Interaction between Metal Ions and NAD(P) Coenzymes. ^{1}H, ^{31}P, ^{13}C and ^{59}Co NMR Spectroscopy and Conformational Analysis

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The interaction of the coenzymes NAD(P) with metal ions Mg^{2+}, Ca^{2+}, Zn^{2+}, Li^{+}, $[Cr(NH_3)_6]^{3+}$, $[Ru(NH_3)_6]^{3+}$ and $[Co(NH_3)_6]^{3+}$ have been studied by ^{1}H, ^{13}C, ^{31}P and ^{59}Co NMR spectroscopy. The complexes with Mg^{2+} have been examined in detail, by performing the conformational analysis of the ribose rings, of the glycosidic moieties, of the chain fragments from C(5') to P(5') atoms, by using H–H and H–P coupling constant values and ^{1}H NOESY experiments. The stability constants of the complexes with diamagnetic metal ions have been obtained from titration experiments, following the chemical shift of the two (three) phosphorus atoms. The effects of association on the coupling constant J(P–O–P) and on the ^{31}P chemical shifts were compared for the different ions. The ligands $[M(NH_3)_6]^{3+}$ have been utilized as mimics of $[Mg(H_2O)_6]^{2+}$ and the paramagnetic ion provided structural information through the measurements of the line-width variation of ^{31}P and ^{13}C signals. The ^{59}Co experiments allowed us to exclude dimeric structures such as $(NADP)_2$–Co. Models of (1:1) complexes of NADPH with $[Mg(H_2O)_6]^{2+}$ and $[Cr(NH_3)_6]^{3+}$ are proposed. The metal ion binds to the three phosphates with interactions of electrostatic nature, through the mediation of NH_3 or H_2O molecules. The hydrogen-bonding of NH_3 and H_2O to the phosphate oxygens contributes to the stabilization of the complexes.

The NAD and NADP coenzyme-dependent dehydrogenases are a fascinating class of enzymes which still has many aspects of interest.[1–9] The mechanism of the enzymatic reductions and oxidations and the stereospecificity of the enzymes present many open questions. Recently, the importance of conformational features in the reaction of dehydrogenase enzymes has also been reconsidered.[1,2]

The effects of metal ions in these processes have not attracted much attention. It has been suggested that divalent cations influence the activity of some NAD^{+}-dependent isocitrate dehydrogenases.[3] Actually these enzymes bind several nucleotides as activators or inhibitors, such as NADH, NADPH, ATP and ADP. Kinetics and binding studies of ADP activation have shown an influence of divalent metal ions. The oxidation of NAD(P)H on the surface of the mitochondrial membrane is also regulated by cations.[4] On the other hand, divalent metal ions have been shown to affect the kinetics and sometimes even the stereochemistry of the reduction of organic substrates by NADH model compounds.[5]

More generally, metal ions and nucleotides are involved in the basic processes of life,[8] but, over the last 10 years, many studies have concerned only the interactions of ATP with metals.[8 10] The coordinating properties of this extraordinary molecule are now relatively well understood and a recent stimulating review on the topic has been written by Sigel.[10] Analogous investigations on NAD(P) coenzymes have not yet appeared. The crystal structure of the NAD^{+} lithium salt[11] and two NMR studies of Mn^{II}–NADP[12] and Co^{II}–NAD(P)[13] complexes have been published. We have thus studied the interactions of these coenzymes with the following metal ions: Mg^{2+}, Ca^{2+}, Zn^{2+}, $[Cr(NH_3)_6]^{3+}$ and $[Co(NH_3)_6]^{3+}$, by using ^{1}H, ^{13}C, ^{31}P and ^{59}Co NMR spectroscopy.

Experimental

Materials.—β-$NADP^{+}$ monosodium salt and NADPH tetrasodium salt 98% pure were from Sigma, β-NAD^{+} monosodium salt and NADH disodium salt 98% pure were from Boehringer. $[Co(NH_3)_6]Cl_3$ and $[Ru(NH_3)_6]Cl_3$ were from Strem Chemicals. $[Cr(NH_3)_6]Cl_3$ was a kind gift from R. J. P. Williams (Inorganic Chemistry Laboratory, Oxford). D_2O low in paramagnetic impurities was from Aldrich. The coenzymes were purified to eliminate traces of metal contaminants, by passing a solution through a column of Chelex 100 (Biorad), previously conditioned with D_2O at pH 7 and then lyophilizing the effluent solution. The samples for NOE experiments were carefully degassed with four freeze–thaw–pump cycles directly in NMR tubes, which were then sealed. Variable concentrations, from 0.4 to 20 mg cm^{-3}, of the coenzyme in D_2O were used for the NMR experiments; a standard concentration of 10 mg per 0.6 cm^3 was utilized to obtain the NMR data for the conformational analysis and for the titration experiments. The concentration of the coenzyme's solution was determined by means of UV absorbance (ε = 14.1 cm^2 μmol^{-1} at alkaline pH, ε = 18.1 at acidic pH). The solutions were adjusted to pH 8.5–9.5 (uncorrected for D_2O solvent) with ammonia vapours, except for the Zn complexes which were studied at pH 4.0 and the coenzymes in the oxidized form, which were studied at pH 7.5. The titrating solutions of Mg^{2+}, Ca^{2+} and Zn^{2+} ions were prepared from the corresponding oxides, which were dried at 150 °C for 4 h, by adding a stoichiometric amount of DCl 38% w/w. The NMR spectra were recorded after addition of increasing amounts of the titrating solution to the coenzyme solution in D_2O. The variation of the line-width during the titration with paramagnetic $[Cr(NH_3)_6]Cl_3$ was normalized with respect to the line-width of a reference.

NMR Techniques.—^{1}H, ^{13}C, ^{31}P and ^{59}Co NMR spectra were recorded at room temperature with a Bruker AMX 600 spectrometer, operating at 600.13 MHz for the ^{1}H nucleus. Chemical shifts are given in ppm, referenced from external 3-(trimethylsilyl)propane-1-sulfonic acid sodium salt hydrate (DSS) for ^{1}H, from external 85% H_3PO_4 for ^{31}P and from external $[Co(NH_3)_6]Cl_3$ for ^{59}Co. The estimated accuracy was ±0.01 ppm for ^{31}P and ^{13}C and ±0.005 ppm for ^{1}H, unless specified in Table 1, where the values for NADP–Mg complexes are reported. ^{13}C Assignments were performed by ^{1}H ^{13}C heteronuclear shift-correlated 2D experiments (Bruker

Table 1 ^{1}H and ^{31}P Chemical shift assignment (ppm) for complexes of Mg^{2+} with NADP coenzymes

	^{1}H Adenine nucleotide[a]								^{1}H Nicotinamide nucleotide[a]										^{31}P[b]		
Complex	2-H	8-H	1′-H	2′-H	3′-H	4′-H	5′-H	5″-H	2-H	4-H	5-H	6-H	1′-H	2′-H	3′-H	4′-H	5′-H	5″-H	P(A)	P(N)	P(2′)
NADPH–Mg	8.17	8.45	6.17	5.02	4.61	4.37	4.21	4.19	6.94	*c*	4.77[d]	5.90	4.74	4.10	4.14	4.02	4.03	4.00	−11.27	−10.98	+3.19
$NADP^{+}$–Mg	8.07	8.41	6.08	5.03	4.62	4.37	4.23	4.19	9.26	8.79	8.14	9.04	6.03	4.41	4.35	4.47	4.30	4.19	−11.16	−11.06	+3.26

[a] From external DSS reference. [b] From external 85% H_3PO_4 reference. [c] The geminal protons at C-4 are diastereotopic and lie at 2.68 and 2.80 ppm. [d] Partially overlapped by HOD signal.

Table 2 Selected coupling constants (Hz) for the complexes of NAD(P) coenzymes with magnesium[a]

	NADPH		$NADP^+$		NADH		NAD^+	
	A[b]	N[b]	A	N	A	N	A	N
J(H1',H2')[c]	5.5	7.2	5.5	5.2	5.1	7.1	5.5	5.5
J(H2',H3')	4.8	5.5	5.1	5.0	4.8	5.5	5.3	5.0
J(H3',H4')	3.8	2.0	3.6	3.2	4.7	2.1	3.7	3.1
J(H4',H5')	3.0	3.0	2.5	2.2	2.6	3.5[d]	2.5	2.5
J(H4',H5'')	2.8	4.0	3.0[d]	2.2[d]	3.0	5.1[d]	3.6	2.2
J(H5',H5'')	−11.8	−11.8	−11.6	−12.0	−11.8	−11.8[d]	−11.6	−12.0
J(P2',H2')	7.3		7.2					
J(P5',H4')	2.2	2.0	2.0[d]	3.0	2.2	2.4[d]	2.3	2.9
J(P5',H5')	4.9	4.8	5.2	4.5	5.0	4.8[d]	4.8	4.4
J(P5',H5'')	5.3	5.6	4.0[d]	5.6[d]	5.4	5.6[d]	5.2	5.4
J(P–O–P)[e]	−17.0		−17.5		*f*		−18.8	
J(P5',C4')	8.8	8.8						

[a] Estimated accuracy ±0.2 Hz, unless otherwise specified. The negative sign of ^2J are assumed, as the spectra are independent on the sign of these coupling constants. [b] A and N indicate adenine and nicotinamide nucleotide unit, respectively. [c] A small coupling (0.5 Hz) between 2'- and 4'-H was observed in the spectrum of NADPH–Mg. [d] Accuracy ±0.5 Hz. [e] Sign assumed (see text). This J was also measured for the following complexes: NADPH–Ca^{2+} (19.5), $NADP^+$–Zn^{2+} (19.0), NADPH–$[Co(NH_3)_6]^{3+}$ (19.4, with R = [M]/[NAD] = 1) and $NADP^+$–$[Cr(NH_3)_6]^{3+}$ (19.0, with R = 0.1). [f] Not measured because ^{31}P resonances overlap.

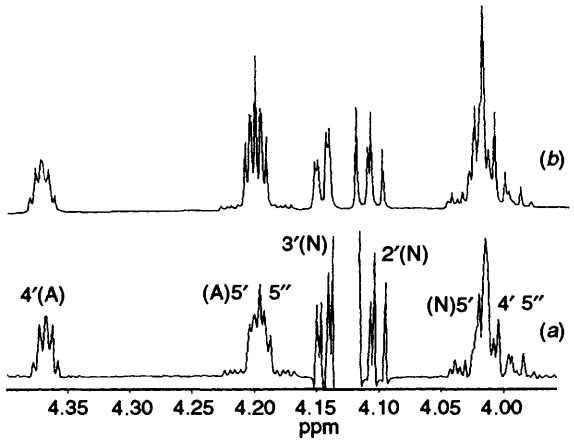

Fig. 1 (*a*) ^1H NMR spectrum (600 MHz, D_2O, pH = 9.0) of the NADPH–Mg^{2+} complex from δ 3.9 to 4.4; (*b*) simulated spectrum

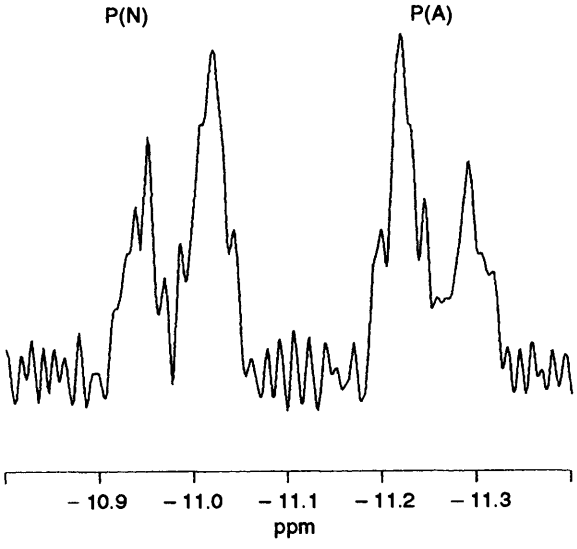

Fig. 2 ^{31}P NMR spectrum (242.94 MHz, D_2O, pH = 9.0) of the pyrophosphate fragment in the NADPH–Mg^{2+} complex

INVBTP program). The ^{13}C chemical shift values are not reported. The reference for line-width measurements was external dioxane. Coupling constants are in Hz, accuracy ±0.2 Hz, unless otherwise specified in Table 2. The assignment of ^1H and ^{31}P resonances was performed following the procedure used for the free coenzymes.[14] Some spectra are reported in Figs. 1 and 2.

1D NOESY and ROESY experiments were performed using a 270° gaussian pulse (1% truncation level, 512 data points, duration 90 ms). 2D NOESY and ROESY experiments were performed with mixing times ranging from 0.3 to 1 s for NOESY and 0.3 s for ROESY (effective spin lock field 7692 Hz, corresponding to θ = 75°) (T_1 relaxation times ranging from 1 to 5 s). 512 × 1 K spectra were acquired in TPPI mode and processed with zero filling to 2 K × 1 K points and weighted with a squared sinebell function. In order to minimize the effects due to direct and NOE-relayed Hartmann–Hahn magnetization transfer, the offset was placed at 9 ppm.[15]

Computational Methods.—The molecular modelling was carried out by using Biosym software (INSIGHT and DISCOVER) on a Silicon Graphics 4D20-GT computer. Octahedral $[Cr(NH_3)_6]^{3+}$ and $[Mg(H_2O)_6]^{2+}$ complexes were built with Cr–N and Mg–O distances of 2.36 and 2.17 Å respectively. The theoretical spectra for the spin systems of ribose protons were simulated by means of a Bruker PANIC program. The peak volumes in the ROESY experiments were calculated using the standard Bruker UXNMR software, the values were then translated into interatomic distances by means of the 'two spin approximation'.[16] The correction of the volumes for the offset effects was performed by using a program performed in our laboratory and written in FORTRAN-77, on a VAX-station 2000.

The relative populations of the different conformers for the pyrophosphate chain were calculated by solving the appropriate linear eqn. (1); where J_{obs} is the experimental coupling constant,

$$J_{obs} = x_1J_1 + x_2J_2 + x_iJ_i \quad (1)$$

x_i denotes the molar fraction of the i-th species and J_i is the coupling constant relative to the pure i-th conformation. The equations are solved as sets of linear equations, with the additional constraints that $\Sigma_i x_i = 1$. The calculation of the preferred conformation β^t from J(P,C4') was performed by using eqn. (2).

$$\beta^t(\%) = (J_{obs} - J_{60})/(J_{180} - J_{60}) \quad (2)$$

The relative populations of S-type conformers for the ribose

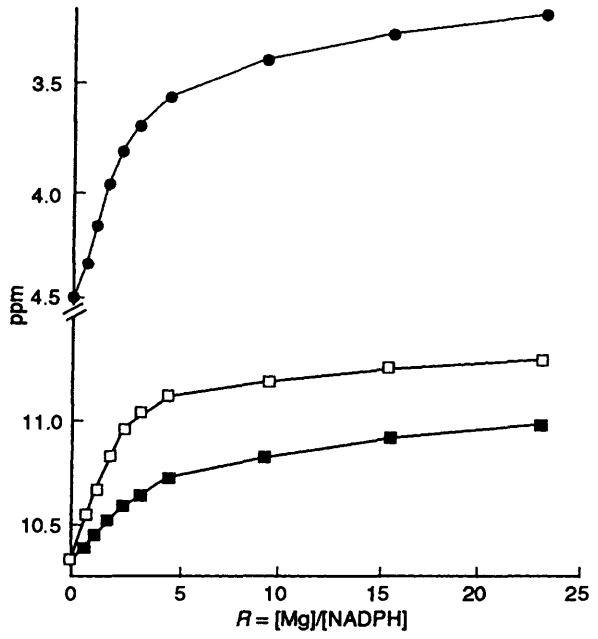

Fig. 3 ^{31}P Chemical shift variation of NADPH, by adding increasing amounts of Mg^{2+} ion: □, P(A); ■, P(N); ●, P(2′)

rings are calculated as arithmetic means of two values, obtained independently from the experimental values of J(H1′–H2′) and J(H3′–H4′). The estimated error on the populations is within ± 5%, unless otherwise specified in Table 5.

Results and Discussion

Stability Constants of the Complexes NAD(P)–M^{2+}.—The existence of intermolecular association involving nucleotide molecules and/or their metal ion complexes might in principle interfere in the interaction process of metal ion with NAD(P) coenzymes. Self-association has been found and quantified in purine and pyrimidine nucleoside 5′-diphosphates.[17] Recently, intermolecular stacking interactions have been recognized by NOE experiments in a sufficiently concentrated solution of NAD^+.[18] We performed 1D and 2D NOE experiments on $NADP^+$ and $NADP^+$–Mg^{2+} complexes, but no interactions between adenine and nicotinamide protons were detected. Dilution experiments did not show any concentration dependence of ^1H chemical shifts. On the basis of these results we concluded that self-association is negligible in our experimental conditions.

Thus the interactions of a metal ion (M) with the nucleotide (NAD) can be described by the following equilibria [(3), (4)], providing that two binding sites have been assumed.

$$NAD + M \rightleftharpoons NAD\text{–}M \quad (3)$$

$$NAD + 2M \rightleftharpoons NAD\text{–}M_2 \quad (4)$$

The stability constants of the corresponding equilibria are thus defined as K_1 and K_2 [eqns. (5), (6)].

$$K_1 = [NAD\text{–}M]/[NAD][M] \quad (5)$$

$$K_2 = [NAD\text{–}M_2]/[NAD][M]^2 \quad (6)$$

The values of K_1 and K_2 were obtained from titration experiments carried out by observing the chemical shift variation of ^{31}P NMR signals of the two (three) phosphorus atoms. As the equilibria are fast with respect to the NMR time-scale, only one averaged signal per ^{31}P atom can be observed, for the bound and the free species. Thus the values of the chemical shift of the bound species can only be obtained by calculations.

The concentrations of the molecular species are related by eqn. (7); where $[NAD]_0$ and $[M]_0$ are the total concentrations

$$\begin{cases} [NAD]_0 = [NAD] + [NAD\text{–}M] + [NAD\text{–}M_2] \\ [M]_0 = [M] + [NAD\text{–}M] + 2[NAD\text{–}M_2] \end{cases} \quad (7)$$

of the nucleotide and of the metal, respectively. The relationship between the macroscopic constants K_1 and K_2 and the microscopic constants K_a and K_b, which take into account the affinities of the ligand with the single sites, are given by eqns. (9) and (11). Eqns. (7) can be rewritten in terms of the concentrations of the free species, thus obtaining a set of non-linear eqns. (12), that can be solved with numerical methods, after substituting suitable values for K_a and K_b. Then these parameters, together with the value of the chemical shift for the bound species, were optimized by a least-squares fitting procedure and by using two models of the complex, *i.e.* with stoichiometric ratios $R = [M]/[NAD]$ of 1 and 2, respectively. The results are reported in Table 3, while Fig. 3 shows the experimental data for the complex of NADPH with Mg^{2+}.

$$\begin{cases} NAD + M \rightleftharpoons NAD\text{–}M(a) \\ NAD + M \rightleftharpoons NAD\text{–}M(b) \end{cases} \quad (8)$$

$$K_1 = K_a + K_b \quad (9)$$

$$\begin{cases} NAD\text{–}M(a) + M \rightleftharpoons NAD\text{–}M(a)M(b) \\ NAD\text{–}M(b) + M \rightleftharpoons NAD\text{–}M(b)M(a) \end{cases} \quad (10)$$

$$K_2 = K_a \times K_b \quad (11)$$

$$\begin{cases} [NAD]_0 = [NAD] + (K_a + K_b)[M] + (K_a + K_b)[NAD][M]^2 \\ [M]_0 = [M] + (K_a + K_b)[NAD][M] + 2(K_a + K_b)[NAD][M]^2 \end{cases} \quad (12)$$

An inspection of the data in Table 3 shows that, for NADP complexes, one constant is always much lower than the other and in some cases the least-squares analysis with model $R = 2$ did not converge or gave large errors. This means that (*i*) there are two very different binding sites, or (*ii*) there are two equivalent binding sites for the first metal ion, that become competitive for the binding to the second one. However, by comparing the Mg complexes of NADP with those of NAD, where one metal ion only can be bound, we observed that the unique association constant in these cases is similar to the higher constants found for NADP complexes. Therefore we decided to neglect in the following discussion the lower constant K_2, since the interaction with a second metal ion is not very likely.

An inspection of Table 3 also shows that the K_1 value for NADP complexes obtained for P(N)† is in many cases lower than those for the other phosphates, especially when the nicotinamide ring is positively charged. This indicates that the association at this phosphate is disfavoured. The interaction at P(A) and P(2′) is similar in the case of Mg^{2+} and Ca^{2+} complexes. For the Zn^{2+} complex with $NADP^+$, the inter-

† P(N), P(A) and P(2′) indicate the phosphorus atom of the nicotinamide and adenine nucleotide unit, respectively, and that bound at C-2′ of the (A)-ribose ring.

Table 3 Stability constants for NAD(P) complexes wth metal ions[a]

		K_1/dm^3 mol^{-1}	K_2/dm^6 mol^{-2}	K_a/dm^3 mol^{-1}	K_b/dm^3 mol^{-1}	K_1/dm^3 mol^{-1}
NADPH–Mg	P(A)[b]	76.0 ± 2.6	5.2 ± 0.4	68.4 ± 2.3	7.7 ± 0.3	74.0 ± 1.4
	P(N)	31.4 ± 0.9	1.2 ± 0.1	26.8 ± 0.6	4.6 ± 0.3	20.8 ± 0.4
	P(2′)	88.6 ± 1.4	1.1 ± 0.1	43.0 ± 1.3	2.6 ± 0.1	37.5 ± 0.4[e]
$NADP^+$–Mg	P(A)	96.3 ± 0.5	14.2 ± 0.3	78.2 ± 0.1	18.1 ± 0.4	64.0 ± 1.3
	P(N)	8.1 ± 0.2	10.6 ± 0.1	6.1 ± 0.1	2.0 ± 0.1	8.9 ± 0.1
	P(2′)	*d*	*d*	*d*	*d*	54.7 ± 1.0
NADH–Mg	P(A,N)[c]					38.0 ± 0.4
NAD^+–Mg	P(A)					40.5 ± 0.7
	P(N)					31.1 ± 0.6
NADPH–Zn	P(A,N)[c]	64.1 ± 3.1	2.0 ± 0.2	60.9 ± 3.0	3.2 ± 0.1	78.2 ± 3.1
	P(2′)	*f*	*f*	*f*	*f*	2.9 ± 0.3[g]
$NADP^+$–Zn	P(A)[h]	35.7 ± 0.7	1.1 ± 0.1	32.5 ± 0.6	3.1 ± 0.1	31.8 ± 0.8
	P(2′)	28.5 ± 6.1	0.6[g]	11.6 ± 3.0	5.2 ± 3.1	14.3 ± 0.6
NADPH–Ca	P(A)	404.6 ± 16.0	56.1 ± 3.4	390.7 ± 15.7	14.4 ± 0.3	371.7 ± 15.0
	P(N)	104.4 ± 2.7	4.8 ± 0.2	99.7 ± 2.6	4.8 ± 0.1	99.1 ± 2.4
	P(2′)	306.6 ± 20.5	27.5 ± 3.9	297.4 ± 19.8	9.3 ± 0.7	226.0 ± 4.9
NADPH–Li	P(A,N)[c]	49.9 ± 2.0	3.8 ± 0.3	40.5 ± 1.8	9.4 ± 0.2	37.4 ± 1.3
	P(2′)	*f*	*f*	*f*	*f*	172.1 ± 15.6

[a] The *K*-values were obtained by using two models of complex, *i.e.* with $R = [M]/[NAD] = 1$ (last column) and with $R = 2$ (other columns). K_a and K_b refer to microscopic constants relative to two different binding sites. Estimated error ± 10% unless specified. [b] The titration experiments (see Experimental) were performed by observing the chemical shift variation of ^{31}P NMR signal of individual phosphorus atoms. [c] P(A) and P(N) show the same chemical shift in either free and bound coenzyme. [d] The calculated standard deviation was greater than the binding constant. [e] Estimated error ± 30%. [f] The calculation did not converge. [g] Estimated error ± 50%. [h] No variation of chemical shift was observed for P(N).

action at P(N) is null, while the association constant obtained at P(2′) is lower than that at P(A). This is interpreted as an increased stability of the complex with the Zn^{2+} ion bound to P(A), due to an additional interaction with the adenine moiety. It is known[8,9a] that the Zn^{2+} ion forms macrochelates with ATP involving the N-7 and -1 atoms of the adenine ring, either directly or through the mediation of water molecules. The ^1H NMR spectra, obtained by titration of NADP coenzymes with Zn^{2+} ion, showed a deshielding for both 8- and 2-H ($\Delta\delta$ +0.08 and +0.12, respectively), which is of the same order as that observed[9a] for ATP. This confirms a small but significant interaction of Zn^{2+} ion with the adenine ring.

No evidence for direct interaction of Mg^{2+} or Ca^{2+} with the adenine ring was observed, in our cases, by ^1H NMR spectroscopy. The chemical shifts' variation for 8-H ($\Delta\delta$ −0.01) and for 2-H ($\Delta\delta$ −0.06) is negative and small, *i.e.* it lies in the range observed for all the other protons (see Table 1 and ref. 14). The upfield effect induced by the Mg^{2+} ion on the ^{31}P chemical shift of the three phosphates in NADP complexes is strong, compared with that induced by Ca^{2+}, Zn^{2+} and Li^+ and also with that observed for Mg^{2+} on the two phosphates of the NAD complexes (see Table 4). It must be noted that the $\Delta\delta$ values reported in Table 4 are the chemical shift differences between two molecular species: one, usually called free coenzyme, is actually NAD(P) with Na^+ as counter-ion, the other, called bound coenzyme, is NAD(P) bound to a metal ion that has replaced sodium. Thus, it is expected that the Li^+ ion does not induce any significant shift variation. On the other hand it is known[19] that many factors other than ionization are responsible for ^{31}P chemical shifts, the most important being the bond angle O–P–O and the P–O ester torsional angle. For instance an increase of the bond angle, as well as a change from a *gauche–trans* to a *gauche–gauche* conformation, induces in phosphates an upfield effect. Thus, ^{31}P chemical shifts must be used with caution. However, in the case of Mg^{2+} the stronger effect with respect

Table 4 ^{31}P Chemical shift difference $\Delta\delta$ (ppm) induced by complexation[a]

	$\Delta\delta = (\delta_{bound} - \delta_{free})$		
Complex	P(A)	P(N)	P(2′)
NADPH–Mg	−0.93	−0.71	−1.43
$NADP^+$–Mg	−0.72	−0.35	−1.13
NADH–Mg	−0.56	−0.56	
NAD^+–Mg	−0.31	−0.21	
NADPH–Zn	−0.11	−0.11	*b*
$NADP^+$–Zn	−0.13	0.00	−0.42
NADPH–Ca	−0.29	−0.33	−0.87
NADPH–Li	+0.10	+0.10	−0.01

[a] The $\Delta\delta$ values were derived by fitting the data from titration experiments; the negative sign indicates upfield shift with complexation. [b] At acid pH this signal became too broad and undefined.

to Ca^{2+}—apparently contrasting with the higher value of the association constants found for Ca^{2+}—suggests that Ca^{2+} and Mg^{2+} bind NAD(P) in a different way. In addition the presence of the phosphate at C-2′ on the adenine ribose ring seems determinant for the mode of binding of magnesium to NADP coenzymes.

Calcium was found to coordinate ATP only at the phosphate chain, whereas magnesium can form macrochelates involving the adenine ring, but only through the mediation of water molecules.[8,9] Thus a direct interaction of Ca^{2+} to the phosphates of NADPH could explain the higher association constant with respect to Mg^{2+}, which on the contrary should prefer the mediation of water molecules. This mediation could also explain the absence of deshielding effects on the protons of the adenine ring, which does not imply that Mg^{2+} is not interacting with the adenine moiety, but indicates that this can only occur through water molecules. The decreasing values of the shielding from P(2′), P(A) towards P(N) is in agreement

Table 5 Population (%) of conformers for NAD(P) coenzymes and for their complexes with magnesium[a]

		Ribose S-type[b]		Around bond C(5')–O(5')						Around bond C(4')–C(5')					
				β^t		β^+		β^-		γ^+		γ^t		γ^-	
		c	*d*	*c*	*d*	*c*	*d*	*c*	*d*	*c*	*d*	*c*	*d*	*c*	*d*
NADPH	A	52	67	75	74	12	14	13	12	63	78	34	14	3	7
	N	94	92	72	73	15	15	13	12	50	65	37	28	13	7
$NADP^+$	A	58	67	75	78	12	8	13	14	62	86	36	13	2	1
	N	85	73	76	74	15	16	9	10	88	89	11	10	1	1
NADH	A	66	58	74	72	14	15	12	13	82	80	17	18	1	2
	N	92	91	73[e]	73	15[e]	15	13[e]	12	60[e]	50	27[e]	37	13[e]	13
NAD^+	A	72	68	75	75	13	13	12	12	73	76	22	23	5	1
	N	84	79	76	76	15	14	9	10	89	89	10	10	1	1

[a] The populations were obtained from the experimental coupling constants of Table 2 and from the data reported in ref. 14 for the free coenzymes NADP. The coupling constant values for the free coenzymes NAD are not reported. Estimated error ± 5% unless specified. [b] Population of S-type conformers, obtained by assuming pseudorotational angles P_S 153° and P_N 9° and puckering angle Φ_m 37°. [c,d] Data for the free (*c*) and bound (*d*) coenzyme respectively. [e] Estimated error ± 10%. Due to signal overlap, the estimated error of the corresponding coupling constants is ± 0.7 Hz (data not reported).

with the association constants, which show a lower interaction at P(N).

Another interesting parameter is the coupling constant ^2J(P–O–P), which changes with complexation from 20.0–20.5 (free coenzymes) to 17–18 Hz (see Table 2), but the variation is more pronounced for magnesium (17.0 Hz) than for the calcium complex (19.5 Hz). These results are in line with those found in an early study[20] on tripolyphosphate complexes with metal ions. The sign of such a coupling has been determined only in the case of the dianion of pyrophosphoric acid [^2J(P–O–P) = −10.7 Hz].[21] As no great change in the hybridization of the phosphorus atoms occurs for these structurally similar phosphate groups, we presume that the relative sign of ^2J(P–O–P) in NAD(P) coenzymes is also negative. In order to have a reversal of sign, a significant change of the substituent's electronegativity and/or hybridization should occur.[19,22] ^2J(P–C–H) Presents a Karplus-like dependence on the H–C–P angle, similar to that observed for ^2J(H–C–H), the values of which increase algebraically with the bond angle.[23] If the same relationship may be postulated also for ^2J(P–O–P), the increase of this coupling in magnesium complexes might be related to an increase of the P–O–P angle. From all these results it follows that the effects induced by magnesium are better explained by conformational changes, which lead to variations of the angles at the three phosphorus atoms.

Conformational Analysis of the Complexes NAD(P)–Mg^{2+}

Conformation of the Ribose Ring.—The ribose rings in nucleotides have been reported to exist as an equilibrium mixture of C(2')-*endo* (S-type) and C(3')-*endo* (N-type) conformers.[24–26] We have calculated, following the procedure of Altona *et al.*,[25,26] the relative population of the S-type *vs.* N-type conformers and the maximum puckering angle Φ_m, by using the coupling constant values for the protons on the ribose rings (Table 2). de Leeuw and Altona[25] have found that the sum of J(H1',H2') + J(H3',H4') is practically independent and J(H2',H3') is only slightly dependent on the position of the N⇌S conformational equilibrium. Φ_m, assumed to be equal for N- and S-type conformation according to Altona, was estimated from the values (4.8–5.5 Hz) of J(H2',H3') as *ca.* 37°, which is in agreement with the majority of X-ray data (Φ_m = 36–38°).

A large number of X-ray analyses also give averaged values of the pseudorotational phase angles P_N = 9° and P_S = 162°. Calculated[25] J-values from model geometries of P_N = 9° and P_S = 153–162° are in agreement with our experimental data for the sum J(H1',H2') + J(H3',H4') (9.1–9.8 Hz). In the case of the nicotinamide moiety of the oxidized coenzymes, the lower values (8.2–8.4 Hz) of this sum are due to the substituent effect of the positively charged nitrogen atom directly attached to C-1', which decreases the value of J(H1',H2'). This effect has been taken into account.

With the values P_N = 9°, P_S = 153° and Φ_m = 37°, we calculated the relative populations of the S-type *vs.* N-type conformers. The results, reported in Table 5, show that the presence of the metal ion does not affect the conformation of the two ribose rings in NAD coenzymes, which display a preference for the S-type conformer, significantly more relevant (80–90%) for the nicotinamide ribose ring. On the contrary, a small increase (10–15%) of the S-type conformation can be observed for the adenine ribose in NADP coenzymes, when a Mg ion is present. Without magnesium, the two puckering modes for this ring are equally populated.

Conformation about Bonds C(5')–O(5') *and* C(4')–C(5').—In the solid phase, the conformation around these bonds has been determined[11] for NAD^+, as lying in the *trans* (β^t) and *gauche–gauche* (γ^+)‡ range, respectively, for both A and N units; a conformation which is usually considered as preferred for 5'-nucleosides.[24]

We derived the relative populations of the three main conformers around C(5')–O(5') (β-conformers) from J(P,H5'), J(P,H5") and, for NADPH, also from J(P,C4'); the populations around C(4')–C(5') (γ-conformers) from J(H4',H5'), J(H4',H5") and ^4J(P,H4'), following usual procedures (see Experimental section).

The limiting coupling constants' values used for J(P,H), in the case of β-conformers, were J_{180} = 23.0 and J_{60} = 2.4 Hz and for J(P,C) were J_{180} = 11 and J_{60} = 0.7 Hz, which are supported by many experimental data.[24,25] For the relationship between dihedral angles and vicinal couplings we have used the Karplus/Altona equations.[19,27] The conformation around C(4')–C(5') bonds was instead studied by using the 'non-classical' model for the three main γ-conformers, as suggested by Altona and co-workers,[28] *i.e.* γ^+ = 53°, γ^t = 182° and γ^- = 292°. The results are reported in Table 5.

‡ The angles β and γ are defined as β = C(4')–C(5')–O(5')–P and γ = C(3')–C(4')–C(5')–O(5'), conforming to the IUPAC–IUB convention. β-*trans* and β-*gauche* orientations refer to the position of C-4' with respect to the phosphorus atom; *gauche–gauche* (γ^+) conformation corresponds to the mutual orientation of O-4', O-5' and C-3'.

Table 6 Angular coefficients (Hz) for the line-width variation of selected ^{13}C resonances for $NADP^+$ induced by $[Cr(NH_3)_6]Cl_3$[a]

Adenine nucleotide						
C-2′	C-5′	C-2[b]	C-4[b]	C-5	C-6	C-8[b]
5.1	2.3	6.5	7.8	4.5	4.7	7.8
Nicotinamide nucleotide						
C-1′	C-2′	C-3′	C-5′	C-2	C-5	C-6
6.0	3.7	4.3	2.2	7.8	3.5	6.0

[a] The values reported are the average from two experiments; estimated accuracy ± 20% unless specified. [b] Estimated accuracy ± 30%.

The preferred (70%–80%) *trans*-conformation, β', also confirmed by J(P,C4′) in the case of NADPH, is independent of the presence of magnesium. The minor conformers β^+ and β^- are, in general, equally populated. On the contrary an increase (15%–25%) can be noted for the main conformer, γ^+, in the case of NADP complexes, which might be related to the small increase of the S-type conformers observed for the ribose of the adenine nucleotide unit in the same complexes. Actually the increase of γ^+-conformers is more significant (25%) in the $NADP^+$ complex and limited to the adenine nucleotide unit, in agreement with the small interaction of Mg^{2+} with the nicotinamide moiety, as shown by the low value of the binding constant measured at P(N).

Determination of the Geometry at the Glycosidic Site by ROESY Experiments for NADPH *and* $NADP^+$–Mg^{2+} *Complexes.*—The geometry at the glycosidic bonds is usually described by the dihedral angle χ, which is defined as O(4′)–C(1′)–N(1′)–C(2) for the pyrimidine and O(4′)–C(1′)–N(9)–C(4) for the purine moiety.[24] NOE interactions were observed between protons of the bases and those of the adjacent ribose ring, specifically 2- and 6-H with 1′- and 2′-H in the nicotinamide moiety; 8-H with 1′-, 2′- and 3′-H in the adenine moiety. The volumes, V_{ij}, of the NOE cross-peaks, corrected for the 'offset effect', were translated into interatomic distances, r_{ij}, by using the 'two spin' approximation eqn. (13) and, as a reference value, the known distance (2.46 Å) between 5- and 6-H of the

$$r_{ij} = r_{ref}(V_{ref}/V_{ij})^{1/6} \quad (13)$$

nicotinamide ring. Adenine and nicotinamide moieties were assumed to have the same correlation time. When the population of the N-type conformer is relevant, as in the case of the ribose in the adenine moiety, the relaxation rates obtained by ROESY experiments were considered as averaged relaxation rates of two conformers, with two different χ values, χ_N and χ_S. Then we performed a best-fitting procedure between experimental and calculated distances as a function of χ_N and χ_S, by using eqn. (14), where P_N and P_S are the population of

$$r^{-6}_{exp} = P_N r^{-6}(\chi_N) + P_S r^{-6}(\chi_S) \quad (14)$$

N- and S-type conformers, obtained from J_S values. The interatomic distances are reported in Table 7; the dihedral angles χ_N and χ_S were very similar, *i.e.* in the range $-150° \pm 20°$, for the free coenzyme as well as for NADPH and $NADP^+$ complexes.

When the population of the N-type conformer is negligible, as for the nicotinamide moiety, we considered a two states model, defined by two glycosidic angles, χ and $\chi + 180°$. The best fitting was obtained for $\chi = -130° \pm 20°$, for both the free coenzyme and the complex with magnesium.

Structure Proposed for the Complex $NADP^+$–Mg^{2+}.—

Table 7 Selected interprotonic distances (Å) for NADP–Mg^{2+} complexes obtained from ROESY experiments[a]

	Adenine nucleotide		Nicotinamide nucleotide			
	8–1′	8–2′	2–1′	2–2′	6–1′	6–2′
NADPH	3.4	2.7	2.3	3.0	2.8	2.6
NADPH–Mg	3.2	2.5	2.3	3.0	3.1	2.5
$NADP^+$–Mg	3.2	2.7	2.4	3.1	2.6	3.0

[a] The known distance between 5- and 6-H of nicotinamide ring (2.46 Å) was taken as reference.

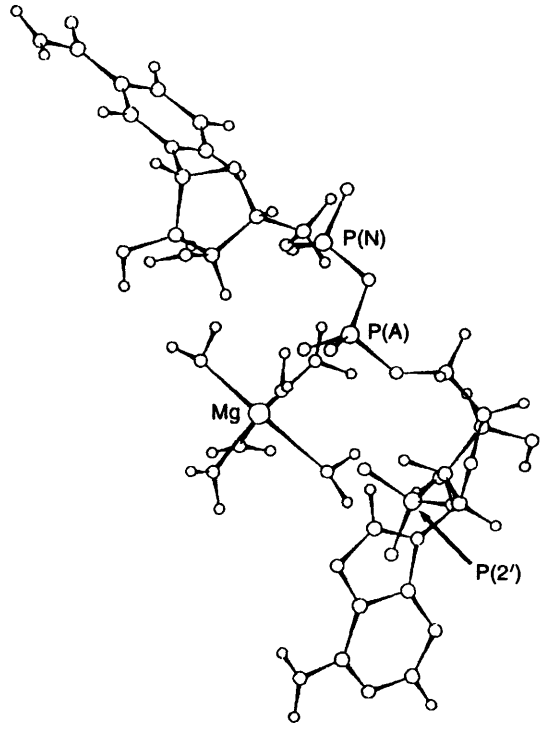

Fig. 4 Tentative structure of one outer-sphere species for the $NADP^+$–Mg^{2+} complex. The distances of the metal ion from P(2′), P(A) and P(N) are 4.57, 5.73 and 6.63 Å, respectively. The distances of the hydrogen atoms of the coordinated water from the phosphate oxygens (1.5–2.0 Å) allow the formation of hydrogen bonds.

Taking into account (*i*) the NOE data, (*ii*) the conformational analysis of the ribose rings, (*iii*) the binding constants' results and (*iv*) the chemical shift variation induced by the association process, we built up structures of the complex $NADP^+$–Mg^{2+}, by molecular modelling calculations. The position of the metal ion was changed manually and fitted with a model geometry corresponding to the most populated conformations of the pyrophosphate chain. The pyrophosphate group is in general rather flexible; the staggered and the *trans*-eclipsed conformations are both energetically allowed. The first is the unique one observed in the solid state;[24] the *trans*-structure was suggested[24] as being especially suited for a bidentate metal complex. Therefore we decided to start with either staggered or *trans* forms as geometrical models.

We have considered an outer-sphere macrochelate, with six water molecules between the magnesium ion and the phosphates, as suggested by the chemical shift variation observed on the three phosphate resonances and on the basis of the comparison with Ca^{2+} complex. The upfield effects of 0.7–1.4 ppm in our case are in the opposite direction, compared with those which occur upon direct binding of the metal to the pyrophosphate anion (low-field shift of 1.6 ppm).[20] After the

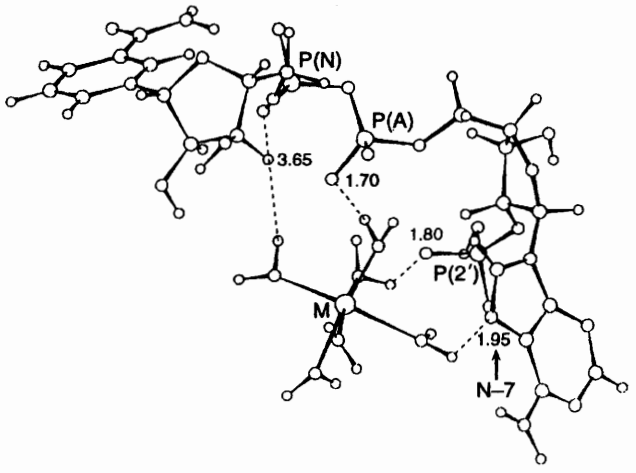

Fig. 5 Tentative structure of one outer-sphere $NADP^+$–M^{2+} species, showing the M^{2+}–N-7 coordination. The distances of the hydrogen atoms of the coordinated water from the phosphate oxygens are given in Å.

energy minimization the pyrophosphate group shows a conformation intermediate between the staggered and the *trans* forms and the octahedral structure of $[Mg(H_2O)_6]^{2+}$ appears perfectly arranged to the tri-dimensional profile of $NADP^+$ obtained from NOE and coupling constants' data. The energy minimization was performed at first by using as constraints the experimental parameters of the coenzyme, in order to find the best position of the metal ion and the proper orientation of the water molecules. Then, a final minimization without constraints was performed and the structure remained almost unchanged. It can be seen from Fig. 4 that two water molecules link Mg^{2+} to P(2') and to P(A), respectively, giving a contribution to the stabilization of the complex, through the formation of hydrogen bonds between the phosphate oxygens and the hydrated magnesium ion. A third water molecule could also mediate the binding to P(N), but the distance between this phosphate group and the metal centre is significantly larger, in agreement with the disfavoured interaction at this phosphate.

We report in Fig. 5 another structure where the metal ion is closer to the adenine ring, showing the interaction with N-7 through one molecule of water. The conformation of the coenzyme was maintained constant; the distances of the water molecules from N-7, P(2') and P(A) are a direct consequence of the hydrogen bonds between oxygen and phosphorus atoms, as indicated in the Figure. This structure might be present in equilibrium, together with many others, contributing to the interaction of Mg^{2+} with NADP coenzymes. In the case of Zn^{2+} such a contribution must be larger, as suggested by the deshielding observed on 8-H.

Interaction of NAD(P) *Coenzymes with* $[Co(NH_3)_6]^{3+}$, $[Ru(NH_3)_6]^{3+}$ *and* $[Cr(NH_3)_6]^{3+}$.—In order to study the effect of ion ligands on the ^{31}P and ^{13}C chemical shifts, in the case of outer-sphere complexes, we utilized metal ions bound to ammonia, *i.e.* $[M(NH_3)_6]^{3+}$, which should form hydrogen bonds to the phosphates, thus stabilizing the complex in the same way as water molecules. The chromium ion in particular is interesting, because, owing to its paramagnetic properties, it can provide important structural information.

The strong local fields produced by the unpaired electrons are coupled to the nuclei by dipole–dipole interactions (through space) and sometimes by scalar interactions (through chemical bonds). The fluctuations of both of these interactions contribute to the relaxation mechanism of the nuclei involved and of those relatively close in space to the metal. The relaxation rates are related to the molecular geometry through the Solomon–

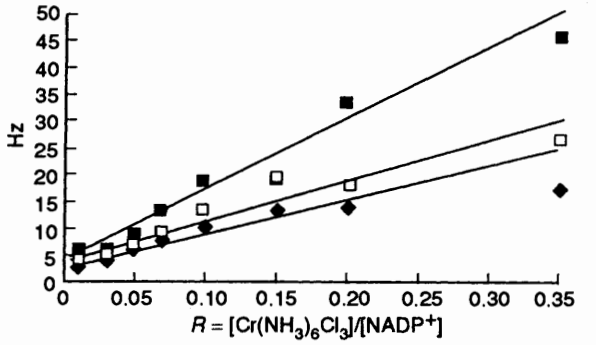

Fig. 6 Line-width variation of ^{31}P NMR signals for $NADP^+$, by adding increasing amounts of $[Cr(NH_3)_6]Cl_3$: ■, P(2'); □, P(A); ◆, P(N)

Bloembergen equations,[29] which contain terms arising from scalar and dipole–dipole interactions, together with the correlation time for the latter. The interatomic distances and the correlation time can be estimated, but not accurately calculated. Usually, line-width data were used to obtain structural information, taking advantage of the r^{-6} dependence of the transverse relaxation rate $1/T_2$. Thus, we performed titration experiments, measuring the variation of the line-width of ^1H, ^{13}C and ^{31}P resonances, by adding increasing amounts of $[Cr(NH_3)_6]Cl_3$ to a solution of the coenzyme. Unfortunately the two phosphorus signals of the pyrophosphate chain overlap in the complex with NADPH, thus preventing us from obtaining the ^{31}P NMR parameters and important information for the phosphate sites; consequently only the complex with $NADP^+$ was analysed. It must be noted that these complexes lie in the fast exchange regime, *i.e.* the relaxation rates are the weighted average of those of the free and bound species. In addition the equilibrium (15) remains significantly shifted to

$$NADP + [Cr(NH_3)_6]^{3+} \rightleftharpoons NADP\text{–}[Cr(NH)_6]^{3+} \quad (15)$$

the left, because we used small quantities of metal ion, in order to observe better a selective broadening of the signals. For instance, ^1H spectra are affected by quantities corresponding to $R = [Cr(NH_3)_6{}^{3+}]/[NADP] = 0.0014$–$0.0018$. The protons of the adenine nucleotide unit showed, in general, larger broadening than those of the other unit. With $R = 0.0018$, the (A)-protons 2-, 8-, 2'- and 3'-H show $\Delta\nu$ values of 2.2–2.5 Hz, whereas the (N)-protons 2-, 4-, 5-, 6-, 1'- and 2'-H show $\Delta\nu$ values of 1.0–1.5 Hz. ^{13}C and ^{31}P spectra start to broaden at $R = 0.1$ and 0.001 respectively. The P(2') signal displays the strongest effect, whereas the broadenings on P(A) and P(N) are lower in this order (Fig. 6). This is confirmed by the ^{13}C results; the signals of the adenine moiety are in general more affected than the others. The line-widths' variation gave, through a fitting procedure, the angular coefficients C (selected values are reported in Table 6), defined by eqn. (16). The same procedure allowed us to obtain D as a number near to zero. The picture

$$\Delta\nu = C[Cr(NH_3)_6{}^{3+}]/[NADP] + D \quad (16)$$

reported in Fig. 7 was then obtained by using the ratios of the interatomic distances estimated from the angular coefficients C and by fitting the position of the Cr atom with a model geometry corresponding to the most populated conformation of the coenzyme[24] *i.e.* S-type for the ribose rings, β^t (*trans*) and γ^+ for the fragments C(4')–C(5')–O(5')–P and C(3')–C(4')–C(5')–O(5'), respectively. As concerns the geometry at the glycosidic bonds, the *anti*-conformation for the (A)-ribose and the *syn* for the (N)-ribose were adopted as starting conformations. For the pyrophosphate group the staggered form was first considered;

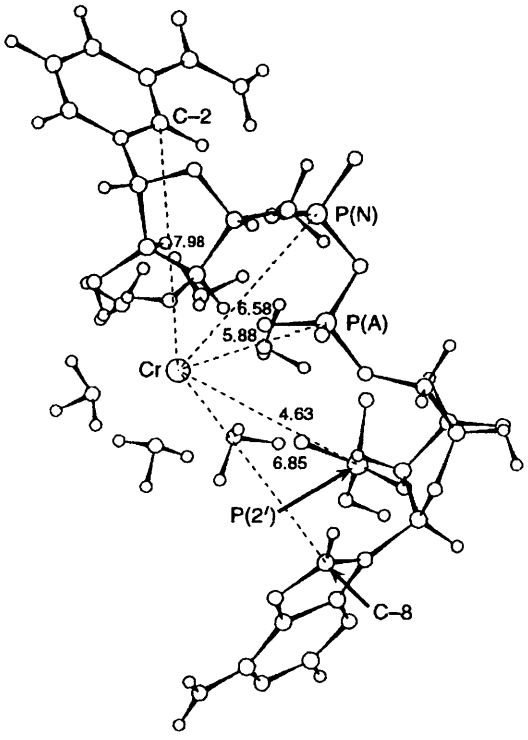

Fig. 7 Tentative structure of one outer-sphere species for the $NADP^+$–$[Cr(NH_3)_6]^{3+}$ complex. The numbers refer to the distances (Å) of the metal ion from the three phosphorus atoms and from C-2 and -8 of nicotinamide and adenine ring respectively.

then the final conformation resulted in an intermediate between the staggered and the *trans*-form.

An inspection of Fig. 7 shows that the position of the metal ion is in agreement with the strong effects on P(A) and P(2'), on the adenine nucleotide carbons C-2', -5', -2, -4, -8 and on the nicotinamide C-2 atom. A χ-value of 175° at the adenine glycosidic site makes it possible to have similar distances from the paramagnetic centre for C-2 and -8, thus justifying similar line-broadening effects. The glycosidic linkage of the nicotinamide moiety can be either *syn* or *anti*; in Fig. 6 it measures 55°. Although the effect of the paramagnetic ion is stronger for the C-2 and -3 side than for C-6 and -5, the base can freely rotate in the complex. This rotation was seen to be possible in a model of a inner-sphere complex NADP–Mn^{2+}, proposed on the basis of T_1 relaxation times values.[12]

The complexes with $[Co(NH_3)_6]^{3+}$ and $[Ru(NH_3)_6]^{3+}$ were prepared in order to perform ^{59}Co experiments and ^1H NOESY experiments at low temperature, that could reveal interactions between the amine protons and the nuclei of the coenzyme. These latter were unsuccessful, as the average life-time of the complexes was too short with respect to the NOESY times. However, the titration experiment performed by addition of NADPH to a solution of $[Co(NH_3)_6]Cl_3$ and by following the ^{59}Co chemical shift variation (Fig. 8), provides evidence that the complex has a 1:1 stoichiometry, thus excluding dimeric structures such as $(NADPH)_2$–$[Co(NH_3)_6]^{3+}$. Fig. 8 shows the decrease of the ^{59}Co chemical shift values, until a plateau was reached, for $R = 1$. The experiment, continued to $R = 10.8$, leaves the ^{59}Co shift unchanged. The changes observed on ^{31}P chemical shifts upon complexation, are small; the $\Delta\delta$ values obtained with $R = 5.4$ are the following: P(2') −0.17, P(A) +0.10 and P(N) +0.25 ppm. These data, compared with those reported[30] for the complex $[Co(NH_3)_3$–$ATP]^-$, where Co ion is directly bonded to the phosphate oxygen atoms and a low field shift of *ca.* 10 ppm was observed, indicates that in our

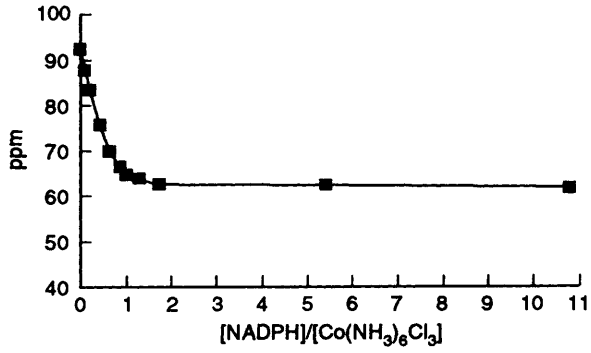

Fig. 8 ^{59}Co Chemical shift variation of $[Co(NH_3)_6]Cl_3$, by adding increasing amounts of NADPH

complex the NH_3 molecules mediate the bond between the coenzyme and the metal, as expected.

Conclusions

The results of the conformational analysis, performed on the free and bound NAD(P) coenzymes, show that the effects of binding to a magnesium ion are small, but significant. An increase (10%–15%) of the S-type conformer was observed for the adenine–ribose moiety, together with an increase (15%–25%) of the γ^+ (*gauche–gauche*) conformer for the same unit. This is actually a further stabilization of the already most stable conformer for these coenzymes without magnesium. The presence of the phosphate at C-2' of the adenine ribose ring seems determinant for the mode of binding of Mg^{2+} to NADP molecules. Mg^{2+} preferentially interacts with P(2') and P(A), while the association at P(N) is in general disfavoured, especially for the coenzyme in the oxidized form. This follows from the binding constants and ^{31}P chemical shift values. However, some influence of the metal ion on P(N) was revealed. In the case of NAD coenzymes, the interaction at the two phosphates is identical. The relatively strong upfield effect, consequent to magnesium coordination, compared for instance with calcium, indicates that some conformational changes at the phosphate sites must occur. This was also suggested by the significant change of the geminal coupling constant J(P–O–P), which is not so greatly relevant for the other ions.

Providing that in aqueous solution several bound species might be present in equilibrium, some structures for the metal–coenzyme complexes can be proposed. In the case of Mg^{2+} and $[Cr(NH_3)_6]^{3+}$ the model structures obtained are similar. Actually the magnesium ion must be present as $[Mg(H_2O)_6]^{2+}$. The metal atom sits in a pocket among the three phosphate groups and binds to the phosphates with interactions of electrostatic nature, through the mediation of H_2O or NH_3 molecules, respectively. The hydrogen-bonding of NH_3 and H_2O to the phosphate oxygen atoms contributes to the stabilization of the complexes. The octahedral structures of $[Mg(H_2O)_6]^{2+}$ and $[Cr(NH_3)_6]^{3+}$ are perfectly matched to the three-dimensional profile of the coenzyme. The pyrophosphate group adopts a conformation intermediate between a staggered and a *trans* form. The aromatic rings of adenine and nicotinamide are shown to be approximately perpendicular to each other; although the pyrimidine can freely rotate around the glycosidic linkage, the planes of the two bases can never be parallel, thus a folded form with close stacking of the two rings appears very unlikely.

Ca^{2+} and Li^+ apparently show a different mode of binding to NADPH with respect to the Mg^{2+} ion. We did not perform a conformational analysis of the ribose–phosphate fragment, as was done for Mg^{2+}, but the variations observed on ^{31}P

chemical shift and coupling constant J(P–O–P) upon complexation are small, thus suggesting that significant conformational changes did not occur. On the other hand, the higher value of the association constants, with respect to magnesium, is in favour of direct interaction of Ca^{2+} and Li^{+} to the phosphates oxygens, without the mediation of water molecules, in line with the results on ATP.[8,9]

The association at P(N) is in general disfavoured for all ions, but this is particularly noticeable in the case of Zn^{2+}, which presents additional interactions with N-7 and -1 atoms of the adenine ring, thus stabilizing the complex with the ion bound to P(A).

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