m*M*)–enzyme(0.0287 m*M*)–acetate anion(100 m*M*)	22.0
$^{25}Mg^{2+}$ (3 m*M*)–ADP(0.4 m*M*)	20.0
$^{25}Mg^{2+}$ (3 m*M*)–ADP(0.4 m*M*)–enzyme(0.0225 m*M*)	57.6
$^{25}Mg^{2+}$ (3 m*M*)–ADP(0.4 m*M*)–enzyme(0.0225 m*M*)–acetate anion (46 m*M*)	35.7
$^{25}Mg^{2+}$ (3 m*M*)–ADP(0.4 m*M*)–enzyme(0.0225 m*M*)–acetate anion (46 m*M*–KNO_3(10 m*M*)	39.7

0020-1693/83/$3.00

TABLE II. ^{31}P NMR Spectra of ATP and ADP Complexes of Acetate Kinase.

Complexes	δ (ppm from 85% H_3PO_4)					
	ATP			ADP		
	α-P	β-P	γ-P	α-P	β-P	
free substrates	10.77	21.94	7.47	10.49	7.15	
free substrates–Mg^{2+}	10.29	18.73	5.26	9.92	6.11	
enzyme–ATP	10.85	21.50	6.39			
enzyme–ATP–Mg^{2+}	10.00	19.45	5.00			
enzyme–ATP–Mg^{2+}–acetate anion	9.84	19.25	4.70			
enzyme ADP				10.33	6.43	
enzyme–ADP–Mg^{2+}				10.00	6.15	4.94
enzyme–ADP–Mg^{2+}–acetate anion				9.92	6.23	5.06
enzyme–ADP–Mg^{2+}–acetate anion–NO_3^-				9.96	6.19	4.86

^{31}P NMR spectra were obtained for 0.25 m*M* ATP and 0.5 m*M* ADP solution in the presence of equimolar or excess Mg^{2+}, enzyme, substrate or KNO_3. These compounds were added to the ATP or ADP solution until spectral changes were saturated. They consisted of collecting 12,000–32,000 transients using 47° pulse (9 μs), 4k data points and an acquisition time of 0.21 s over 10,000 Hz spectral window in quadrature detection mode.

gel electrophoresis. NMR spectra were obtained in 50 m*M* N-2-hydroxyethylpiperazine-N′-2-ethenesulfonic acid(HEPES)-10% glycerol buffer (pH 7.2) at 25 °C. ^{25}Mg and ^{31}P NMR spectra were accumulated on a Bruker CXP-300 FT NMR spectrometer at 18.36 MHz and 121.48 MHz, respectively, with external D_2O for the frequency lock [4].

Results

Figure 1 shows typical ^{25}Mg NMR spectra of aqueous Mg^{2+}, Mg^{2+}–ATP, Mg^{2+}–ATP–acetate kinase and Mg^{2+}–ATP–acetate anion–acetate kinase solutions. Table I summarizes selected ^{25}Mg NMR findings observed for acetate kinase solutions. The half-band width ($\Delta\nu_{1/2}$) (4.2 Hz) of aqueous $^{25}Mg^{2+}$ increased linearly by adding ATP. Mg^{2+}–ATP (1:1) solution must have $\Delta\nu_{1/2}$ more than 600 Hz, not detectable under our experimental conditions. Addition of acetate kinase to the Mg^{2+} (3 m*M*)–ATP (0.2 m*M*) solution markedly increased the $\Delta\nu_{1/2}$ of the solution. The broadened $\Delta\nu_{1/2}$ of the Mg^{2+}–ATP–enzyme solution was decreased by adding acetate anion. The decrease of $\Delta\nu_{1/2}$ of the ternary solution caused by adding acetate anion seemed likely to be a distinctive feature for the quaternary solution. Mg^{2+}–ADP solution provided ^{25}Mg NMR spectral features similar to those observed for the Mg^{2+}–ATP solution. Thus, the broadened $\Delta\nu_{1/2}$ of the Mg^{2+} (3 m*M*)–ADP(0.4 m*M*) solution was further enlarged by adding the enzyme, whereas the $\Delta\nu_{1/2}$ of the Mg^{2+}–ATP–enzyme solution was decreased by adding acetate anion (Table I). It is known that some anions such as nitrate or thiocyanate form a transition state analogue in the Mg^{2+}–ADP–substrate–kinase quaternary complex by mimicking the migrating phosphoryl group [5, 6]. The $\Delta\nu_{1/2}$ of the Mg^{2+}–ADP–acetate anion–acetate kinase solution was enlarged by adding NO_3^-. The ^{25}Mg NMR change caused by adding NO_3^- is in contrast with that observed for the creatine kinase solution, where addition of NO_3^- decreased $\Delta\nu_{1/2}$ [4]. The $\Delta\nu_{1/2}$ of the acetate kinase solutions measured here all decreased as temperature was lowered.

^{31}P NMR spectra of the Mg^{2+}–ATP, ADP–acetate kinase complexes are summarized in Table II. The shift of the γ-peak of ATP caused by adding the enzyme was more than 1 ppm, which is decidedly larger than those of the α- and β-peaks. The marked ^{31}P NMR shifts of ATP caused by adding kinases were not observed for creatine kinase [7] and arginine kinase [8, 9]. The shift of the α-peak of the ATP–acetate kinase complex caused by adding Mg^{2+} was almost twice that of free ATP, while the shifts of the β- and γ-peaks of the ATP–enzyme complex were almost two-thirds of those of free ATP. The α- and β-peaks of ADP shifted to down-field by 0.16 ppm and 0.72 ppm, respectively, by adding acetate kinase to the ADP solution. These shifts were not so large as those observed by adding creatine kinase and arginine kinase [7–9]. The β-peak of ADP bound to acetate kinase was split into doublets by adding Mg^{2+} to the solution. The split of the β-peak of ADP bound to creatine kinase was seen only in the presence of both Mg^{2+} and creatine [7]. The down-field shift of the β-peaks caused by adding NO_3^- to the Mg^{2+}–ADP–acetate anion–acetate kinase

complex was different from those observed for creatine kinase and arginine kinase, since the β-peaks of the Mg^{2+}–ADP–creatine kinase and Mg^{2+}–ADP–arginine–arginine kinase complexes moved to upper-field upon adding NO_3^- [7, 9].

Discussion

The $\Delta\nu_{1/2}$ values of the ^{25}Mg NMR, which are associated with the transverse relaxation rate, are attributed to various causes such as (1) chemical exchange rate of Mg^{2+} (between free Mg^{2+} and Mg^{2+} bound to ATP(ADP) or protein; (2) reorientational motion of the Mg^{2+} nucleus, Mg^{2+}–ATP-(ADP) complex or whole protein molecule; (3) symmetry around the Mg^{2+} nucleus. However, from temperature dependence studies on the ^{25}Mg NMR spectra, it seems likely that chemical exchange mechanism dominates the $\Delta\nu_{1/2}$ of ^{25}Mg NMR of the Mg^{2+}–ATP(ADP)–acetate kinase solutions [4, 10]. The exchange rate of Mg^{2+} in the Mg^{2+}–ATP(ADP)–acetate anion–acetate kinase solution may be lower than that in the Mg^{2+}–ATP(ADP)–acetate kinase solution. A symmetrical factor, which the quadrupole coupling constant in part contributes to, may influence the $\Delta\nu_{1/2}$ of the ^{25}Mg NMR. Thus, the symmetry around Mg^{2+} in the quaternary solution may be higher than those of the corresponding ternary solution. Likewise, the exchange rate and/or the symmetry of Mg^{2+} in the NO_3^--bound quaternary ADP solution of acetate kinase may be different from those of creatine kinase and arginine kinase.

^{31}P NMR spectral features observed for acetate kinase are different from those observed for creatine kinase and arginine kinase in some respects. Mg^{2+} may specifically interact with the α-phosphorus group of ATP in acetate kinase under the conditions studied here. The change of the α-phosphorus environment of ATP was not observed for the ternary creatine kinase and arginine kinase complexes. In addition, the β-phosphorus group of ADP will be situated at two environmentally different binding sites in the Mg^{2+}–ADP–acetate kinase ternary complex and they exchange slowly in the NMR time scale. For creatine kinase and arginine kinase, the two binding sites of the β-phosphorus of ADP on the enzyme exist only in the quaternary complex.

^{25}Mg and ^{31}P NMR findings of the Mg^{2+}–ADP–substrate–enzyme–NO_3^- solution for acetate kinase were in contrast with those observed for creatine kinase and arginine kinase. Thus, it seems likely that NO_3^- cannot form the transition state analogue in acetate kinase. EPR spectrum of the Mn^{2+}–ADP–acetate anion–acetate kinase–NO_3^- solution was different from that of the corresponding creatine kinase or arginine kinase solution, but was the same as that of the Mn^{2+}–ADP solution (unpublished results).

Therefore, from comparative ^{25}Mg and ^{31}P NMR studied, it is suggested that the active-site structure of acetate kinase, especially of the Mg^{2+}–ADP–substrate–enzyme–NO_3^- complex, is different from those of corresponding creatine kinase and arginine kinase complexes. Kinases having oxygenous substrates such as acetate (CH_3COO^-) may have active-site structures different from those having nitrogenous substrates such as creatine($H_2NC(NH)N(CH_3)CH_2COOH$) and arginine ($H_2NC(NH)_2CH_2CH_2CH_2CH(NH_2)COOH$) [7, 9]. It should be emphasized again that ^{25}Mg NMR can provide quite new qualitative information on the active-site of the enzyme and that ^{31}P NMR can provide complementary knowledge and confirmed the suggestion deduced from ^{25}Mg NMR studies.

Acknowledgements

We are grateful to Unitika, Co. for providing the enzyme. This work was supported in part by a grant from Nissan Science Foundation and by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan to M.H.

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