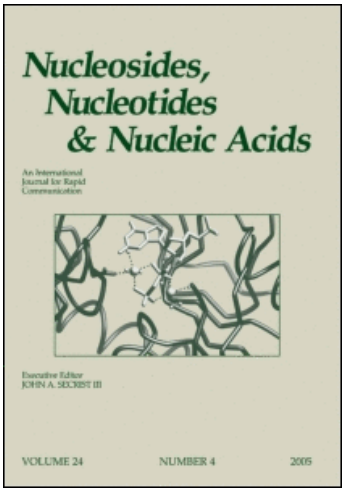

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Characterization and Elucidation of Coordination Requirements of Adenine Nucleotides Complexes with Fe(II) Ions

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Characterization and Elucidation of Coordination Requirements of Adenine Nucleotides Complexes with Fe(II) Ions

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ABSTRACT

In spite of the significant role of iron ions-nucleotide complexes in living cells, these complexes have been studied only to a limited extent. Therefore, we fully characterized the ATP:Fe(II) complex including stoichiometry, geometry, stability constants, and dependence of Fe(II)-coordination on pH. A 1:1 stoichiometry was established for the ATP:Fe(II) complex based on volumetric titrations, UV and SEM/EDX measurements. The coordination sites of ferrous ions in the complex with ATP, established by ^{1}H-, ^{31}P-, and ^{15}N-NMR, involve the adenine N7 as well as Pα, Pβ, and Pγ. Coordination sites remain the same within the pH range of 3.1–8.3. By applying fluorescence monitored Fe(II)-titration, we established a log K value of 5.13 for the $Fe(ATP)^{2-}$ complex, and 2.31 for the $Fe(HATP)^{-}$ complex. Ferrous complexes of ADP^{3-} and AMP^{2-} were less stable (log K 4.43 and 1.68, respectively). The proposed major structure for the $Fe(ATP)^{2-}$ complex is the 'open' structure. In the minor 'closed' structure N7 nitrogen is probably coordinated with Fe(II) through a bridging water molecule. The electronic and stereochemical requirements for Fe(II)-coordination with ATP^{4-} were probed using a series of modified-phosphate or modified-adenine ATP analogues.

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We concluded that: Fe(II) coordinates solely with the phosphate-oxygen atom, and not with sulfur, amine, or borane in the cases of phosphate-modified analogues of ATP; a high electron density on N7 and an *anti* conformation of the adenine-nucleotide are required for enhanced stability of ATP analogues:Fe(II) complexes as compared to ATP complexes (up to more than 100-fold); there are no stereochemical preferences for Fe(II)-coordination with either Rp or Sp isomers of ATP-α-S or ATP-α-BH_3 analogues.

Key Words: ATP; ATP analogues; Fe(II); log K.

INTRODUCTION

An important factor in the functionality of adenine nucleotides is their ability to participate in many biological reactions in the form of metal ion complexes.[1] These reactions include hydrolysis of ATP, enzyme activation, and enzyme inhibition.[2–4] Investigation of the crucial role of ATP metal-ion complexes yielded numerous reports describing both metal-ion binding properties of adenine nucleotides, and their detailed role in enzymatic reactions.

The field of adenine nucleotide complexes with metal ions, especially transition metal ions, has been thoroughly investigated providing important data on both the properties of natural complexes, and on complexes of nucleotides with metal ions that do not exist in organisms. Over the years, a substantial amount of information has been accumulated regarding the stability[5] and structure of various metal ion complexes formed with nucleotides.[6,7]

Among transition metal ions present in humans, iron ions are the most abundant.[8] ATP was proven to be the dominant ligand for iron in reticulate cytosol,[9] affecting the chelation of iron first entering the cell before it is incorporated into heme and ferritin.[10] The involvement of complexes of Fe(II) or Fe(III) ions with ATP in iron toxicity was also proposed.[11–13] On the other hand, those complexes were explored as inhibitors of cytotoxicity induced by oxygen radical precursors.[14–19] Furthermore, the participation of complexes of ATP with iron ions in various biochemical reactions is most likely, although minimal evidence has been provided to date.[20,21]

In spite of the significant role of nucleotide:Fe(III) complexes in living cells, the chemical characteristics of these complexes have been studied only to a limited extent. ATP:Fe(III) complex was studied by Mössbauer[22] and Raman[23] spectroscopies, and recently by ^{1}H NMR,[24] for deducing the geometry of the complex. However, nucleotide:Fe(II) complexes,[25,26] and especially ATP:Fe(II) complex[20,21,27] have been hardly studied.

Here, we characterize ferrous complexes of adenine nucleotides. Specifically, we report on the stoichiometry, geometry, dependence of Fe(II)-coordination on pH, and stability constants of natural adenine nucleotide complexes with Fe(II) ions. Furthermore, we report on the electronic and stereochemical requirements for Fe(II)-coordination with ATP probed by a series of modified-phosphate or modified-adenine ATP analogues.

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RESULTS

Stoichiometry

The empirical formula of solid ATP:Fe(II) complex was determined by UV measurements and volumetric titration of the resulting solution. In addition, stoichiometric data were obtained from Scanning Electron Microscope/ X-ray Energy Dispersive Spectroscopy (SEM-EDX).

Specifically, the nucleotide:Fe(II) complex was precipitated from aqueous solutions of Na_2H_2ATP and $FeSO_4$, of varying molar ratios, upon the addition of acetonitrile under nitrogen (Table 1). The precipitate was filtered, washed with water, and re-dissolved in water after vigorous stirring for 2 h. Titration with standard potassium dichromate solution[28] (eq. 1), and UV measurements were used to determine the Fe(II) and nucleotide concentration,[29] respectively.

$$Cr_2O_7^{2-} + 6Fe^{2+} + 14H^+ \longrightarrow 2Cr^{3+} + 6Fe^{3+} + 7H_2O \quad (1)$$

For initial ATP:Fe(II) ratios of 1:2 or 1:1, the established stoichiometry was 1:1. Further support of this stoichiometry was obtained by the SEM-EDX technique. The precipitate obtained from a 2:1 ATP:Fe(II) solution was dried in vaccuo and analyzed. Three repeating measurements indicated a ratio of 3:1 between phosphorous and Fe, thus proving a 1:1 stoichiometry of the complex (Table 1).

Geometry

Coordination sites in the $Fe(ATP)^{2-}$ complex were investigated by ^1H-, ^{31}P-, and ^{15}N-NMR experiments.

A paramagnetic ion such as Fe(II), in complexes, broadens NMR lines of the NMR active nuclei to which the ion is directly or indirectly bound. This broadening of NMR lines occurs because the magnetic field of the ion changes the relaxation time of the magnetically active nuclei, and because of the exchange between the complexed and free molecule in solution.[30,31] Therefore, NMR studies enable us to determine which magnetically active nuclei are in the metal ion vicinity. For this

Table 1. Determination of empirical formula of ATP:Fe(II) complex.

Initial ATP : Fe(II) ratio	ATP:Fe(II) ratio determined experimentally
1:1	1.1:1.0[a]
1:2	1.2:1.0[a]
2:1	1.0:1.0[b]

[a]Established by volumetric titration and UV measurements.
[b]Established by SEM-EDX.

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purpose, Fe(II)-titration of ATP solution in water was monitored by ^{1}H-, ^{15}N-, and ^{31}P-NMR.

Aqueous solutions of ATP, at pH 4.5, 6.5, and 8.5, were titrated with $FeSO_4$ solutions (Fig. 1). The effect of paramagnetic ferrous ions on the chemical shifts of ATP protons or phosphorous nuclei was negligible. Yet, these ions significantly affected the line-width of certain NMR signals. The dependence of line-width on Fe(II) concentration was explored. Maximal line broadening of ATP (^{1}H and ^{31}P signals) could be observed after the addition of 0.07 eq $FeSO_4$ at pH 8.5. At higher Fe(II) concentrations the signals disappeared into the baseline. ^{1}H NMR signal for H8 was broadened from 2.5 Hz, up to 35 Hz. Line-broadening for H8 was more significant than for H2 (Fig. 1A), indicating that N7 rather than N1 coordinates with Fe(II). ^{31}P NMR spectrum indicated line-broadening for all three phosphorous signals. The ^{31}P NMR signal for Pβ, which was separated from the other phosphate signals, broadened from 5 Hz, up to 137 Hz (Fig. 1B). In acidic medium, pH 4.5, a more significant line-broadening was measured, up to 440 Hz, with 0.07 eq Fe(II) (Fig. 2). Smaller line broadening at pH 8.5 is due to the lesser amount of available ferrous ions owing to a hydroxylation reaction at this pH.

Line broadening in the ^{31}P NMR spectra was identical for Pα, Pβ, and Pγ (Fig. 1B). This broadening might be due to coordination of all three phosphate groups, or due to the averaging of equilibrating bidentate phosphate-chelating species. The first explanation is supported by the finding that the longer the phosphate chains in adenine nucleotides were, the higher were the stability constants of the corresponding Fe(II) complexes (see below).

Line broadening was both pH-dependent and dependent on the molar ratio of ATP and Fe(II) ions. The lower the molar ratio of the nucleotide: Fe(II), the greater the line broadening. For instance, with the nucleotide: Fe(II) ratios 14:1 and 20:1, at pH 4.5, Pβ line broadening was 180 and 89, and H8 line–broadening was 14 and 4, respectively.

To identify the adenine-ring nitrogen atoms that are involved in the coordination of Fe(II), ATP solutions, at pH 2.0, 4.5, and 6.5, were titrated with $FeSO_4$ and monitored by ^{15}N NMR (Fig. 1C, D, data for pH 2.0 is not shown). Significant line broadening was observed for N7, indicating its role in chelation both in acidic and neutral medium. N9 signal has also broadened, at the pH range 2.0–6.5. This observation is unexpected since the lone pair of N9 is not available for coordination. Apparently, this line-broadening is due to N9 spatial proximity to Fe(II), affecting the relaxation time of N9.

Dependence of Complex Geometry on pH

^{1}H NMR spectra of ATP solutions at the pH range 3.1–8.3, measured 10 min after the addition of Fe(II), showed clear broadening of H8 only (Fig. 3A, B, C). This finding indicates that in either acidic or basic pH, N7 is involved in Fe(II) coordination. Likewise, ^{31}P NMR spectra measured under these conditions showed most significant line broadening for all phosphates in both acidic and basic pH (Fig. 3D, E, F). When spectra of those samples were measured again after 2 h at room temperature, the same observations were made except for the pH range 3.7–4.2. At this

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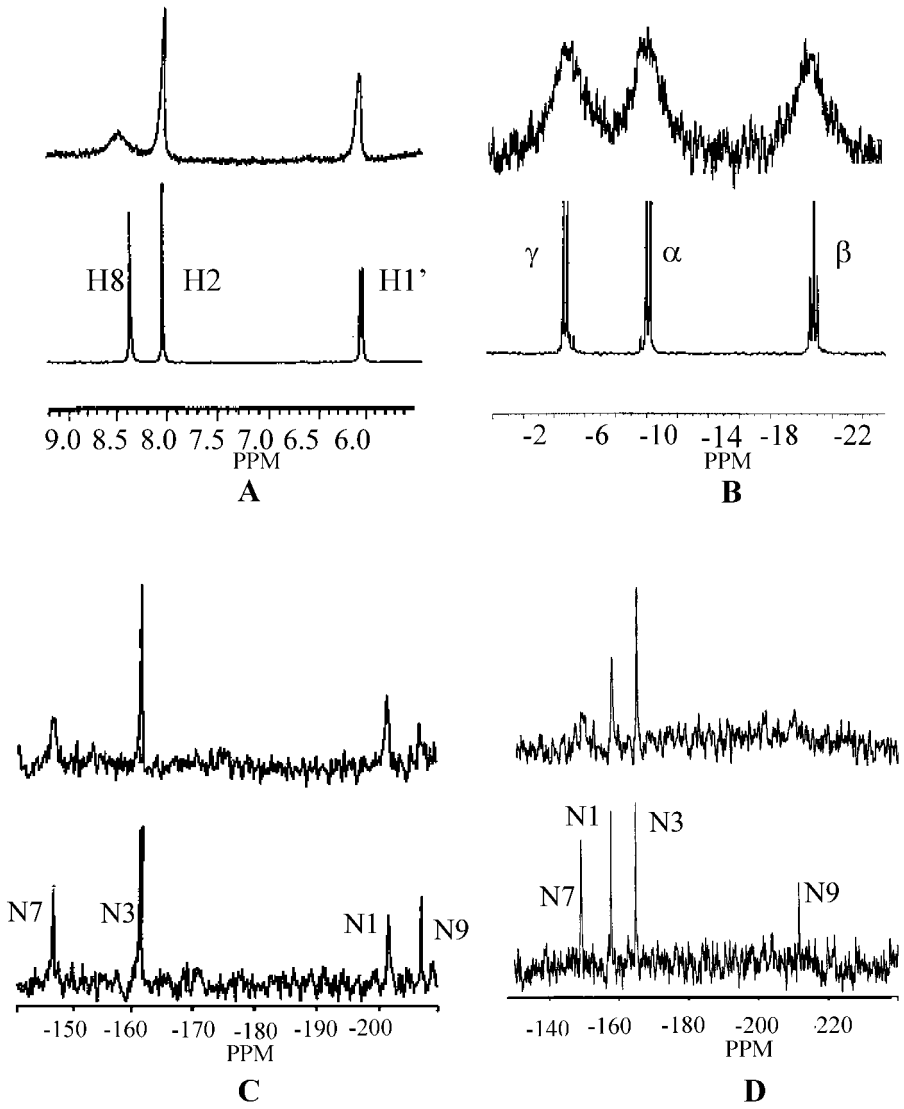

Figure 1. Fe(II) titration of ATP monitored by ^{1}H, ^{31}P, and ^{15}N NMR. Aqueous solutions of ATP, at several pH values were titrated with $FeSO_4$ solution, at 298 K, and monitored by ^{1}H, ^{31}P, and ^{15}N NMR. A. ^{1}H NMR (200 MHz), pH 8.5, bottom: ATP (60 mM), top: ATP + 4 mM mM $FeSO_4$. B. ^{31}P NMR (80.3 MHz), pH 8.5, bottom: ATP (60 mM), top: ATP + 4 mM $FeSO_4$. C. ^{15}N NMR (60.8 MHz), pH 4.5, bottom: ATP (1.5 M), top: ATP + 0.02 eq Fe(II). D. ^{15}N NMR, pH 6.5, bottom: ATP (1.5 M), top: ATP + 0.004 eq Fe(II).

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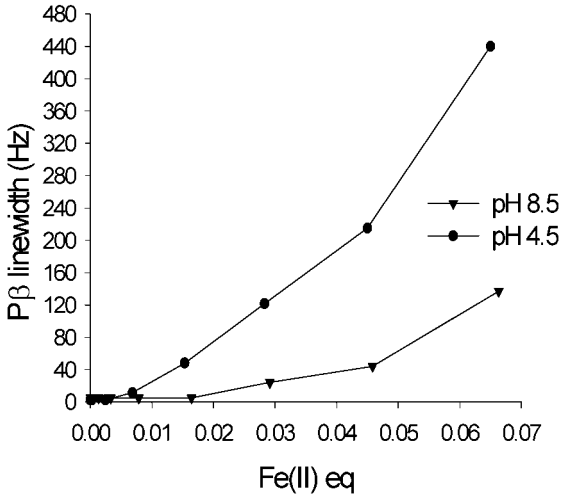

Figure 2. Fe(II)-dependence of ATP's Pβ line-width at pH 4.5 and 8.5. ATP solutions (60 mM) at pH 4.5 and 8.5 were titrated with $FeSO_4$ from 0.02 to 0.07 eq, and the line-width was measured at half peak's height in Hz.

pH range, which approximates the pK_a values for the N1 nitrogen of free ATP,[32,33] and possibly of the metal-ion-bound nucleotide, line broadening was observed also for H2 (Fig. 4). Apparently, under thermodynamic control, with pH around the pK_a value, a mixture of species, which does not exist at other pH values, is obtained due to N1 protonation equilibrium. NMR spectrum lines are broadened, reflecting the existence of this mixture of Fe(II)-coordinated species.

The involvement of N1 in coordination at pH range of 3.7–4.2 is less likely due to the preferred *anti* conformation of ATP in solution (see below). Indeed, ^{15}N NMR spectrum at pH 4.5 indicates that N1 is not involved in coordination (Fig. 1C). Some line broadening of N1 observed is due to protonation[32] and not to metal coordination, as compared to the change of N7 at the same pH.

Stability Constants of Fe(II) Complexes of Natural Adenine Nucleotides

We determined the stability constants of Fe(II) complexes with natural adenine-nucleotides: ATP^{4-}; $HATP^{3-}$; ADP^{3-}; and AMP^{2-}. For this purpose we applied metal-ion-titration monitored by fluorescence measurements, following a method reported by Watanabe et al.[34] Adenine nucleotides have a fluorescence spectrum with a maximum intensity between 390–396 nm. The addition of metal ions to the nucleotide sample decreases the fluorescence intensity. This phenomenon may be due to the partial neutralization of the phosphate-groups charges, allowing self-stacking of the nucleotides, thus causing quenching of the fluorescence. The fluorescence intensities of adenine nucleotides at pH 2.8, which were approximately maximal, were studied as a function of metal ions concentration. We used 0.5 M

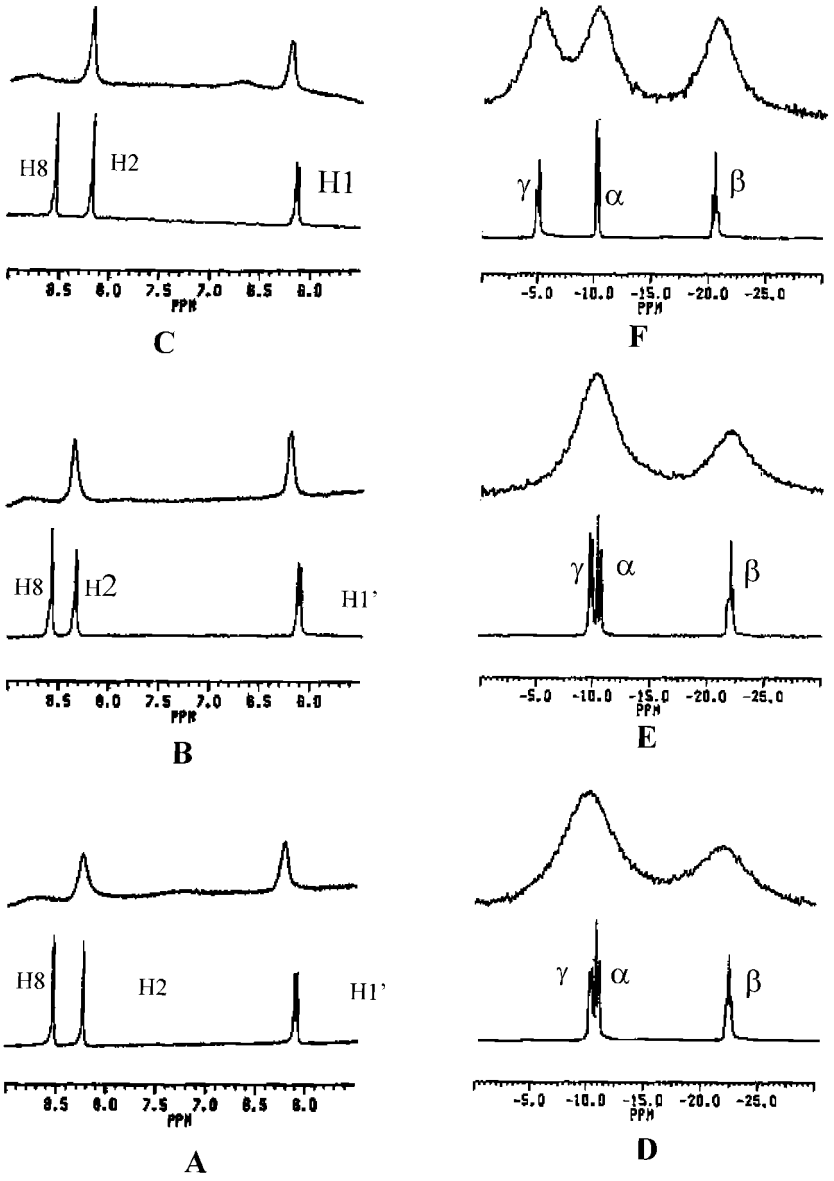

Figure 3. pH-dependence of Fe(II)-coordination with ATP N7 and phosphate groups, monitored by ^{1}H, and ^{31}P NMR. Measurements were performed at various pH values in 90% H_2O:10% D_2O, 10 min after Fe(II) addition. A–C: ^{1}H NMR spectra at pH 3.1, 4.7, and 8.3, respectively, bottom panels; ATP solutions (170 mM), top panels; ATP solutions with 3.2 mM Fe(II). D–F: ^{31}P NMR at pH 3.1, 4.7, and 8.3, respectively, bottom panels; ATP solutions (170 mM), top panels; ATP solutions with 3.2 mM Fe(II).

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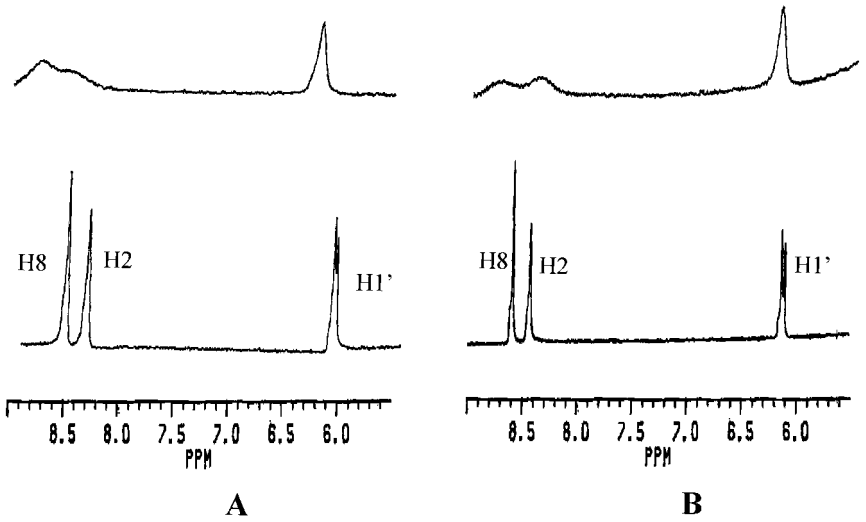

Figure 4. pH-dependence of Fe(II)-coordination with ATP N7 monitored by ^{1}H NMR. Bottom panels present ^{1}H NMR spectra of ATP solutions (170 mM) at various pH values in 90% H_2O:10% D_2O, measured 2 h after Fe(II) addition. Top panels present ^{1}H NMR spectra of ATP solutions at various pH values with 3.2 mM Fe(II). A. pH 3.7 B. pH 4.2.

NH_4Cl solution as a supporting electrolyte for disregarding the variations of ionic strength and the resulting changes of fluorescence, due to metal ion addition.[34]

Free ATP at pH 2.8, is monoprotonated at the ATP terminal phosphate,[33] as well as at the adenine ring at N1[35–40] (93% of N1 is protonated at this pH). Neutralization of the phosphate chain due to protonation is expected to enhance base stacking of free ATP. Self-association constants of ca. 200 M^{-1},[41] or 158 M^{-1},[42] were determined for ATP at pH 2.8. Although the largest self-association tendency is reached with $H_2(ATP)^{2-}$, yet, in our case, fluorescence measurements were performed at micromolar concentrations. At these concentrations the degree of self-association was negligible.

Metal ion (M^{2+}) coordination with ATP renders the latter more acidic. For instance, in an $M(ATP)^{2-}$ complex the pK_a of Pγ, which is 6.5 in free ATP, is reduced by 2.0–2.8 log units.[43] Namely, under our measurement conditions (pH 2.8 and the presence of metal ions) the population of nucleotide:metal-ion complexes includes $M(HATP)^-$ as the major species and $M(ATP)^{2-}$ as the minor species (Sch. 1).

To evaluate the accuracy of fluorescence-monitored metal-ion-titration method for the determination of stability constants, we first applied it for the reproduction of log K values obtained before by potentiometric,[43] and UV-monitored[44] titrations, for $Mg(ATP)^{2-}$ and $Fe(ATP)^-$ complexes, respectively. Indeed, for the $Mg(HATP)^-$ and $Mg(ATP)^{2-}$ complexes, we obtained log K values of 1.96 and 5.00, respectively, which are within the range of log K values reported in the literature.[5] Likewise, a $\log K_1$ value of 6.50 was obtained for the Fe(III) complex, $Fe(ATP)^-$, by this

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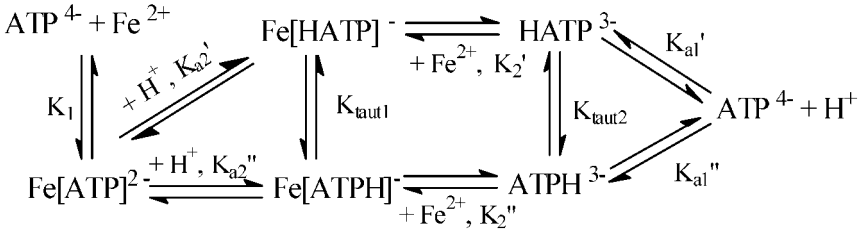

Scheme 1. Complex formation and acid-base equilibria in a solution of ATP and Fe(II) ions.

fluorimetric titration. This log K value was almost identical with the reported value (6.59).[44]

After validation of the fluorimetric method for the determination of log K values of the ATP:metal ion complexes, we determined log K values for complexes of adenine nucleotides with Fe(II), as follows:

Aqueous solution of ATP was titrated with Fe(II) at constant ionic strength, and fluorescence spectra were measured at each concentration at pH 2.8. The nucleotide sample was excited at 286 nm with a maximum emission at 396 nm. A plot of fluorescence intensity at 396 nm vs. log [Fe(II)] yielded a sigmoid curve with two inflection points (Fig. 5A). The inflection points are indicative of the existence of several species in solution (Sch. 1). In addition to the major protonated species, $Fe(HATP)^{-}$, there is a minor species, $Fe(ATP)^{2-}$, at pH 2.8. The inflection points, which were determined by the second derivative of the fitted sigmoid function, were the logarithm of the stability constants (log K) of complexes of $HATP^{3-}$ and ATP^{4-} with ferrous ions.

The inflection point at $-\log [Fe(II)] = 5.13$ (Fig. 5B) is the $\log K_1$ value for $Fe(ATP)^{2-}$ complex. The latter complex is a minor species existing under the measurement conditions (pH 2.8). For the protonated species, $Fe(HATP)^{-}$, $\log K_2$ value of 2.31 was determined (Fig. 5C). The latter complex is the major species at pH 2.8. $Fe(HATP)^{-}$ species is protonated at Pγ.[43] Yet, we cannot completely rule out the presence of a tautomeric species (protonated at N1 rather than at Pγ).

These stability constants obtained by us are consistent with previous reports.[21] Log K for the complex of ATP with Fe(II) is 1.37 log units lower than that of the corresponding Fe(III) complex.[44] A higher log K value is expected for a trivalent metal ion that forms tighter interactions with the same ligand.

In the same way, we determined $\log K_1$ values of 1.68, and 4.43 for Fe(II) complexes with AMP^{2-} and ADP^{3-}, respectively (Table 2). Namely, the longer the phosphate chain of adenine nucleotide, the higher the stability of its complex with Fe(II). This finding is in agreement with a similar increase of stability constants of complexes of mono-, di-, and tri-phosphate monoesters with a variety of divalent ions, and with predictions regarding stability constants of phosphate monoesters complexes with Fe(II).[45] A difference of one order of magnitude of formation constant was also found between the related Fe(III) complexes with ATP^{4-} and ADP^{3-}, $3.9 \times 10^6\ M^{-1}$, and $4.6 \times 10^5\ M^{-1}$, respectively.[44]

Based on $\log K_1$ values for ferrous complexes of AMP^{2-}, ADP^{3-}, and ATP^{4-}, it is suggested that all three phosphate groups in ATP take part in coordinating with

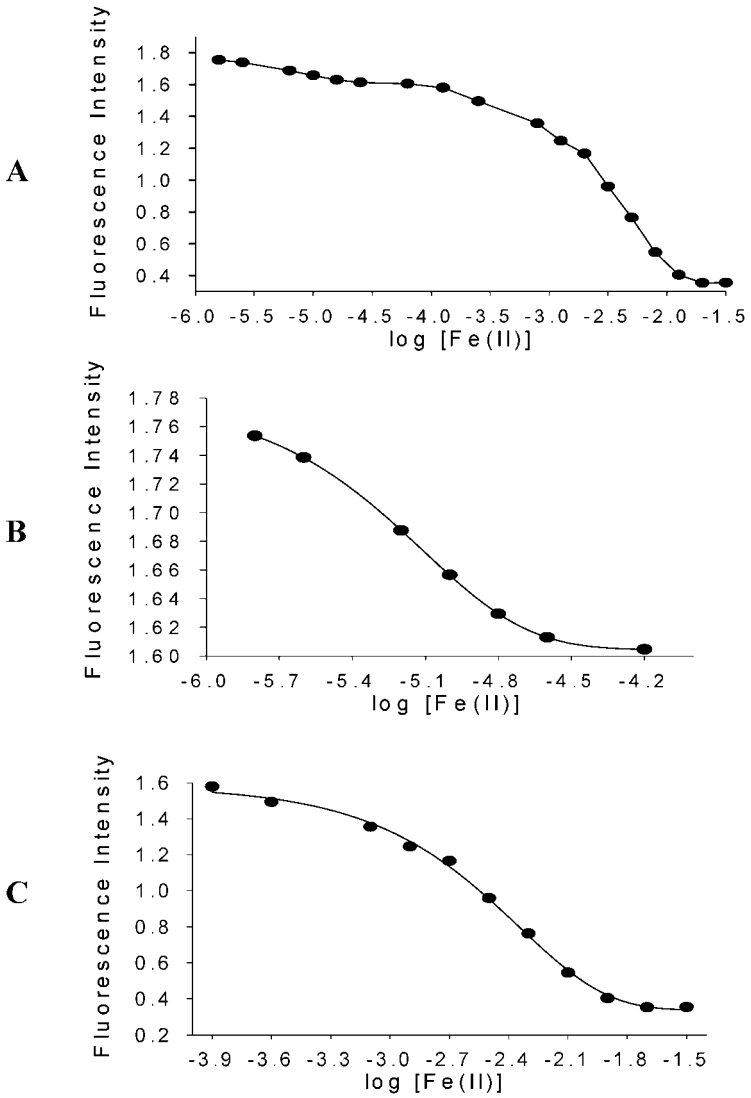

Figure 5. Determination of log K value of ATP:Fe(II) complex by fluorescence spectroscopy. ATP solution (5 μM) at pH 2.8 at 0.5 M NH_4Cl ionic strength was titrated with $FeSO_4$ from 1.6 μM to 30 mM and fluorescence intensity was measured. The sample was excited at 286 nm, and maximum emission was measured at 396 nm. Panel A. Titration curve shows two inflection points. Panel B. The inflection point at −log[Fe(II)] 5.13 corresponds to the log K_1 value of $Fe(ATP)^{2-}$. Panel C. The inflection point at -log[Fe(II)] 2.31 corresponds to the log K_2 value of $Fe(HATP)^{-}$.

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Table 2. Log K_1 values of Fe(II): phosphate chain modified nucleotide complexes.

Compound	Analogue	Log K_1[a]
1	ATP	5.13 ± 0.05
4	ATP-γ-S	4.93 ± 0.02
5a	ATP-α-S (isomer A)[b]	5.36 ± 0.03
5b	ATP-α-S (isomer B)[b]	5.06 ± 0.02
6	ATP-α-BH_3 (isomer B)[b,c]	5.43 ± 0.09
7	AMP-PNP	5.44 ± 0.01
2	ADP	4.43 ± 0.02
3	AMP[d]	1.68 ± 0.01
15	AMP-NH_2	1.74 ± 0.07
16	AMP-S	2.13 ± 0.04

[a]Log K_1 values were obtained from fitting a five parameter sigmoid curve to the data (see materials and methods), with errors noted as the deviation from the average value.
[b]Isomers are named by their elution order from HPLC.
[c]A similar log K_1 value was obtained for isomer A.
[d]0.5 M NaCl was used as supporting electrolyte.

Fe(II). This suggestion is consistent with the above-mentioned ^{31}P NMR data (Fig. 1B).

Evaluation of Fe(II)-Coordination Requirements with Adenine Nucleotides – Selection of Nucleotide Probes

For the purpose of analyzing the stereochemical, conformational, and electronic requirements of the coordination of Fe(II) with adenine nucleotides, we evaluated log K_1 values for two sets of synthetic adenine nucleotide probes (Sch. 2, Tables 2–3). The first set consisted of adenine nucleotide analogues modified at the phosphate chain (Table 2), and the second set consisted of nucleotides modified at the adenine ring (Table 3).

Specifically, possible enhancement of Fe(II)-coordination to ATP^{4-} due to increased electron density on N7 by C8 substitution was probed by derivatives **9**–**12**. In addition, based on our previous results, we used probe **14** as another means of increasing the availability of the N7 lone-pair, by substituting C2-position with a thioether group.[32] As controls, we prepared the corresponding derivatives bearing electron-withdrawing groups, e.g., 8-Br-ATP and 2-Cl-ATP (**8** and **13**). To evaluate the Fe(II)-coordination requirements with the nucleotide's phosphate chain, we replaced the 'hard' oxygen atoms of the phosphate by 'soft' sulfur atoms, as in ATP-γ-S and ATP-α-S (**4**, **5**), which may have high affinity to 'borderline' ferrous ion. As a control, we chose ATP-α-BH_3 analogue (**6**) that bears a borane group incapable of coordinating metal ions.[46] Furthermore, for the evaluation of the stereochemical requirements of Fe(II) coordination, we investigated both Rp and Sp diastereoisomers of ATP-α-S and ATP-α-BH_3. In addition, effects of chemical modifications on the adenine or phosphate moieties were also evaluated for AMP analogues (**15**–**19**).

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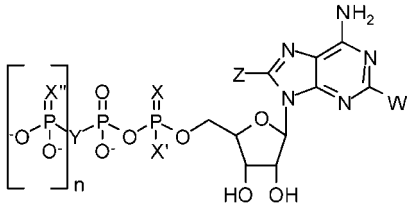

1	X = O	X' = O^-	X'' = O	Y = O	Z = H	W = H	n = 1
2	X = O	X' = O^-	X'' = O	Y = O	Z = H	W = H	n = 0
4	X = O	X' = O^-	X'' = S	Y = O	Z = H	W = H	n = 1
5	X = S	X' = O^-	X'' = O	Y = O	Z = H	W = H	n = 1
6	X = O	X' = BH_3^-	X'' = O	Y = O	Z = H	W = H	n = 1
7	X = O	X' = O^-	X'' = O	Y = NH	Z = H	W = H	n = 1
8	X = O	X' = O^-	X'' = O	Y = O	Z = Br	W = H	n = 1
9	X = O	X' = O^-	X'' = O	Y = O	Z = NH_2	W = H	n = 1
10	X = O	X' = O^-	X'' = O	Y = O	Z = NHBu	W = H	n = 1
11	X = O	X' = O^-	X'' = O	Y = O	Z = OMe	W = H	n = 1
12	X = O	X' = O^-	X'' = O	Y = O	Z = Shex	W = H	n = 1
13	X = O	X' = O^-	X'' = O	Y = O	Z = H	W = Cl	n = 1
14	X = O	X' = O^-	X'' = O	Y = O	Z = H	W = $SCH_2C(CH_3)_3$	n = 1

Scheme 2A. Phosphate-modified and adenine-modified adenosine nucleotides used for evaluation of stereo-electronic requirements for coordination with Fe(II) ions.

Evaluation of Fe(II)-Coordination Requirements with Adenine Nucleotides – Determination of Stability Constants of Nucleotide Probes Complexes with Fe(II)

The role that both bridging and non-bridging phosphate oxygen atoms, play in metal coordination, was evaluated by adenine nucleotide analogues where phosphate oxygen atoms were substituted by isoelectronic groups such as nitrogen, sulfur, or borane. When a Pγ oxygen atom was substituted by a sulfur atom, the $\log K_1$ value of Fe(ATP-γ-S)$^{2-}$ slightly decreased compared with Fe(ATP)$^{2-}$ complex (Table 2). Likewise, for both diastereoisomers of ATP-α-S only 0.1-0.2 $\log K$ units differences were obtained, compared with the corresponding value for ATP.

Whereas there is only a minor effect of the sulfur atom in ATP-α-S or ATP-γ-S on the coordination with Fe(II), the effect of sulfur-substitution on AMP is more significant compared to AMP, with a difference of 0.45 $\log K$ units. However, the replacement of phosphate oxygen with an amino group, as in AMP-NH_2, **15**, resulted in practically no change of $\log K_1$. A related amino substitution of ATP

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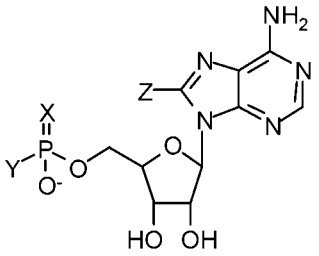

3	X = O	Y = O^-	Z = H
15	X = O	Y = NH_2	Z = H
16	X = S	Y = O^-	Z = H
17	X = O	Y = O^-	Z = OBu
18	X = O	Y = O^-	Z = NHBu
19	X = O	Y = O^-	Z = SBu

Scheme 2B. Phosphate-modified and adenine-modified AMP analogues used for evaluation of stereo-electronic requirements for coordination with Fe(II) ions.

bridging oxygen, as in AMP-PNP, **7**, resulted in a small increase in the $\log K_1$ value of 0.3 log units. Substitution of phosphate-oxygen by a borane group increased $\log K_1$ slightly (0.13–0.30) for both diastereoisomers of ATP-α-BH_3 (Table 2).

Table 3. $\log K_1$ Values of Fe(II): adenine ring modified nucleotide complexes.

Compound	Analogue	$\log K_1$[a]
1	ATP	5.13 ± 0.05
8	8-Br-ATP	4.80 ± 0.05
9	8-NH_2-ATP	5.15 ± 0.08
10	8-NHBu-ATP	6.82 ± 0.11
11	8-OMe-ATP	7.27 ± 0.06
12	8-Shexyl-ATP	5.29 ± 0.07
13	2-Cl-ATP	5.17 ± 0.03
14	2-$SCH_2C(CH_3)_3$-ATP	5.06 ± 0.09
3	AMP[b]	1.68 ± 0.01
17	8-OBu-AMP	2.99 ± 0.06
18	8-NHBu-AMP	3.07 ± 0.09
19	8-SBu-AMP	2.13 ± 0.10

[a]$\log K_1$ values were obtained from fitting a five parameter sigmoid curve to the data (see materials and methods), with errors noted as the deviation from the average value.
[b]0.5 M NaCl was used as supporting electrolyte.

The effect of the adenine ring modifications at either C8 or C2-positions, upon the coordination of Fe(II), was also explored (Table 3). Thus, when C8-position of AMP was substituted by electron-donating groups such as OBu, NHBu, and, SBu, **17**–**19**, higher stability constants were obtained as compared to AMP, up to ca. 20-fold increase in the case of 8-OBu-AMP.

A similar observation was made for the corresponding ATP analogues, **10**–**11**. NHBu-, and OMe-groups at C8 position affected a tighter coordination of Fe(II) to analogues **10**–**11**, as compared to the coordination with ATP, resulting in ca. 100-fold increase of stability constants. Yet, surprisingly, the $\log K_1$ value of Fe(8-amino-ATP)$^{2-}$ complex has not significantly changed (5.15) compared to the corresponding values of ATP (5.13), and 8-Br-ATP (4.80) complexes.

The substitution of C2 by $-SCH_2C(CH_3)_3$, shown to increase electron density on N7,[32] did not enhance N7 coordination with Fe(II). This is indicated by the $\log K_1$ value (5.06) as compared to 2-Cl-ATP (5.17) and ATP (5.13).

Evaluation of the Stability of Fe(II) Ions and ATP Under Measurements Conditions

To establish that the above log K values were obtained for ATP:Fe(II) complexes with no contaminants of Fe(III) ions due to air-oxidation of ferrous ions, we analyzed the solutions used for fluorescence measurements, for the presence of Fe(III).

To detect the presence of Fe(III), an excess KSCN was added to $FeSO_4$ solutions in 0.5 M NH_4Cl, at pH 2.8. The presence of any Fe(III) is indicated by the formation of colored complexes, $[Fe(SCN)_n]^m$, with a major peak at 460 nm. Changes in absorbance intensities at 460 nm, were monitored at 10 min intervals for 5 h. Based on a standard curve, the percentage of formed Fe(III) was plotted vs. time. After 5 h, under conditions simulating fluorescence measurements, only 0.35% of Fe(II) ions were oxidized. We conclude that the above measurements of log K values were performed under conditions free of Fe(III) contamination.

Furthermore, the hydrolytic stability of the nucleotide ligand during log K measurements was also investigated. Thus, any possible hydrolysis products that might be formed under conditions simulating fluorescence measurements, were monitored by tlc and ^{31}P NMR. After 5 h, no degradation products (ADP, AMP, or adenine) were detected.

Although divalent metal ions have been shown to catalyze the hydrolysis of ATP,[47,48] under the conditions used here for log K determination, no Fe(II)-promoted ATP hydrolysis was detected.

DISCUSSION

The determination of the geometry of the 1:1 ATP:Fe(II) complex based on X-ray crystallography was impossible, due to major difficulties in the crystallization of this complex. Therefore, the simplified macrochelate geometry for the Fe(ATP)$^{2-}$ complex we propose here (Sch. 3), is based on analysis of the spectral findings obtained by us and others.

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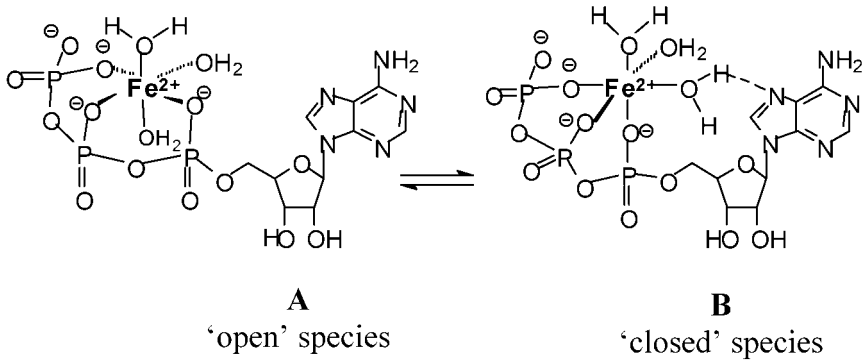

Scheme 3. Proposed geometry for the $Fe(ATP)^{2-}$ complex.

For ATP:Fe(II) complex, we concluded, based on ^{31}P NMR (Figs. 1, 3) and log K values (Table 2), that Fe(II) interacts with all three phosphate groups. Apparently, this mode of phosphate coordination is a characteristic feature of the ATP:Fe(II) complex. Generally, there is no rule regarding the number and identity of ATP's phosphate groups coordinating with divalent metal ions. For instance, although, Co^{2+} and Mn^{2+} interact with all three phosphate groups, NMR measurements indicated that Mg^{2+}, Ca^{2+}, and Zn^{2+} form complexes with the ATP β and γ phosphate groups, while ADP interacts with the α and β groups.[49]

^{1}H and ^{15}N NMR spectra indicated the involvement of N7 in Fe(II) coordination (Figs. 1, 3). This is consistent with reports on ATP complexes with other borderline metal ions. Metal ions such as Cd^{2+}, Co^{2+}, and Ni^{2+} form macrochelates with ATP involving N7.[50] Such metal ions have a rather pronounced affinity for both borderline aromatic N-sites, and phosphate groups.

For the $Fe(ATP)^{2-}$ complex N7-coordination is more likely than N1 coordination, since ATP adopts preferentially an *anti* conformation. Furthermore, no interaction with the pyrimidine ring, i.e., N1 nitrogen atom, was detected at a pH range of 2.0–8.3 (Figs. 1, 3).

Du et al. recently provided ^{1}H NMR evidence that for the related ATP:Fe(III) complex, both H2 and H8 were affected upon pH-titration, especially at a pH range of 4.5–5.5.[24] These authors proposed that at acidic pH (up to pH 3.5), N1 was preferentially coordinated with Fe(III) rather than proton-bound. At a basic pH (5–8.5), Fe(III)-coordination is solely with N7; and at an intermediate pH region (4–4.5), a large conjugated system involving both N7 and N9, was proposed to be involved in Fe(III)-chelation (π-complex).

For the ATP:Fe(II) complex, we provided a direct evidence for the pH-dependence of metal-ion coordination to nitrogen atoms, by ^{15}N NMR (Fig. 1C, D). The addition of soft or borderline metal ions generally results in significant Δδ of adenosine N1 or N7 signals, respectively. In the case of Fe(II), due to extensive line-broadening of the coordinating adenine nitrogen, we were able to add at most 0.04 eq Fe(II), and therefore no significant Δδ was observed for the nitrogen signal. We further noticed that at pH 2.0, the N1 signal, which appeared at –222 ppm due to

protonation, remained sharp upon the addition of Fe(II). The same observation was made for N1 at pH 6.5 (Fig. 1D). At pH 4.5, at which a formation of a π-complex was proposed by Du et al., for the ATP:Fe(III) complex, we noticed that N1 and N3 signals of the corresponding ferrous complex remained sharp (Fig. 1C). Namely, these nitrogen atoms did not participate in metal coordination, either directly or through π-complex.

At acidic pH, at which some of the nucleotide is N1-protonated, N7 is still interacting with Fe(II) (Figs. 1C, 3). Yet, the interaction of N7 with Fe(II) is weak as implied in the UV-spectrum of the complex, at either pH 4.5 or 8.5, which is almost identical with the spectrum of free ATP.

Another important structural evidence for deducing the geometry of the ATP:Fe(II) complex was obtained from Mössbauer spectrum at acidic pH indicating that there are only oxygen ligands around Fe(II).[22] Based on this evidence and the facts discussed above, we propose that the preferential structure for the ATP:Fe(II) complex at both acidic and basic pH is the 'open' structure, whereas the 'closed' structure exists at a low percentage. Furthermore, N7 nitrogen in the 'closed' structure, at acidic pH, is probably coordinated with the metal ion through a bridging water molecule ('outer sphere' coordination) (Sch. 3B).

Addition of Fe(II) ions to an acidic solution of ATP (pH 2.8), gives rise to several complex-formation and acid-base equilibria (Sch. 1). Complex-formation equilibria include: formation of $Fe(ATP)^{2-}$ [K_1], and formation of $Fe(HATP)^{-}$ or $Fe(ATPH)^{-}$ [protonated at Pγ or N1, with $K_{2'}$ and $K_{2''}$, respectively]. Acid-base equilibria include: protonation of ATP^{4-}, yielding $HATP^{3-}$ or $ATPH^{3-}$ [protonated at Pγ or N1, with $K_{a1'}$ and $K_{a1''}$, respectively], and of $Fe(HATP)^{-}$ or $Fe(ATPH)^{-}$ [protonated at Pγ or N1, with $K_{a2'}$ and $K_{a2''}$, respectively]. In addition, tautomeric equilibria K_{taut1} and K_{taut2} may exist as well. Protonated species such as $H_2(ATP)^{2-}$ and $Fe(H_2ATP)$ may also exist, with the corresponding equilibria constants.

Here, we evaluated by fluorescence measurements the stability constant for $Fe(ATP)^{2-}$ complex [K_1]. This includes both the 'open' (only phosphates' coordination) and 'closed' (both phosphates' and N7 coordination) structures. Likewise, we evaluated log K for the Pγ-protonated species, $Fe(HATP)^{-}$, [$K_{2'}$], which also includes both 'open' and 'closed' structures. The value of $K_{2''}$ is assumed to be negligible.[43] Likewise, log K for the $Fe(H_2ATP)$ complex is expected to be very small.

Log K values of 5.13 and 2.31 obtained for the $Fe(ATP)^{2-}$ and $Fe(HATP)^{-}$ complexes, correspondingly, were consistent with potentiometric measurements for these complexes.[20,21] Log K_1 for the $Fe(ATP)^{2-}$ complex is within the log K range expected for divalent metal ion complexes (4–6.5).[43] The stability constant of $Fe(ATP)^{2-}$ complex is ca. 1000-fold higher than the constant of the corresponding $Fe(HATP)^{-}$ complex; which is in agreement with the log K of $M(HATP)^{-}$ complexes, with other divalent ions.[43]

The contribution of the triphosphate moiety of ATP to the stability of the complex with Fe(II) was probed using a series of modified-phosphate analogues (Table 2).

Substitution of the ATP's bridging-oxygen atom between Pβ and Pγ, with NH (AMP-PNP) or of non-bridging phosphate-oxygen atoms with sulfur atoms, either at Pα or Pγ; or with BH_3 group at Pα, had no significant effect on the log K_1 values of their corresponding complexes with Fe(II).

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The minor effect of sulfur substitution (0.07–0.2 log K units difference), might be indicative of a preferential interaction of the metal-ion with the phosphorothioate's oxygen, rather than the sulfur atom. Although metal-ions such as Mg^{2+}, Ba^{2+}, and Ca^{2+} have equal binding properties towards a thiophosphate and phosphate group, the borderline character of Fe(II) enables coordination with medium bases phosphate oxygen, but not with soft ligands such as phosphorothioates. Yet, the effect of sulfur-substitution on AMP is more significant (difference of 0.45 log K units), leading to a more stable complex. Sigel has proved that alkaline earth ions are solely oxygen-coordinated to the thiophosphate group in AMP-S, whereas in AMP-S complexes of Mn^{2+}, Cd^{2+}, Pb^{2+}, Cu^{2+}, and Zn^{2+}, the metal ions are partially or completely S-coordinated to the thiophosphate group.[51] Stability increases of 0.2 and 0.7 log units for Mn(AMP-S) and Zn(AMP-S), were attributed to the approximate percentages of 30, and 80 for sulfur-coordinated species, respectively.[51] Thus, in our case, for Fe(AMP-S) we may assume that stability increase of 0.45 log unit relative to AMP, may be due to major contribution of sulfur-coordinated species.

The negligible effect of the borane group, substituting the non-bridging phosphate oxygen, in **6**, is due to solely oxygen-coordination, since a borane moiety does not coordinate with metal ions.[46]

Both Rp and Sp diastereoisomers of ATP-α-BH_3, **6**, and ATP-α-S, **5**, were used to probe the stereochemical requirements of Fe(II)-coordination. We found no significant preference for either the Rp or Sp isomer for metal-ion coordination (Table 2).

The electronic requirements for N7-coordination with Fe(II) were probed by a series of ATP analogues substituted by either electron donating or electron-withdrawing groups at either C8 or C2 positions. These substituents were chosen to affect electron density on N7. Furthermore, these molecular probes were designed to also probe conformational requirements of metal-ion coordination with the nucleotide (namely, *syn/anti* preference of the nucleotide).

For AMP^{2-} and ATP^{4-} analogues substituted by electron-donating groups at C8, we established stability constants that are up to 15 and 100-fold higher than those of Fe(II) complexes with AMP^{2-} and ATP^{4-}, respectively (Table 3). These stability constant values support the contribution of N7-coordination to the stability of the Fe(AMP) and $Fe(ATP)^{2-}$ complexes. Indeed, $\log K_1$ value for $Fe(8\text{-Br-ATP})^{2-}$ complex was lower by 0.33 log units relative to the $\log K_1$ value for the corresponding ATP complex. Yet, the $Fe(8\text{-Shexyl-ATP})^{2-}$ complex was only 0.16 log unit more stable than the corresponding ATP complex.

In this case, conformational preferences play a role in addition to electronic effects. The relatively minor increase of the $\log K_1$ value for the complex of 8-Shexyl-ATP may be due to the fact that most of this nucleotide exists in solution in the *syn* conformation,[52] where Fe(II) coordination with N7 is impossible.

Participation of C8-substituents themselves in metal coordination is less likely to occur since the lone pairs of –NHBu; -OMe; -Shexyl in analogues **10**–**12**, are expected to be less available to metal-ion coordination, due to their delocalization into the aromatic adenine ring. Furthermore, ^{1}H NMR spectra of **10** and **11** with Fe(II) indicated that -OMe and –NHBu signals have not broadened upon Fe(II)-addition (data not shown), implying no involvement of C8-substituent in metal-ion coordination.

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The relatively low stability of Fe(8-NH_2-ATP)$^{2-}$, $\log K_1$ 5.15, compared with the corresponding complex with 8-NHBu-ATP, $\log K_1$ 6.82, is rather unexpected based on structural considerations. Yet, the reason for this relatively low stability may stem from the enhanced protonation of 8-NH_2-ATP at N7 under the measurement conditions (pH 2.8), compared to 8-NHBu-ATP.[53] This enhanced protonation makes the former N7 less available to coordination with Fe(II).

2-Cl- and 2-$SCH_2C(CH_3)_3$–ATP analogues were also explored as probes that are solely in the *anti*-conformation,[52] and yet may change electron distribution on N7. The substitution of C2 by -$SCH_2C(CH_3)_3$, in **14**, shown to increase electron density on N7,[32] did not enhance N7 coordination with Fe(II) in this case.

Modified-adenine analogues **8**–**14** were used also to probe coordination requirements regarding conformation of the nucleotide. 8-Br-ATP adopts preferentially *syn* conformation in solution.[54] The facts that the $\log K_1$ for Fe(8-Br-ATP)$^{2-}$ complex was close to that for Fe(ATP)$^{2-}$ complex, and that no line broadening was observed for H2 in the ^{1}H NMR spectrum of the former complex, imply that 8-Br-ATP is forced to adopt preferentially an *anti* conformation in the complex to maintain N7-coordination. Indeed, an *anti* conformation for the cobalt complex of 8-Br-ATP in hexokinase was reported earlier.[55] Previously, we found that a *syn* conformation is preferred for 8-OBu adenine-nucleotides in D_2O solution, whereas, 8-NHBu-adenine nucleotides adopt preferentially the *anti* conformation.[52] Yet, based on $\log K_1$ value for the ferrous complex of 8-OMe-ATP, it appears that the latter is forced to remain preferentially in *anti* conformation to maintain N7 coordination, as in 8-NHBu-ATP.

Concluding Remarks

A 1:1 stoichiometry was established for the ATP:Fe(II) complex. The coordination sites of ferrous ions in the complex with ATP in solution involve the adenine N7 as well as Pα, Pβ, Pγ, with water molecules occupying the remaining octahedral positions. The coordination sites remain the same within the pH range 2.0–8.3. At acidic pH coordination of Fe(II) with N7 is probably through a bridging water molecule (outer-sphere coordination). Furthermore, we concluded that at both acidic and basic pH the 'open' form of the ATP:Fe(II) complex is the predominant one. The Fe(ATP)$^{2-}$ complex was more stable than Fe(HATP)$^-$ complex ($\log K$ 5.13 and 2.31, respectively), and Fe(ADP)$^-$ and Fe(AMP) complexes ($\log K$ 4.43 and 1.68, respectively).

The borderline ferrous-ion coordinates solely with the phosphate-oxygen atom, rather than with sulfur, amine, or borane in the cases of phosphorothioate, phosphoramidate, or boranophosphate analogues of ATP. An *anti* conformation together with the enhancement of N7-electron density by C8 electron donating group are the requirements for highly stable Fe(II) complexes of an ATP analogue. In addition to the conformational requirements, we concluded that there are no stereochemical preferences for Fe(II)-coordination with either Rp or Sp isomers of ATP-α-S or ATP-α-BH_3 analogues.

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EXPERIMENTAL SECTION

General Methods

NMR spectra were measured on a Bruker AC-200 instrument (200.2 and 80.3 MHz for ^{1}H and ^{31}P, respectively). ^{1}H and ^{31}P NMR spectra were measured in D_2O, and the chemical shifts were relative to HOD (4.78 ppm) as an internal standard, and 85% H_3PO_4 as an external standard respectively. ^{15}N NMR spectra were recorded on a Bruker DMX-600 instrument (60.8 MHz for ^{15}N), and nitromethane ($\delta = 0$) was used as an external standard. Negative shifts were upfield from nitromethane. Emission spectra were measured using an Aminco-Bowman series 2 Luminescence Spectrometer (slit: 8 nm, sensitivity: 780–800 V). Absorption data were measured on a Shimadzu UV-VIS recording spectrophotometer UV-2401PC. Measurements of pH were performed with Hanna instruments pH-meter (HI 8521) equipped with a FC 200 Hanna or a biotrode 238140 Hamilton electrode. The pH values of the solutions were adjusted with diluted HCl / NaOH solutions. EDS measurements were done on an X-ray microanalyzer (Oxford scientific) built on a JSM-840 Scanning Electron Microscope (JEOL).

Materials. $FeSO_4 \cdot 7H_2O$ was purchased from J. T. Baker Inc. (Phillipsburg, NJ, USA). Adenosine 5′-triphosphate disodium salt was purchased from Aldrich Chemicals Co. Adenosine 5′-diphosphate sodium salt, 8-Br-adenosine 5′-triphosphate sodium salt, adenosine 5′-O-(3-thiotriphosphate) tetralithium salt, adenosine 5′-monophosphate sodium salt, and adenosine 5′-monophosphoramidate sodium salt were purchased from Sigma Chemicals Co. Adenosine 5′-[β-γ-imino] triphosphate tetralithium salt was purchased from Fluka Chemicals Co. Adenosine 5′-O-monophosphorothioate and adenosine 5′-O-(1-thiotriphosphate) were synthesized according to Hillaire-Buys et al.[56] 8-(*n*-Butylamino)-adenosine nucleotides, 8-(*n*-butylthioether)-adenosine 5′-monophosphate, 8-(*n*-butoxy)-adenosine 5′-monophosphate, and 8-(*n*-hexylthioether)-adenosine 5′-triphosphate were synthesized according to Halbfinger et al.[52] Adenosine 5′-O-(1-boranotriphosphate) was synthesized according to Nahum et al.[57] 2-substituted adenine nucleotides were synthesized according to Major et al.[32] All commercial adenine nucleotides were used without additional purification. $FeSO_4 \cdot 7H_2O$ was re-crystallized twice from water-ethanol before use. Aqueous solutions of nucleotides and $FeSO_4 \cdot 7H_2O$ were freshly prepared with deionized, ultrapure water (Seral co., Paris). Chelex-100 (Biorad, Richmond, CA) was used to remove traces of divalent ions from water (or D_2O) used for the samples preparation.

Stoichiometry Determination. A. $FeSO_4$ (250 mg) was added to Na_2H_2ATP solutions (0.08 M or 0.16 M; 2:1 or 1:1 ratio). Acetonitrile (3 mL) was added to the clear solution. After two days at room temperature under nitrogen atmosphere, a greenish precipitate was obtained. The precipitate was filtered (no need for severe exclusion of air; see below 'Determination of stability of Fe(II)'), washed with water, and then re-dissolved in water after vigorous stirring for 2 h. The ATP concentration was determined by UV-measurements, based on the absorbance at 259 nm, and ε

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15849 $M^{-1}cm^{-1}$ (free and Fe(II)-bound ATP have the same ε value). Fe(II) concentration was determined by volumetric titration with 1.7 mM $K_2Cr_2O_7$.[28]

B. $FeSO_4$ (12.5 mg) was added to Na_2H_2ATP solution (0.15 M; 1:2 ratio). Acetonitrile (100 μL) was added to the clear solution. After two days at room temperature under nitrogen atmosphere, a greenish precipitate was obtained. The precipitate was filtered, washed with water, and dried under vacuum. ATP and Fe(II) ratio was determined by Scanning Electron Microscope/ X-ray Energy Dispersive Spectroscopy (SEM-EDX) measuring phosphorous and iron ratio in triplicate.

NMR Studies. Na_2H_2ATP and Fe(II) solutions used in this study were prepared by dissolving the nucleotide and re-crystallized $FeSO_4$ in D_2O which was deoxygenated prior to spectral measurements. Oxygen was removed from D_2O by 3 cycles of freezing under N_2 atmosphere and thawing in *vacuo*. NMR spectra were measured to monitor the titration of ATP (0.06 M for ^1H, ^{31}P NMR and 1.5 M for ^{15}N NMR) with 1–6 mM Fe(II) solutions.

Determination of Stability Constants of Nucleotide:Fe(II) Complexes. The stability constants of adenosine-nucleotide:Fe(II) complexes were determined by fluorescence measurements following a method reported by Watanabe et al.[34] The fluorescence intensity of adenine nucleotides at pH 2.8, which is approximately maximal, was studied as a function of Fe(II) concentration. The measurements were performed in 0.5 M NH_4Cl as a supporting electrolyte. Varying Fe(II) concentrations (from 0.1 mM to 10 nM) were added to seventeen 5 mL samples of 1 or 5 μM nucleotide analogue solutions. To achieve kinetic equilibrium, these samples were prepared at least 1 h prior to measurements. Excitation was performed at $\lambda = 286$–290 nm, and maximum emission was measured at $\lambda = 386$–396 nm. Fluorescence spectra were obtained for all seventeen samples, and fluorescence intensities for these samples were plotted as a function of the logarithm of Fe(II) concentrations. For fitting a sigmoid function to the data only 6–10 data points, which were close to the inflection point, were taken. A five-parameter sigmoid function was fitted to the data using SigmaPlot 2000 (SPSS, Inc.):

$$I = I_0 + \frac{a}{\left(1 + e^{\frac{(\log[Fe(II)] - \log[Fe(II)]_0)}{b}}\right)^c}$$

Where I is the fluorescence intensity, $\log[Fe(II)]$ is the independent variable, I_0, a, b, c, and $\log[Fe(II)]_0$ are the dependent variables. The inflection point, determined by the second derivative of the fitted sigmoid function, was the logarithm of the stability constant of the complex.

Determination of Stability of Fe(II) and ATP Under the Measurements Conditions. The percentage of oxidation of Fe(II) solutions under the conditions used for log K determinations was evaluated in two steps as follows: A. An excess of KSCN (20 eq) was added to 0.01–0.5 mM $FeCl_3$ solutions at pH 2.8 ($I = 0.5$ M, NH_4Cl). UV spectra were measured for these solutions, containing a mixture of $[Fe(SCN)_n]^m$ species,[58] showing peaks at 460–480 nm. A standard curve was plotted for initial

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Fe(III) concentration vs. OD at 460 nm, at which the largest peak for $[Fe(SCN)_n]^m$ species was observed ($R^2 = 0.96$). B. An excess of KSCN (20 eq) was added to 5 mM $FeSO_4$ solution at pH 2.8 ($I = 0.5$ M, NH_4Cl). UV spectra were measured at 5–10 min intervals for 5 h, and the resulting OD at 460 nm, indicative of oxidation of Fe(II) and formation of $[Fe(SCN)_n]^m$ species, was plotted vs. time. Finally, based on the standard curve, the percentage of Fe(III) obtained under the fluorescence measurement conditions was plotted vs. time.

The stability of Na_2H_2ATP in the presence of Fe(II) (1 eq) at pH 2.8 was evaluated by tlc (H_2O:isopropanol:NH_4OH 7:11:2) during 5 h.

pH Dependence of Fe(II) Coordination with ATP. $FeSO_4$ (0.02 eq) was added to six Na_2H_2ATP solutions in 9:1 H_2O: D_2O (0.17 M), at the following pH values: 3.1; 3.7; 4.2; 4.7; 6.5; 8.3. After ca. 10 min, ^1H- and ^{31}P NMR spectra were recorded.

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