Biophysical Chemistry 16 (1982) 79–88
Elsevier Biomedical Press

THE BINDING OF Ni^{2+} TO ADENYLYL-3',5'-ADENOSINE AND TO POLY(ADENYLIC ACID)

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Received 23rd February 1982
Revised manuscript received 6th May 1982
Accepted 14th May 1982

Key words: Ni^{2+}-nucleotide complex; Poly(A); Thermodynamic stability; Relaxation kinetics

Studies of the binding of Ni^{2+} to adenylyl-3',5'-adenosine (ApA) at pH 6.0 by ultraviolet spectrophotometry indicate the formation of a 1:1 complex in the presence of a large excess of metal ion. At 25°C, and ionic strength $\mu = 0.5$ M, the stability constant of Ni(ApA) is evaluated to be $K = 2.6\ (\pm 0.6)\ M^{-1}$. The low stability is taken as evidence that the predominant complex species is one in which the ApA acts as a monodentate ligand, mainly through the adenine group. The rate constants for complex formation and dissociation, $k_f = 1430\ M^{-1}\ s^{-1}$ and $k_b = 665\ s^{-1}$ (25°C, $\mu = 0.5$ M), determined by the temperature-jump relaxation technique, are consistent with this interpretation. The binding strength of Ni^{2+} to poly(adenylic acid) [poly(A)] has been studied at pH 7.0 using murexide as an indicator of the concentration of free Ni^{2+}. Within the concentration range $[Ni^{2+}]_{free} = 1 \times 10^{-5} - 1 \times 10^{-3}$ M the data can be represented in the form of a linear Scatchard plot, i.e., the process can be described as the binding of Ni^{2+} to one class of independent binding sites. The number of binding sites per monomer is 0.26, and the stability constant $K = 8.2 \times 10^{3}\ M^{-1}$ (25°C, $\mu = 0.1$ M). In kinetic studies of the reaction of Ni^{2+} with poly(A), two relaxation effects due to complex formation were detected, one with a concentration-independent time constant of about 0.4 ms, the other with a concentration-dependent time constant in the millisecond range. The concentration dependence of the longer relaxation time can be accounted for by a three-step mechanism which consists of a fast second-order association reaction followed by two first-order steps. There is evidence, however, that the overall process is more complicated than expressed by the three-step mechanism.

1. Introduction

In recent years, the interactions of metal ions with nucleotides and related species have been the subject of many investigations and the results have been summarized in several review articles [1–7]. Most of the work was concerned with the sites of the interactions both in solution and in the solid state. Thermodynamic and kinetic aspects of these interactions have been studied less extensively. In the kinetic studies adenosine phosphates have been the preferred nucleotides and Mg^{2+} and Ni^{2+} the preferred metal ions [2,8–10]. Other nucleotides such as cytidine phosphates and inosine monophosphate and metal ions like Co^{2+} and Mn^{2+} have also been used in kinetic studies [2,11–14].

The kinetics of the interactions of divalent metal ions with mononucleotides are usually rather complex. For the reactions involving nucleoside diphosphates (NDP) and nucleoside triphosphates (NTP), curved plots are obtained if the reciprocal of the observed relaxation time is plotted vs. the reactant concentrations, indicating that the reaction is not a simple complex-formation process. The curvature can be rationalized by taking into account that at higher metal ion concentrations NDP and NTP form 2:1 complexes (M_2L) in addition to 1:1 complexes (ML) [2,9,11]. Curved plots of $1/\tau$ vs. reactant concentration have also been reported for the reactions of Ni^{2+} and Co^{2+} with various nucleoside monophosphates (NMP)

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[8,10,12,13] although NMP apparently is not able to form complexes of the type M_2L [8]. In these cases the curvature is accounted for by a 1:1 complex-formation mechanism in which the transition metal ion binds stepwise to the phosphate group and to the nucleotide base of the ligand. The experimental data usually allow a complete kinetic evaluation of such a two-step substitution process [8,13].

The results obtained so far with mononucleotides should provide a reasonable basis for the study of more complex nucleotides. Among the simplest oligonucleotides is adenylyl-3′,5′-adenosine (ApA) which has two adenine bases and one phosphate group as potential binding sites. Far more complex are polynucleotides like poly (adenylic acid) [poly(A)]. This compound, which exists in the single-stranded form above pH 6, has been frequently used as a model for a biological macromolecule. Equilibria and dynamics of the interactions of Ni^{2+} with ApA and with poly(A) are reported in this study.

2. Materials and methods

ApA, poly(A) (Boehringer), $Ni(ClO_4)_2 \cdot 6H_2O$ (Fluka), $NaClO_4$ (p.a., Merck), cacodylic acid (Fluka), murexide (Merck) and other chemicals were of the highest quality commercially available and were used without further purification. All solutions were prepared in water which was doubly distilled from a quartz apparatus. Stock nickel solutions were standardized by titration with EDTA using bromopyrogallol red as indicator [15]. The EDTA was standardized by titration with either standard zinc solution (Eriochrome Black-T as indicator) or nickel solutions prepared by diluting Merck Titrisol concentrate.

All measurements were carried out at 25.0 (±0.1)°C. Measurements of pH were made using a Radiometer PHM 52 digital pH-meter equipped with a Metrohm EA 125 combined electrode. The pH-meter was calibrated using Merck Titrisol buffer solutions. For measurements involving solutions containing $NaClO_4$, the KCl solution in the reference compartment of the electrode was replaced by an NaCl solution. The pH of all solutions for both equilibrium and kinetic studies was stabilized by adding 1×10^{-3} M cacodylate buffer. At this concentration the buffer components do not interact noticeably with Ni^{2+} [8].

2.1. Reactions of Ni^{2+} with ApA

The ionic strength (μ) of all solutions was adjusted to 0.5 M with $NaClO_4$. The equilibrium constant for the binding of Ni^{2+} to ApA was determined by ultraviolet spectrophotometry using a Cary 118 spectrophotometer. The free ligand has an absorption maximum at 258 nm, with $\epsilon_{258} = 2.78 \times 10^4$ M^{-1} cm^{-1} in neutral solution [16]. Addition of Ni^{2+} shifts the absorption band slightly to longer wavelengths. In order to increase the accuracy, difference absorbance measurements have been carried out at a high sensitivity of the instrument (0.1 A/full scale). Relatively high concentrations of Ni^{2+} are required in order to achieve appreciable complex formation. The contribution of free Ni^{2+} to the absorbance was compensated by using two-compartment cells in each path, i.e., by measuring (Ni^{2+} + ApA)(H_2O) vs. (Ni^{2+})(ApA).

Kinetic measurements were carried out by means of the temperature-jump relaxation technique with spectrophotometric detection [17]. The cell with the solution was thermostatically controlled to 19.7°C before raising the temperature to 25.0°C by discharging a 0.05 μF capacitor of 40 kV. A minimum of 10 min were allowed to elapse between successive jumps in order to provide sufficient time for temperature re-equilibration. The relaxation signal was recorded by means of a Datalab DL 905 transient recorder. In all cases a single exponential of relatively small amplitude was observed. Its time constant was determined using an analogue device (constructed by C.R. Rabl) which produces exponential curves of variable time constant and amplitude. The relaxation times given for this system are the mean of 5–9 individual measurements.

2.2. Reactions of Ni^{2+} with poly(A)

Concentrations of poly(A) were determined spectrophotometrically at pH 7, $\epsilon_{257} = 1.01 \times 10^4$

M^{-1} cm^{-1} (in terms of monomer concentration) [18]. The equilibrium constant for the binding of Ni^{2+} to poly(A) was evaluated spectrophotometrically, in this case, however, not in the ultraviolet range but in the visible region, using murexide as an indicator of the concentration of free metal ions. (Addition of Ni^{2+} to solutions of murexide (mur) produces very large spectral changes, e.g., $\epsilon_{mur}/\epsilon_{Nimur} \approx 9$ at 520 nm). This procedure was adopted since the ultraviolet absorption changes on addition of Ni^{2+} not only reflect direct Ni^{2+}-base interactions but also shifts in the degree of base stacking [19], and these two contributions are not easily separated. Solutions of murexide were titrated spectrophotometrically against standardized Ni^{2+} solutions in the presence of poly(A) and, for calibration, in the absence of poly(A), each series of spectra showing excellent isosbestic points. In these titrations, small amounts of the Ni^{2+} solution were added to the solution in the spectrophotometer cell by means of a micrometer syringe unit. The cell holder was equipped with a variable-speed d.c. motor in order to operate a micro magnetic stirring bar in the cell. Separate tests showed that the spectrum of murexide is also sensitive to higher concentrations of alkali metal ions, the effect of K^+ being considerably smaller than that of Na^+. The ionic strength of all solutions was therefore adjusted to 0.1 M using KNO_3. Additional tests revealed that the spectrum of murexide is not affected by poly(A) in the absence of Ni^{2+}. Because of the instability of murexide in aqueous solution, the spectrum was recorded only over a narrow wavelength range around 520 nm after each addition of Ni^{2+}, and all measurements of individual runs were completed within 1 h. It was previously demonstrated that the decomposition of murexide under our conditions is negligible over a period of 1 h.

The kinetic measurements were carried out by the temperature-jump technique as described above (no murexide present, observation in the ultraviolet). The helix-coil equilibration is very rapid [20,21], it occurs within the heating time (a few microseconds) of the apparatus used and contributes to a fast initial absorbance change. The observed relaxation effects could not be fitted by a single exponential. Therefore, the measured signals were transferred from the transient recorder to the Univac 1108 computer of the Gesellschaft für Wissenschäftliche Datenverarbeitung, Göttingen (GWDG). This was done either directly via a PDP 11/34 minicomputer or alternatively using magnetic tapes. The data were subsequently processed using the Provencher method [22]. The curve-fitting routine was a standard non-linear least-squares method as used in the GWDG. The time constants given represent the average of 9–15 individual measurements.

3. Results

3.1. Ni^{2+} + ApA

In the spectrophotometric determination of the binding constant the concentrations of total ligand (L_0) and total metal (M_0) were $L_0 = 3 \times 10^{-5}$ M and $M_0 = 0.03$–0.17 M. The spectrophotometric data for 1 : 1 complex formation (ML) can be represented by the relationship

$$\frac{1}{\Delta A} = \frac{1}{\Delta\epsilon L_0} + \frac{1}{\Delta\epsilon L_0 K} \cdot \frac{1}{M_0} \tag{1}$$

where ΔA = difference absorbance per cm light path, $\Delta\epsilon = \epsilon_{ML} - \epsilon_L$ = difference of the extinction coefficients of complex and ligand and K = stability constant of ML. In deriving eq. 1, use has been made of the condition $M_0 \gg L_0$, and, conse-

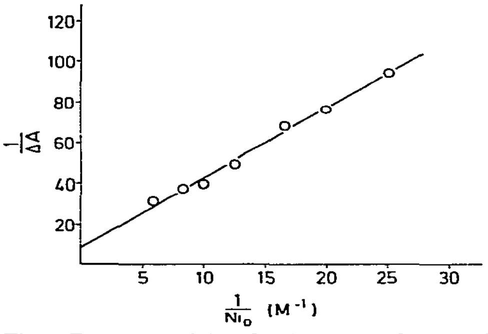

Fig. 1. Experimental data for the spectrophotometric evaluation of the stability constant of the Ni-(ApA) complex (total ApA concentration = 3×10^{-5} M, pH 6.0, λ = 270 nm, 25°C, μ = 0.5 M).

quently, of $M_0L_0 \gg [ML]^2$, i.e., of the Benesi-Hildebrand approximation [23].

Experimental data obtained at pH 6.0 and $\lambda = 270$ nm are shown in fig. 1 in the form of a plot of $1/\Delta A$ vs. $1/M_0$ (eq. 1). A least-squares evaluation yields for the stability constant K,

$$K = \frac{[NiApA^+]}{[Ni^{2+}][ApA^-]}$$

$$= 2.58\ (\pm 0.65)\ M^{-1}\ (25°C, \mu = 0.5\ M).$$

The kinetics of the complex-formation reaction between Ni^{2+} and ApA^- were studied by the temperature-jump relaxation technique. In all experiments the observed signal corresponded to a single exponential, its time constant being dependent on the reactant concentrations. The expression for the reciprocal relaxation time which is expected for a simple complex-formation process is of the form

$$\frac{1}{\tau} = k_f([Ni^{2+}] + [ApA^-]) + k_b \quad (2a)$$

where k_f is the second-order association rate constant and k_b the first-order dissociation rate constant. The total concentration of Ni(II) in the kinetic studies was again very large compared to that of ApA ($L_0 \simeq 5.5 \times 10^{-5}$–$7.0 \times 10^{-5}$ M); therefore, $[Ni^{2+}] \simeq Ni_0$, and

$$\frac{1}{\tau} = k_f Ni_0 + k. \quad (2b)$$

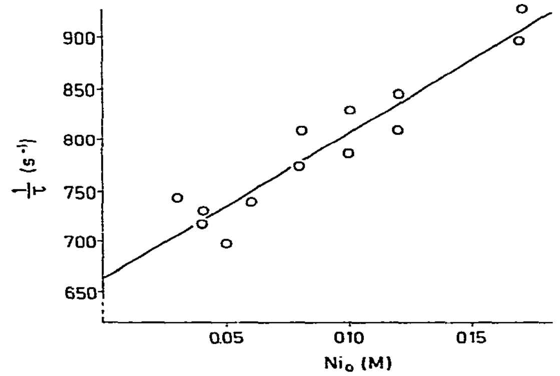

Fig. 2. Concentration dependence of the reciprocal relaxation time for the reaction $Ni^{2+} + ApA^- \rightleftharpoons Ni(ApA)^+$ (pH 6.0, $\lambda = 265$ nm, 25°C, $\mu = 0.5$ M).

Experimental data obtained at pH 6.0 are shown in fig. 2 ($\lambda = 265$ nm). A least-squares fit of the data gives $k_f = 1428\ (\pm 145)\ M^{-1}\ s^{-1}$ and $k_b = 665\ (\pm 20)\ s^{-1}$ (25°C, $\mu = 0.5$ M). The ratio $k_f/k_b = K_{(kin)} = 2.15\ (\pm 0.3)\ M^{-1}$ is in fair agreement with the value of 2.58 (±0.65) obtained from the spectrophotometric data.

3.2. Ni^{2+} + poly(A)

In order to evaluate the strength of binding of Ni^{2+} to poly(A), we have used the metallochromic indicator murexide (ammonium 5,5′-nitrilodibarbiturate) to determine the free Ni^{2+} concentration of solutions containing murexide, poly(A) and Ni(II). The equilibrium between Ni(II) and murexide alone was investigated at pH 6.0, 6.5 and 7.0 in 0.1 M KNO_3. An apparent stability constant K_{app} for the binding of Ni^{2+} to murexide was obtained from spectrophotometric data (460 or 520 nm) via eq. 3 by an iterative procedure (the second term on the left-hand side of eq. 3 was neglected at first and approximate values of K_{app} and $\Delta\epsilon$ were evaluated; the approximate value of $\Delta\epsilon$ was then introduced into the left-hand side of eq. 3 to get better values of K_{app} and $\Delta\epsilon$, etc.).

$$\frac{M_0L_0}{\Delta A} + \frac{\Delta A}{(\Delta\epsilon)^2} = \frac{1}{K_{app}\Delta\epsilon} + \frac{M_0 + L_0}{\Delta\epsilon} \quad (3)$$

M_0, L_0, ΔA and $\Delta\epsilon$ denote total metal (3×10^{-5}–1.2×10^{-3} M Ni(II)), total ligand (6.7×10^{-5} M murexide), the change in absorbance, and the difference of the apparent extinction coefficients of complex and ligand, respectively. The stability constants thus obtained show an appreciable pH dependence: $K_{app} = 2.48 \times 10^3\ M^{-1}$ at pH 6.0, 4.47×10^3 at pH 6.5 and 1.03×10^4 at pH 7.0 (25°C, $\mu = 0.1$ M). This pH dependence indicates that Ni^{2+} does form a complex not only with the mono-negative murexide anion (HL^-, stability constant K_{MHL}) but also with the deprotonated form L^{2-} (K_{ML}). Therefore (charges neglected)

$$K_{app} = \frac{[ML] + [MHL]}{[M]([HL] + [L])} \quad (4)$$

and

$$K_{app}([H^+] + K_a) = K_aK_{ML} + K_{MHL}[H^+] \quad (5)$$

with

$K_a = [H^+][L^{2-}]/[HL^-] = 6.3 \times 10^{-10}$ M [24].

A plot of $K_{app}([H^+] + K_a)$ vs. $[H^+]$ (not shown) yields a good straight line from which is obtained $K_{ML} = 1.43 \times 10^6$ M^{-1} and $K_{MHL} = 1.59 \times 10^3$ M^{-1} (25°C, $\mu = 0.1$ M). This gives a value of K_{app} at pH 4.0 of 1.6×10^3 M^{-1}, compared to the value of 2.3×10^3 reported by Geier [25] at a temperature of 12°C.

If the fraction of murexide converted to its nickel complex is α, eq. 4 may be rewritten as eq. 6.

$$K_{app} = \alpha/[Ni^{2+}](1-\alpha) \tag{6}$$

Thus, if K_{app} is known, the free Ni^{2+} concentration can be determined–also in the presence of poly(A)–from absorbance meaurements with the aid of eq. 7.

$$\alpha = (A_L - A_m)/(A_L - A_{ML}) \tag{7}$$

where A_m is the absorbance measured at a given Ni(II) concentration, $A_L = \epsilon_L L_0$ is the initial absorbance in the absence of Ni(II), and $A_{ML} = \epsilon_{ML} L_0$ is the absorbance if all murexide has been converted to the Ni^{2+} complex. The concentration of Ni^{2+} bound to poly(A), $[Ni_{poly(A)}]$, is now obtained from the mass balance relationship [8],

$$Ni_0 = [Ni_{free}] + [Ni(mur)] + [Ni_{poly(A)}], \tag{8}$$

with $[Ni(mur)] = \alpha L_0$.

The ratio $n = [Ni_{poly(A)}]/poly(A)_0$ denotes the average number of Ni^{2+} ions bound per mononucleotide (the concentration of total polynucleotide, $poly(A)_0$, given in terms of monomeric units). Under our conditions, with $L_0 = 6.7 \times 10^{-5}$ M, $poly(A)_0 = 1.8 \times 10^{-3}$ M and $Ni_0 = 5.6 \times 10^{-5}$–$1.1 \times 10^{-3}$ M, n varies between 0.021 and 0.23 (25°C, $\mu = 0.1$ M, pH 7.0); i.e., at the lowest Ni(II) concentration 1 Ni^{2+} is bound per approx. 50 mononucleotides, at the highest Ni(II) concentration 1 Ni^{2+} per 4.4 mononucleotides.

The simplest representation of site-binding data for polyelectrolytes is that of Scatchard [26]. Assuming that binding occurs only at certain binding sites S, eq. 9 is obtained (the Scatchard equation),

$$n/[Ni^{2+}] = Km - Kn, \tag{9}$$

in which m is the number of binding sites per monomer and K is the stability constant for binding to S, i.e., $K = [NiS]/[Ni^{2+}][S]$.

Fig. 3 shows a plot of $n/[Ni^{2+}]$ vs. n according to eq. 9 for the reaction of Ni^{2+} with poly(A) at pH 7.0 (25°C, $\mu = 0.1$ M). Within the experimental accuracy the relationship can be represented by a straight line. From the slope and intercept the results $K = 8.2 \times 10^3$ M^{-1} and $m = 0.264$ are obtained.

The kinetics of the reaction between Ni^{2+} and poly(A) were investigated at pH 7.0 under the

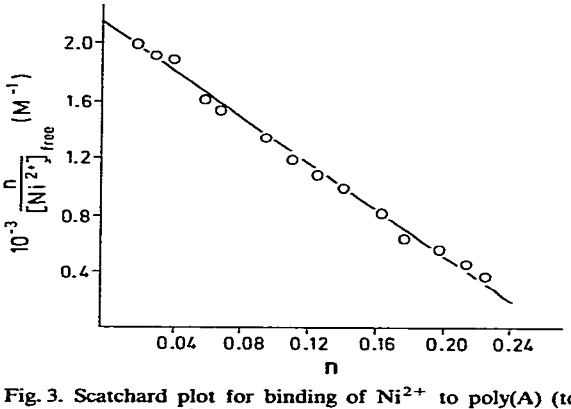

Fig. 3. Scatchard plot for binding of Ni^{2+} to poly(A) (total poly(A)$= 1.8 \times 10^{-3}$ M, total Ni(II)$= 5.6 \times 10^{-5}$–1.1×10^{-3} M, pH 7.0, 25°C, $\mu = 0.1$ M).

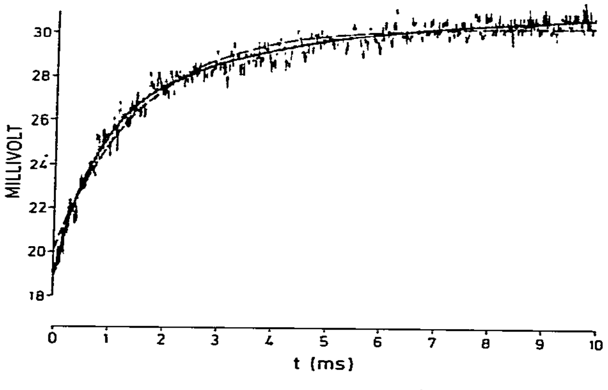

Fig. 4. Relaxation effect in the system Ni^{2+} + poly(A) (total poly(A)$= 8.2 \times 10^{-5}$ M, total Ni(II)$= 1.2 \times 10^{-3}$ M, pH 7.0, 25°C, $\mu = 0.1$ M, $\lambda = 264$ nm). Broken line: best fit with one time constant. Solid line: best fit with two time constants.

concentration conditions, poly$(A)_0 = 4.1 \times 10^{-5}$–$8.2 \times 10^{-5}$ M, $Ni_0 = 2.5 \times 10^{-5}$–$1.5 \times 10^{-3}$ M (25°C, $\mu = 0.1$ M, $\lambda = 264$ nm). The total amplitude of the relaxation process is rather small, corresponding to a relative change in light intensity, $\Delta I/I$, of 2×10^{-3}–4×10^{-3}. The observed relaxation signals were always clearly fitted better by two overlapping exponentials than by a single exponential. This is demonstrated in fig. 4 which shows an experimental curve, the best fit with one time constant (broken line), and the best fit with two time constants (solid line). The relaxation time constants and amplitudes of the two contributions were resolved as described in section 2. The time constant τ_f of the faster process is always approx. 0.4 ms and does not show a pronounced concentration dependence but considerable scatter (up to ±35%). This lack of precision is due to the small amplitude of this effect which contributes about 30–40% to the total amplitude. The slower relaxation effect has a larger amplitude and its time constant τ_s can be evaluated with better accuracy. Its concentration dependence is shown in fig. 5 in form of a plot of $1/\tau_s$ vs. $[S]_{free} + [Ni^{2+}]_{free}$, where $[S]_{free}$ denotes the concentration of free binding sites and is given by $[S]_{free} = m\text{poly}(A)_0 - [Ni_{poly(A)}]$ (with $m = 0.26$, see above). The sum of the reactant concentrations, $[S]_{free} + [Ni^{2+}]_{free}$, was always close to Ni_0.*

At low reactant concentrations $1/\tau_s$ varies approximately linearly with increase in reactant concentration (fig. 5). At higher reactant concentrations it rapidly levels off and becomes independent of reactant concentration. This type of behaviour suggests that the reaction proceeds by a multi-step reaction mechanism.

Several mechanistic models of increasing complexity were tested and their rate parameters evaluated using a non-linear curve-fitting technique. A two-step mechanism involving a rapid pre-equilibrium, applicable to many complex-formation reactions of metal ions with simple ligands, is clearly inconsistent with the experimental data: The broken line in fig. 5 shows the best fit obtained for this mechanism with an overall equilibrium constant of $K = 8.2 \times 10^3$ M^{-1}. The simplest mechanism consistent with the experimental data is given by the scheme:

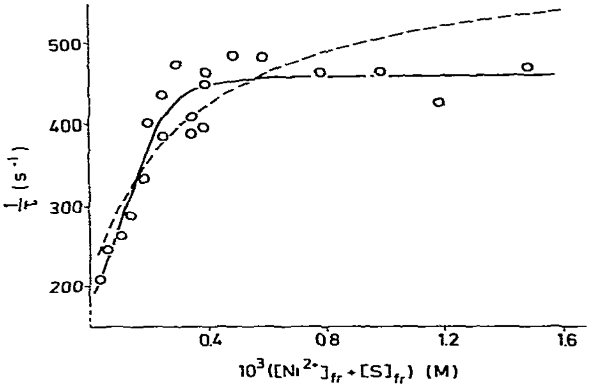

Fig. 5. Concentration dependence of $1/\tau_s$ for the reaction Ni^{2+} + poly(A) (pH 7.0, 25°C, $\mu = 0.1$ M). Broken line: best fit with two-step mechanism. Solid line: best fit with three-step mechanism; see text).

$$Ni^{2+} + S \overset{K_0}{\rightleftharpoons} Ni,S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} NiS \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} NiS'. \tag{10}$$

In this three-step mechanism, a rapid association process is followed by two first-order reaction steps. The first product in this sequence, Ni,S, is an outer-sphere state, whereas NiS and NiS′ are considered to be inner-sphere states. Thus, K_0 is a rapidly establishing pre-equilibrium (time constant < 1 μs) compared to the following steps and two more relaxation time constants are expected for this mechanism. The expressions for these two time constants can be calculated [17] and are of the form shown by eq. 11:

$$\frac{1}{\tau_{+,-}} = 0.5\Big[(a_{11} + a_{22}) \pm \sqrt{(a_{11} + a_{22})^2 - 4(a_{11}a_{22} - a_{12}a_{21})}\Big] \tag{11}$$

with

$$a_{11} = k_{-1} + k_{-1}\frac{(K - K_0)\cdot C}{1 + K_0 C}\cdot\frac{a_{22} - a_{21}}{a_{22}}$$

$$a_{12} = k_{-1};\ a_{21} = k_2;\ a_{22} = k_2 + k_{-2}$$

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* It was pointed out by a referee that at very low values of n each monomeric unit might act as a binding site. If this model is adopted, the points of fig. 5 at low reactant concentrations (initial steep increase) are shifted to the right by a small amount (3×10^{-5} M) but otherwise the shape of the plot in fig. 5 is not affected.

and where C is the reactant concentration ($= [Ni^{2+}]_{free} + [S]_{free}$), and K the overall stability constant ($= 8.2 \times 10^3$ M^{-1}). K is related to the stepwise equilibrium constants by the relationship:

$$K = K_0[1 + K_1(1 + K_2)] \tag{12}$$

where $K_1 = k_1/k_{-1}$ and $K_2 = k_2/k_{-2}$.

The experimentally observed concentration dependence of τ_s is consistent only with the minus sign of the square root in eq. 11, i.e., $\tau_- = \tau_s$. Keeping K constant and allowing K_0, a_{12}, a_{21} and a_{22} to vary simultaneously, the following values were obtained from the curve-fitting process: $K_0 = 780$ M^{-1}, $a_{12} = k_{-1} = 173$ s^{-1}, $a_{21} = k_2 = 8.9$ s^{-1}, $a_{22} = k_2 + k_{-2} = 463$ s^{-1}; i.e., $k_{-2} = 454$ s^{-1}. From k_{-1} and $K_1 = (K - K_0)/K_0(1 + K_2)$ (by rearranging eq. 12) results $k_1 = 1.61 \times 10^3$ s^{-1}.

The concentration dependence of $1/\tau_-$ calculated with these values is shown as the solid line in fig. 5; the fit to the experimental data is fairly good. However, the minimum of the sum of the square deviations (relative errors) is a rather shallow one for this four-parameter fitting process, and fits which are almost as good as that shown in fig. 5 may be obtained also with other data sets in which K_0 can vary approximately between 10^2 and 10^3 M^{-1}, e.g., with $K_0 = 100$ M^{-1}, $k_{-1} = 184$ s^{-1}, $k_2 = 38$ s^{-1}, $k_{-2} = 434$ s^{-1}, and therefore $k_1 = 1.35 \times 10^4$ s^{-1}.

4. Discussion

4.1. Ni^{2+} + ApA

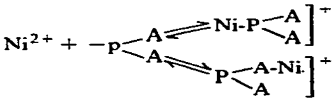
The pK of the phosphate group of ApA in aqueous solution is very low (≤ 1.0) while the apparent pK for the addition of protons to the adenine bases is 3.5 [27]. Thus, at pH 6.0 ApA exists in the mono-negative form, ApA^- (charge at the phosphate).

The stability constant for complex formation of Ni^{2+} with ApA^- was found to be 2.6 M^{-1} by spectrophotometric techniques (25°C, $\mu = 0.5$ M). This value appears to be rather low if compared to that for $Ni^{2+} + AMPH^-$, $K = 11$ M^{-1} at 25°C, $\mu = 0.1$ M [8]. (At an ionic strength of 0.5 M this constant is expected to be close to 6 M^{-1}.) ApA^- is of the same charge type as $AMPH^-$ but has one more potential binding group (an adenine). The stability data apparently indicate that the predominant Ni^{2+}-ApA^- species is not a chelate complex, contrary to the situation with $AMPH^-$ as ligand. This difference in behaviour is probably a consequence of the conformational properties of ApA^- in solution. ApA^- prefers conformations in which the two adenines are in a stacked position. Recent studies gave evidence for the presence of two different stacked conformations in solution [28,29]. Literature data regarding the degree of stacking in ApA^- at room temperature vary considerably, reported values include 50% stacking [30], 62% [27] and 84% [31]. The most likely conformation of unstacked ApA^- is one with a base above a ribose moiety [29]. In all three conformations of ApA^- mentioned the phosphate group is rather far away from the adenines [29] and simultaneous binding of a metal ion to phosphate and a base is not possible without drastic conformational changes. Similarly, the arrangement of the adenines in these conformations does not allow the formation of a chelate structure involving the two bases. On the other hand, the two adenines are fairly close to each other in all three conformations [29] and their N_7 atoms (the preferred binding sites for transition metal ions) can certainly not be taken as independent binding sites. It is probably more correct to consider the region of the two adenines as one reaction site, allowing only the binding of one Ni^{2+} to one of the bases.

Metal ion binding to the phosphate group is also possible, of course. However, under our conditions of high ionic strength (0.5 M) and with the steric hindrance from the two adenosines, binding of Ni^{2+} to the mono-negative phosphate group is rather weak; the binding constant is estimated (from the data of Table 3 of ref. 8) to be less than 2 M^{-1}. This estimate is consistent with stability constants of 3–6 M^{-1} which have been reported [8,32] for complex formation of Ni^{2+} with P_i, $H_2PO_4^-$ and ribose phosphate, RPO_3H^-, at 15 or 25°C, $\mu = 0.1$ M, with little or no steric hindrance effects.

In view of these considerations the observed overall stability constant for complex formation of Ni^{2+} with ApA^- of $K = 2.6$ M^{-1} appears quite reasonable if one takes into account that the stability constant for complex formation of Ni^{2+} with adenosine is 2.0 M^{-1} [8]. As far as we are aware, no other equilibrium data exist for the reaction of transition metal ions with ApA. Pörschke [33] reported the following stability constants for complex formation of Mg^{2+} with the next members of this nucleotide series: $Mg^{2+} + A(pA)_2$, $K = 12\ M^{-1}$ and $Mg^{2+} + A(pA)_3$, $K = 370\ M^{-1}$ (20°C, low ionic strength).

Despite the apparent variety of species in Ni^{2+}/ApA^- solutions, the kinetics of the complex-formation process exhibit a simple pattern: only one relaxation effect is observed, the reciprocal relaxation time increases linearly with the reactant concentrations (fig. 2). In this context it is of relevance that the conformational transitions between stacked and unstacked forms of oligonucleotides are known to be rapid. For ApA^-, the time constants for the equilibration processes are less than or equal to 0.5 μs [20b,34], too short to be observed with the present apparatus (in those studies two relaxation effects were detected, indicating the presence of (at least) three forms of ApA^-, see above). The conformational transitions of monodentate Ni-Apa complexes are presumably just as rapid. Because of these fast conformational pre-equilibria, the complex-formation process can be described by the simple reaction scheme,



(13)

In principle, such a mechanism should give rise to the appearance of two relaxation effects, whereas only one is actually observed. The spectrophotometric amplitude of one of the effects is apparently very low and is not detected experimentally. Two factors may contribute to this behaviour: (a) binding of Ni^{2+} to the phosphate group of ApA^- has no noticeable effect upon its ultraviolet absorption; (b) the rates of Ni^{2+}-phosphate binding and dissociation are appreciably faster than the corresponding reactions of Ni^{2+} with an adenine group. Therefore, there is little coupling between the upper and lower reaction path of scheme 13, i.e., the upper reaction step is, to a good approximation, a pre-equilibrium to the lower step. Point b is illustrated by the following data: the rates of reaction of Ni^{2+} with $H_2PO_4^-$ and RPO_3H^- are $3.3 \times 10^4\ M^{-1}\ s^{-1}$, and the dissociation rates are 1.1×10^4 and $0.7 \times 10^4\ s^{-1}$, respectively (15°C, $\mu = 0.1$ M) [32]). Under our conditions (25°C, $\mu = 0.5$ M) these rate constants will not be very different. The corresponding rate constants for the reaction with adenosine, however, are $1.1 \times 10^3\ M^{-1}\ s^{-1}$ and 650 s^{-1} [8]. Therefore, it is to be expected that the faster effect has a negligible ultraviolet amplitude.

It is also consistent with this interpretation that the apparent rate constants for the complexation reaction of Ni^{2+} with ApA^- which have been evaluated from fig. 2, $k_f = 1.4 \times 10^3\ M^{-1}\ s^{-1}$ and $k_b = 665\ s^{-1}$, are remarkably similar to those just mentioned for the complex-formation reaction of Ni^{2+} with adenosine.

4.2. Ni^{2+} + poly(A)

At pH 7 poly(A) exists in the single-stranded form [35] and carries one negative charge per phosphate group. Because of the high charge density, polynucleotides interact very strongly with metal ions. These interactions may be of two types. Firstly, there is 'diffuse' binding in which the metal ions are attracted by the electrostatic potential of the polyelectrolyte to form a mobile cloud around the polymer. Secondly, there is 'site' binding in which the metal ions are bound to specific binding sites at the polymer [33,36].

The equilibrium studies reported here for the binding of Ni^{2+} to poly(A) do not distinguish between these binding modes. For the reasons given above (section 2), the metal ion indicator murexide was used to evaluate the binding equilibra. Murexide can be used to determine the concentration of free Ni^{2+} with sufficient accuracy approximately between 1×10^{-5} M and 1×10^{-3} M $[Ni^{2+}]_{free}$. Within this concentration range the Scatchard plot shows good linearity (fig. 3) and thus indicates that the process can be described as the binding of Ni^{2+} to one class of independent binding sites. There are $m = 0.26$ binding sites per monomeric unit, and the stability constant for binding of Ni^{2+} to these sites is $K = 8.2 \times 10^3$ M^{-1} (25°C, $\mu = 0.1$ M). It should be realized that this is only a formal description which probably would break down (i.e., the Scatchard plot deviates from linearity) if the studies were extended to higher concentrations of Ni^{2+} (which is not possible with murexide).

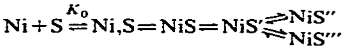
The lack of evidence for anti-cooperative effects is not astonishing, particularly at an ionic strength of 0.1 M, in view of the fact that even at the highest Ni^{2+} concentration used in this study only one Ni^{2+} is bound per 4.4 nucleotide units. When our binding data are subjected to the approach used by Wagner [37], the nearest-neighbour interaction energy evaluated via eq. 5 of ref. 37 is indeed vanishing small, about 250 J mol^{-1}.

Linear Scatchard plots have also been reported for a few other binding reactions between divalent metal ions and polynucleotides, e.g., for the reactions of Mn^{2+} with poly(A) and other homopolyribonucleotides [38], but also for the binding of Mn^{2+} to some natural nucleic acids, like yeast RNA and some DNAs, at nucleic acid concentrations below 1×10^{-3} M [39]. The Scatchard plots for binding of Mg^{2+} by polynucleotides also show a linear region at low degrees of binding ($n < 0.3$) [40].

The stability constants depend very strongly upon the ionic strength. For Mg^{2+} + poly(A), K increases from 2.7×10^4 M^{-1} at medium ionic strength (20 mM Tris buffer) to 2.7×10^7 M^{-1} at very low ionic strength (no buffer) [20a]. The number of binding 'sites' per nucleotide is 0.2–0.3, but according to kinetic studies [20a] inner-sphere site binding does not take place in this system, and only diffuse binding is observed. For the binding of Mn^{2+} to poly(A) the following values for the stability constant and for the number of binding sites per nucleotide have been reported: $K = 4 \times 10^4$ M^{-1}, $m = 0.45$ (20°C, 10^{-3} M NaCl) [39], $K = 8.2 \times 10^3$ M^{-1}, $m = 0.38$ (22°C, $\mu = 0.1$ M) [38] and $K = 5.2 \times 10^3$ M^{-1}, $m = 0.41$ ($\mu = 0.1$ M) [41]. The values of the stability constant for $\mu = 0.1$ M are very similar to that obtained in the present study for Ni^{2+} + poly(A).

From ^1H- and ^{31}P-NMR relaxation studies, Yamada et al. [41] concluded that 48% of the Mn^{2+}-poly(A) complexes are bound only via the phosphate groups (the authors assume that each Mn^{2+} is coordinated to two phosphates), 39% are coordinated additionally to N_7 of the adenine ring, and 13% to two phosphates and to N_1 or N_3. Similar conditions may be expected to apply also to the binding of Ni^{2+} to poly(A). It was shown above that the observed concentration dependence of $1/\tau_s$ can be simulated by a three-step mechanism. However, only the rate constants k_{-1} and k_{-2} can be evaluated fairly accurately by the fitting procedure (section 3), whereas the uncertainties of K_0, k_1 and k_2 are large, up to about one order of magnitude. Still, a value of 10^2–10^3 M^{-1} for the outer-sphere association constant K_0 appears quite reasonable at an ionic strength of 0.1 M, and a value of 10^3–10^4 s^{-1} for k_1 is fully consistent with that for a first substitution step at Ni^{2+} (see table 4 of ref. 8). If k_2 represents a second substitution step at Ni(II), its rather low rate can be rationalized if a conformational change of the poly(A) ligand is necessary before substitution can occur.

The observation of a second relaxation effect with $1/\tau_f \approx 2500$ s^{-1} indicates that the three-step mechanism is not sufficient to describe the binding process ($1/\tau_f$ is not identical with $1/\tau_+$ of eq. 11 for which values between 470 and 1040 s^{-1} are calculated). If Yamada et al. [41] are correct, the reaction scheme could be of the form (charges neglected)



$$\mathrm{Ni} + \mathrm{S} \overset{K_0}{\rightleftharpoons} \mathrm{Ni,S} \rightleftharpoons \mathrm{NiS} \rightleftharpoons \mathrm{NiS'} \begin{matrix} \rightleftharpoons \mathrm{NiS''} \\ \rightleftharpoons \mathrm{NiS'''} \end{matrix} \qquad (14)$$

in which the complex species have the following binding modes: NiS (one phosphate), NiS′ (two phosphates), NiS″ (two phosphates and N_7), NiS‴ (two phosphates and N_3 or N_1). The second relaxation effect (τ_f) may be due to the transition NiS′ ⇌ NiS″ or NiS′ ⇌ NiS‴. If this step equilibrates rapidly compared to NiS ⇌ NiS′, the fitting of the experimental data to the three-step mechanism is formally correct and the considerations given above are still valid. The complete description of reaction scheme 14 would require the determination of eight unknowns. This cannot be done with the experimental data available at present.

Acknowledgements

We express our thanks to the CIPA-GEIGY Fellowship Trust for a Senior Fellowship to M.J.H., to M. Junge of this institute for help with the computer evaluations, to Dr. D. Pörschke for discussions, and to Dr. R.S. Taylor who did some preliminary experiments on these systems in our laboratory several years ago.

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