The conformation of ATP in the presence of Mg$^{2+}$ and/or spermine was studied by $^{31}$P and $^1$H NMR to clarify how polyanines interact with ATP. Spermine predominantly interacted with the $\beta$- and $\gamma$-phosphates of ATP in the presence of Mg$^{2+}$. A conformational change of the $\beta$- and $\gamma$-phosphate of ATP with spermine could not be observed in the absence of Mg$^{2+}$ by $^{31}$P NMR. It was found by $^1$H NMR that the conformation of adenosine moiety of ATP was not influenced significantly by spermine. The binding of Mg$^{2+}$ to ATP was slightly inhibited by spermine and vice versa. The results indicate that the binding sites of Mg$^{2+}$ and spermine on ATP only partially overlap. The PotA protein, an ATP-dependent enzyme, was used as a model system to study the biological role of the ATP-Mg$^{2+}$-spermine complex. The ATPase activity of PotA was greatly enhanced by spermine. Double reciprocal plots at several concentrations of spermine as an activator indicate that spermine interacts with ATP, but not with PotA. The activity of protein kinase A was also stimulated about 2-fold by spermine. The results suggest that a ternary complex of ATP-Mg$^{2+}$-spermine may play an important role in some ATP-dependent reactions in vivo and in the physiological effects of endogenous polyanimes.

Adenosine 5'-triphosphate (ATP) plays a central role in cell physiology, for example as an energy source and as part of signal transduction cascades involving phosphorylation of proteins. However, the conformation of ATP under physiological conditions is not clear, and several different conformations of ATP have been reported. X-ray crystal analysis of Na$_2$ATP showed that the adenine base is syn oriented toward the ribose moiety when the crystal contains eight ATP molecules, sixteen sodium ions and twenty-four water molecules in a unit cell (1). It has been reported that the syn and anti conformations are present in equal proportions in the absence of monovalent and divalent cations at neutral pH, but that the adenine base shifted to the anti orientation in the presence of Mg$^{2+}$ as determined using $^1$H, $^{13}$C and $^{31}$P NMR (2–6). This was most probably because of an ionic interaction between Mg$^{2+}$ and the $\beta$- and $\gamma$-phosphates of ATP. The anti orientation of the base toward the ribose moiety was also shown in ATP-Mg$^{2+}$-enzyme complexes (7, 8). Therefore, it is clear that the conformation of ATP is strongly influenced by cations, and such a conformational restriction of ATP by cations is probably important for efficient recognition of ATP by some enzymes.

Polyamines (putrescine, spermidine, and spermine), which are divalent and polyvalent cations, are important for cell growth and are present at millimolar concentrations, comparable with the concentrations of ATP and Mg$^{2+}$ in animal cells (9, 10). It is also known that polyamines can interact with ATP (11–13). However, in the presence of physiological concentrations of K$^+$ and Mg$^{2+}$ the interaction between spermine (or spermidine) and ATP is weak, and it was thought that polyamines compete with Mg$^{2+}$ for binding to ATP (11, 12). Recently, it was reported that polyamines interact with an ATP-Mg$^{2+}$ complex as well as with ATP (13). Thus, we have carried out experiments to study in detail the physico-chemical characteristics of the ATP-Mg$^{2+}$-spermine complex and the physiological significance of this complex. We found that some biological reactions involving ATP and Mg$^{2+}$ are enhanced by an anti oriented ternary complex containing ATP, Mg$^{2+}$, and spermine.

**EXPERIMENTAL PROCEDURES**

**NMR Analysis**—ATP was dissolved in water and NaOH was added to adjust pH to 7. Then sample solutions (0.4 ml), containing 3 mM ATP, 10 mM Tris-d$_{11}$ (99 atom %D)-HCl, pH 7.8, in the absence or presence of 3 mM Mg$^{2+}$, were prepared. The solutions were evaporated to dryness in vacuo and redissolved in 0.1 ml D$_2$O (99.85 atom %D). The solution was evaporated again and dissolved in the original volume of D$_2$O (99.996 atom D%). Spermine titrations were performed by adding microliter amounts of 0.1 mM spermine in D$_2$O directly to the sample in a 5-mm NMR tube. All spectra were taken using a Bruker DRX500 spectrometer at the operating frequency of 500 MHz for $^1$H and a probe temperature of 298 K. The operation conditions for one-dimensional spectra were as follows: sweep width, 6 kHz, data points, 32,000, and acquisitions, 40. The coupling constant $^{3}$J$_{H1H2}$ was obtained from the splitting of the H$^1$ resonance, whereas the coupling constant $^{3}$J$_{H2H3}$ was not clearly determined. The value of $^{3}$J$_{H2H3}$ was therefore assumed to be equal to 10 Hz as found for a number of nucleotides, and the fractional populations of the C2'-endo conformer and the C3'-endo conformer were obtained with the formulas C2'-endo = $^{3}$J$_{H1H2}$ / ($^{3}$J$_{H1H2}$ + $^{3}$J$_{H2H3}$) and C3'-endo = 1 - C2'-endo (14).

Two-dimensional double quantum filtered-chemical shift correlated spectroscopy, and nuclear Overhauser effect spectroscopy (NOESY) were recorded in the phase-sensitive mode. For all two-dimensional experiments, 512 free induction decays of 2,000 data points were collected by using the states-TPPI (time proportional phase incrementation) method (15), and spectra of 1,000 × 2,000 were obtained with zero-filling, after which a two-dimensional Fourier transformation with $\pi/2$ shifted squared sine-bell window function for F2 dimension and $\pi/2$
shifted sine-bell window function for F1 dimension was performed. The mixing time for NOESY measurement was 500 ms.

\[ \text{31P NMR experiments were performed with a } 31 \text{P frequency of 202.46 MHz at a probe temperature of 298 K. All } 31 \text{P spectra were recorded by using 45° pulse without proton decoupling. The operation conditions were as follows: pulse repetition delay, 0.5 s; sweep width, 7 kHz; data points, 8 k; and acquisitions, 512. Before the fast Fourier transformation an exponential multiplication (line broadening factor of 3 Hz) was applied. Chemical shifts were indicated by ppm from the signal of } \text{H}_2\text{PO}_4^- \text{as an external standard.} \]

**Determination of the Binding Constants of Spermine and Mg}^{2+} \text{ for ATP}**—The binding constant in the presence of 100 mM K\(^+\) was determined by the spectrophotometric resin competition procedure of Jenkins (16) with some modifications (11). Binding of ATP to cationic resin and to cations is a competitive interaction. 20 mg of AG1-X2 (200–400 mesh, Bio-Rad) were used as a cationic resin. The resin, ATP (100 \(\mu\)M), and various amounts of spermine and/or Mg\(^{2+}\) were added in 50 ml of Buffer I (10 mM Tris-HCl, pH 7.5, and 100 mM KCl). Absorbance at 260 nm (A\(_{260}\)) was measured after stirring for 30 min at room temperature. When the binding constant of spermine for ATP-Mg\(^{2+}\) or Mg\(^{2+}\) for ATP-spermine was determined, 1 \(\mu\)M Mg\(^{2+}\) or 2 mM spermine was included in Buffer I, so that more than 85% of 100 \(\mu\)M ATP can make a complex with Mg\(^{2+}\) or spermine.

**Assays for PotA ATPase and Protein Kinase A—PotA**, a spermidine uptake protein that has ATPase activity, was purified to homogeneity as described previously (17). The reaction mixture (0.025 ml) for ATPase activity containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1.5 mM magnesium acetate, 0.5 mM \([\gamma-\text{32P}]\text{ATP} \text{(specific activity, 40 to 60 cpm/pmol)}, \) and 0.5 \(\mu\)g of purified PotA, was incubated at 30 °C for 20 min. The released \(\text{32Pi} \) was determined by the spectrophotometric resin competition procedure of Jenkins et al. (18). Kinetic analysis to examine whether spermine binds to ATP or enzyme was performed according to the method of Dixson and Webb (19).

Protein kinase A activity was measured by the method of Denis et al. (20). The reaction mixture (0.05 ml) containing 50 mM MOPS-NaOH, pH 7.5, 30 mM KCl, 1 mM magnesium acetate, 1 mM dithiothreitol, 0.3 mM \([\gamma-\text{32P}]\text{ATP} \text{(specific activity, } 800–1,200 \text{ cpm/pmol)}, 0.1 \text{ mg/ml bovine serum albumin}, 160 \mu\text{M kemptide (amino acid sequence; LR-RASLG)}, \) and 0.01 milliunits of protein kinase A, catalytic subunit (Boehringer Mannheim), was incubated at 30 °C for 10 min. Phosphorylated kemptide was collected on P81 filter (Whatman), and the radioactivity was counted in a liquid scintillation counter.

**RESULTS**

*Formation of a Complex Containing ATP, Mg}^{2+}, \text{and Spermine}—Intracellular concentrations of ATP and polyamines are 2 mM or more in bovine lymphocytes and rat liver (11). Thus, NMR experiments were performed using 3 mM ATP, Mg\(^{2+}\), and spermine under standard conditions. The \(31 \text{P signals of } \alpha-, \beta-, \text{ and } \gamma\)-phosphates of ATP in the presence of Mg\(^{2+}\) appeared at \(-10.5, -18.8, \text{ and } -5.2 \text{ ppm, respectively. When spermine was added, the } \beta\)-phosphate resonance broadened significantly and the \(\gamma\)-phosphate resonance also broadened (Fig. 1). This broadening probably indicates that the conformation around the \(\beta\)-phosphate is exchanging slowly. In contrast, in the presence of 3 mM spermine and absence of Mg\(^{2+}\), the signals of all three phosphates remained sharp, although a slight change of chemical shifts was observed compared with those without spermine (Fig. 2, A and B). Thus, a significant conformational change of ATP by spermine was observed only in the presence of Mg\(^{2+}\). The change of \(31 \text{P} \) spectra of ATP was almost identical under the conditions Tris-DCl (pH 7.8), D\(_2\)O (pH 7.0), and D\(_2\)O (pH 7.8). In contrast, under acidic conditions (pH 3.3), the chemical shifts of \(\alpha-, \beta-, \text{ and } \gamma\)-phosphates of ATP in the presence of Mg\(^{2+}\) were not changed significantly during titration with spermine. Spectra recorded in the absence and presence of 3 mM spermine are shown in Fig. 2, C and D. The results suggest that a ternary complex of ATP, Mg\(^{2+}\), and spermine is formed at neutral pH but not at acidic pH.*

We further investigated how spermine interacts with an ATP-Mg\(^{2+}\) complex. The \(1 \text{H} \) NMR signals of ATP and spermine, in a solution containing ATP, Mg\(^{2+}\), and spermine (3 mM each) at pH 7.8, were assigned by the analysis of double quantum filtered-chemical shift correlated spectroscopy and NOESY spectra. Each of the \(1 \text{H} \) signals of spermine showed little...
change following the addition of Mg$^{2+}$ or ATP-Mg$^{2+}$ (data not shown), suggesting that spermine interaction with $\beta$- and $\gamma$-phosphates of ATP does not influence the conformation of adenosine moiety. In the NOESY spectra, intramolecular NOE cross peaks were observed only between adjacent protons of spermine, i.e., 1,1'/2,2', 2,2'/3,3', and 4,4'/5,5' (Fig. 3A), indicating that upon the interaction with ATP, spermine exists primarily in a linear conformation, as was reported for the conformation of spermidine in the PotD-spermidine complex (21). Only weak intermolecular NOE cross peaks between H1,1'/3,3' signals of spermine and H8, H5/5' of ATP were observed for the NOESY spectrum recorded at 10 °C, indicating that spermine mainly interacts with the phosphate groups of the ATP-Mg$^{2+}$ complex (data not shown).

As for ATP in the complex, strong NOE between H8 and H2' was observed, whereas NOE between H8 and H3' was less strong (Fig. 3B). Only weak NOE between H8 and H1' was observed for the NOESY spectrum. These results indicate that the glycosidic bond adopts an anti conformation in the ATP-Mg$^{2+}$-spermine complex at neutral pH, similar to the conformation seen in the binary ATP-Mg$^{2+}$ complex. The three-bond coupling, $^{3}J_{H\text{1}/H\text{1}'}$, in the ribose moiety of the ATP-Mg$^{2+}$-spermine complex was observed at 5.6 Hz in the $^1$H NMR spectra, whereas the value in the ATP-Mg$^{2+}$ binary complex was at 6.0 Hz. Thus, the fractional populations of the C2'-endo conformer and the C3'-endo conformer were only slightly affected by the formation of the complex (from 60 to 56%). In addition, the chemical shifts of only H5' and H5 in the ribose moiety were changed by the addition of spermine (Fig. 4). These results confirm that spermine binds to the phosphate groups rather than adenine or ribose moiety of ATP.

Molar Ratio in the Complex of ATP, Mg$^{2+}$, and Spermine—Fig. 5 shows the relative intensity of $^{31}$P signals of $\beta$- and $\gamma$-phosphates to $\alpha$-phosphate, whose signal is constant during the spermine titration. The titration curve showed that the saturation of both the $\beta$- and $\gamma$-phosphate signals of ATP occurred at approximately 2:1 molar ratio of ATP-Mg$^{2+}$ to spermine. This value is consistent with the charge ratio at neutral condition; −2 for ATP-Mg$^{2+}$ and +4 for spermine.

Determination of Binding Constants of Spermine and Mg$^{2+}$ for ATP—The binding constants of spermine and Mg$^{2+}$ for ATP were determined in the presence of 100 mM K$^+$ at pH 7.5 by the spectrophotometric resin competition procedure. The binding constants of spermine and Mg$^{2+}$ for ATP were $2.70 \times 10^{3}$ M$^{-1}$ and $6.94 \times 10^{3}$ M$^{-1}$, respectively (Fig. 6, A and B). Thus, the affinity of Mg$^{2+}$ for ATP was about 2- to 3-fold higher than that of spermine for ATP. Decrease in the binding of spermine to ATP by Mg$^{2+}$ was not significant, and the binding constant of spermine for ATP-Mg$^{2+}$ was $1.06 \times 10^{3}$ M$^{-1}$ (Fig. 6C). Decrease in the binding of Mg$^{2+}$ to ATP by spermine was also not significant, and the binding constant of Mg$^{2+}$ for ATP-spermine was $2.83 \times 10^{3}$ M$^{-1}$. The results indicate that the binding sites of Mg$^{2+}$ and spermine on ATP only partially overlap and support an idea that spermine can make a ternary complex with ATP-Mg$^{2+}$.

Effect of Spermine on the Activities of PotA ATPase and Protein Kinase A—We examined how the ternary complex of ATP-Mg$^{2+}$, and spermidine influences biological activity. The effect of spermine on the ATPase activity of PotA, one of the four components of a spermidine uptake system (22), was first examined. As shown in Fig. 7, A and B, ATPase activity was...
greatly stimulated by spermine in the presence of 1–10 mM Mg^{2+}. In the presence of 1–2 mM Mg^{2+}, close to the physiological concentration of Mg^{2+} in mammalian cells, little or no activity was observed in the absence of spermine. Mg^{2+} alone (3–10 mM) could enhance ATPase activity (Fig. 7B), but spermine, at concentrations up to 10 mM, did not enhance ATPase activity in the absence of Mg^{2+} (data not shown). The addition of 50 mM K^{+} inhibited ATPase significantly when ATP-Mg^{2+} complex was used. The results suggest that the ATP-Mg^{2+}-spermine complex is more stable than the ATP-Mg^{2+} complex at the physiological concentrations of K^{+}. The optimal pH was shifted from 6.9 to 8.1 in the presence of spermine (Fig. 7C). The pH dependence of ATPase was similar to that for formation of the ATP-Mg^{2+}-spermine complex, because the complex formation weakened with decreasing pH.

DISCUSSION

Polyamines are known to function as cell-proliferating factors through their interaction with nucleic acids, phospholipids, and nucleotides, including ATP. However, it is still not clear how polyamines interact with these acidic substances. In this study, we found that spermine can form a ternary complex with ATP and Mg^{2+}. The increased broadening of both the β-
and γ-phosphate resonances in the 31P NMR and the decrease in the ratio of the peak heights of δα and γα suggest that spermine interacts with Mg2+-ATP predominantly at the β- and γ-phosphates of ATP. As indicated above, this broadening probably indicates that the conformation around the β-phosphate is exchanging slowly. Because there was no significant binding of Mg2+ to spermine in our 1H NMR experiments, the binding of spermine to ATP can be attributed to the ATP-Mg2+ binary complex. The fixation of adenine to the anti with respect to the ribose moiety by Mg2+ may be important for the conformational change of β- and γ-phosphates of ATP by spermine.

Because NOE was observed only between adjacent protons of spermine, the conformation of spermine is likely linear in the ATP-Mg2+-spermine complex. Because intramolecular NOE cross peaks between spermine and the adenosine moiety of ATP were not observed, it is concluded that spermine interacts with the triphosphate group but not with the adenosine moiety of ATP. In the ATP-Mg2+-spermine complex, adenine was mainly fixed anti with respect to the ribose moiety, which was in the equilibrium between the C2′-endo and C3′-endo conformers as found in the ATP-Mg2+ complex. The different orientation of the β- and γ-phosphates in the ATP-Mg2+-spermine complex may be important for recognition of the ATP complex at the active site of enzymes and/or for the subsequent hydrolysis of ATP. A similar structure, as for the relative position of phosphate, metal, and NH2 (or NH) group, has been reported in the Co(NH3)4-ATP ternary complex (24) and the Zn2+-ATP-2,2′-bipyridyl complex (25).

It is hypothesized that polyamines modulate reactions involving ATP, such as those of ATPases and protein kinases, through a ternary complex containing ATP, Mg2+, and spermine. Indeed, we found that the activities of PotA ATPase and protein kinase A were enhanced by spermine in in vitro systems. In the case of PotA ATPase, stimulation occurred through spermine binding to an ATP-Mg2+ complex, and strong stimulation was observed when about 10–20% of the ATP-Mg2+ was complexed with spermine. Enhancement of PotA ATPase by spermidine and putrescine was also observed, but 2 times more spermidine and 100 times more putrescine were necessary to cause the similar effect to spermine (data not shown). Accordingly, the important questions are whether an ATP-Mg2+-polyamine complex is made in vivo and whether the complex can modulate reactions involving ATP in vivo. We estimated the cellular distribution of polyamines from their binding constants for DNA, RNA, phospholipids, and ATP, and their total concentration in cells (11). The binding constants of spermine and spermidine for ATP were estimated to be 8.93 × 102 M−1 and 1.06 × 103 M−1, respectively, in the presence of 2 mM Mg2+ and 100 mM K+, close to the physiological ionic conditions (11). These are consistent with the present results; the binding constants of spermine (1.06 × 103 M−1) and spermidine (5.59 × 102 M−1) for ATP-Mg2+ in the presence of 100 mM K+. Thus, it was calculated that about 10% ATP (equivalent to 0.3 mM) exists as the ATP-Mg2+-spermine (or spermidine) complex in ConA-activated bovine lymphocytes. The percentage of the ternary complex is parallel with polyamine contents in cells. Thus, it seems very likely that some reactions involving ATP are regulated by polyamines in rapidly growing cells, in which polyamine contents greatly increase (9, 10).

We have proposed that the cell proliferative effects of polyamines in both Escherichia coli (26, 27) and animal cells (28, 29) are mainly caused by the stimulation of protein synthesis through interactions of polyamines with RNA (11). However, polyamines may also be involved in cell proliferation and differentiation through reactions, such as phosphorylation and dephosphorylation involving ATP. It has been reported that protein kinase CK2 activity is stimulated by polyamines through interactions with the β-subunit of CK2 (30). In this system, however, the contribution of structural changes of ATP-Mg2+ by polyamines has not been studied. There is also a report that induction of ornithine decarboxylase, a rate-limiting enzyme of polyamine biosynthesis, causes an enhanced
level of tyrosine phosphorylation, particularly of p130CAS (31). Bachrach and co-workers recently reported in the meeting that activities of tyrosine kinase and mitogen-activated protein kinases were stimulated by polyamines. It has been reported that phosphorylation of Okazaki-like DNA fragments in mammalian cells is strongly stimulated by polyamines (32). Thus, the complex of ATP-Mg<sup>2+</sup>-spermine may also be involved in the phosphorylation of DNA or RNA.

In *E. coli*, we estimated that at least 30% of ATP exists as the ATP-putrescine complex (33). Now, the complex is thought to be the ATP-Mg<sup>2+</sup>-putrescine complex. Thus, there is also a possibility that ATP-involved reactions in *E. coli* are regulated by polyamines.

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