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A combined experimental and theoretical study of the pH-dependent binding mode of NAD^+ by water-soluble molecular clips

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The highly selective recognition process of NAD^+ and NADH (as important cofactors of many redox enzymes) by molecular clips in aqueous solution is studied systematically by a combined experimental and quantum-chemical approach. The strongly pH-dependent complexation-induced ^{1}H NMR shifts of the guest molecule indicate that in buffered aqueous solution at pH = 7.2 the nicotinamide ring, the active site of NAD^+, is preferentially bound inside the cavity of the molecular clip, whereas in pure water under slightly acidic conditions both units (the nicotinamide ring as well as the adenine moiety) are located outside the cavity. The latter finding is explained by a competing self-aggregation of NAD^+ which prohibits the recognition process. In addition, the investigation of the NAD^+ fragments NMNA, NMN, and AMP as well as the comparison of measured and computed chemical shieldings provides information on possible binding modes. Under equal conditions the binding of NADH to the molecular clip is significantly weaker than that of NAD^+, so that the oxidation states (NAD^+/NADH) can be distinguished by the molecular clips. The nucleotides NMN and AMP are bound less strongly by the molecular clips than NAD^+. The weaker binding indicates that multiple aromatic $\pi-\pi$ and cation$-\pi$ host–guest interactions only possible in NAD^+ have a synergetic effect on the complex stability. In addition to the inhibition of the cofactor NAD^+, a further implication is the development of sensors since a quenching of fluorescence is observed for specific molecular clips by the addition of NAD^+.

Supporting information may be found in the online version of this article.

Keywords: molecular clips; enzyme cofactors; nucleotide; supramolecular chemistry

INTRODUCTION

Many enzymes use cofactors to catalyze, *inter alia*, redox reactions, decarboxylations, or methyl transfer reactions.[1,2] The biologically active part of these cofactors comprises an electron-poor, often cationic heterocyclic ring system or a trialkylsulfonium salt. Direct external interference with the catalytic process hence requires blocking of the cofactor site, e.g., by synthetic receptor molecules. A perfect design for this purpose is a rigid molecular clip with an electron-rich interior for cation inclusion and solubilizing functional groups at its periphery.[3] As a prototype, host family **1a–d** with naphthalene sidewalls and a central benzene spacer unit provide an extremely electron-rich cavity and retain water-solubility due to their pendant methanephosphonate, hydrogenphosphate, or phosphate groups (Scheme 1). It is therefore expected that these artificial cofactor traps chemoselectively bind their guests in aqueous solution by embracing their most electron-deficient sites and thereby affect enzymatic reactivity. Recently, we reported the synthesis of **1a–d** and the complex formation of **1a** as host molecule with various pyridinium, thiazolium, and sulfonium salts such as *N*-methylnicotinamide iodide (NMNA **3**), nicotinamide adenine dinucleotide (NAD^+ **2**), and thiamine diphosphate (TPP) as guest molecules.[4–9] Even non-aromatic *S*-adenosylmethionine (SAM) carrying a tetrahedral sulfur cation was found to be bound by the molecular clips **1b** and **1d** substituted with lithium phosphonate or phosphate groups with its active *S*-methylsulfonium side-chain inside the clip cavity just as predicted.[6] However, in the host–guest complexes of NAD(P)$^+$ with the molecular clip **1a** (substituted by *tetra-n*-butylammonium methanephosphonate groups in the central benzene spacer-unit) the comparison of the experimental and calculated ^{1}H NMR shifts of the guest protons clearly indicates that neither the nicotinamide ring nor the adenine unit of NAD(P)$^+$ is included inside the clip cavity in slightly acidic aqueous solution.[4,5] In this work, we focus on the structures and stabilities of the host–guest complexes of clips

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1b–d substituted by lithium methanephosphonate or phosphate groups with NAD^+ **2**, NMNA **3**, and the nucleotides NMN **4**, AMP **5**, and NADH **6** as guest molecules in buffered aqueous solution at neutral pH value. Under these conditions either the nicotinamide ring or the adenine unit of these guest molecules is, indeed, bound inside the clip cavity. NMNA **3**, NMN **4**, and AMP **5** represent fragments of the NAD^+-molecule whose behavior toward the molecular clips yields additional information about the binding of NAD^+ **2** within clips **1b–d**. The experimental results are supported by quantum-chemical calculations of complexation-induced NMR chemical shifts of the guest protons which allow us to gain further insight into the binding modes of these host–guest complexes. With these findings a new door is opened for the development of a potential supramolecular control of enzymatic reactions. First experiments with the clips **1b** and **1d** and the enzymes alcohol dehydrogenase (AD) and glucose-6-phosphate dehydrogenase (G6PD) using $NAD(P)^+$ as cofactors look very promising.[10,11]

RESULTS AND DISCUSSION

ITC and NMR measurements: self-assembly of NAD^+ and molecular clips

Before discussing the host–guest binding of NAD^+ **2**, NMNA **3**, and the nucleotides **4–6** by the molecular clips **1b–d**, it is important to describe the individual properties of these compounds in aqueous solution: the pH-dependent self-aggregation of the nucleotides (NAD^+ and AMP) and the formation of self-assembled clip dimers.

Commercially available NAD^+ **2** dissolved in pure water reacts slightly acidic (pH = 3.3) and is, presumably, protonated at nitrogen atom N-1 of the adenine unit.[12]. Dilution experiments followed by isothermal titration calorimetry (ITC) provide good evidence that this form of NAD^+ aggregates in aqueous solution (Figure 1(a)).[5] Attempts to fit the experimental heat evolved during the dilution of NAD^+ with a curve calculated for the

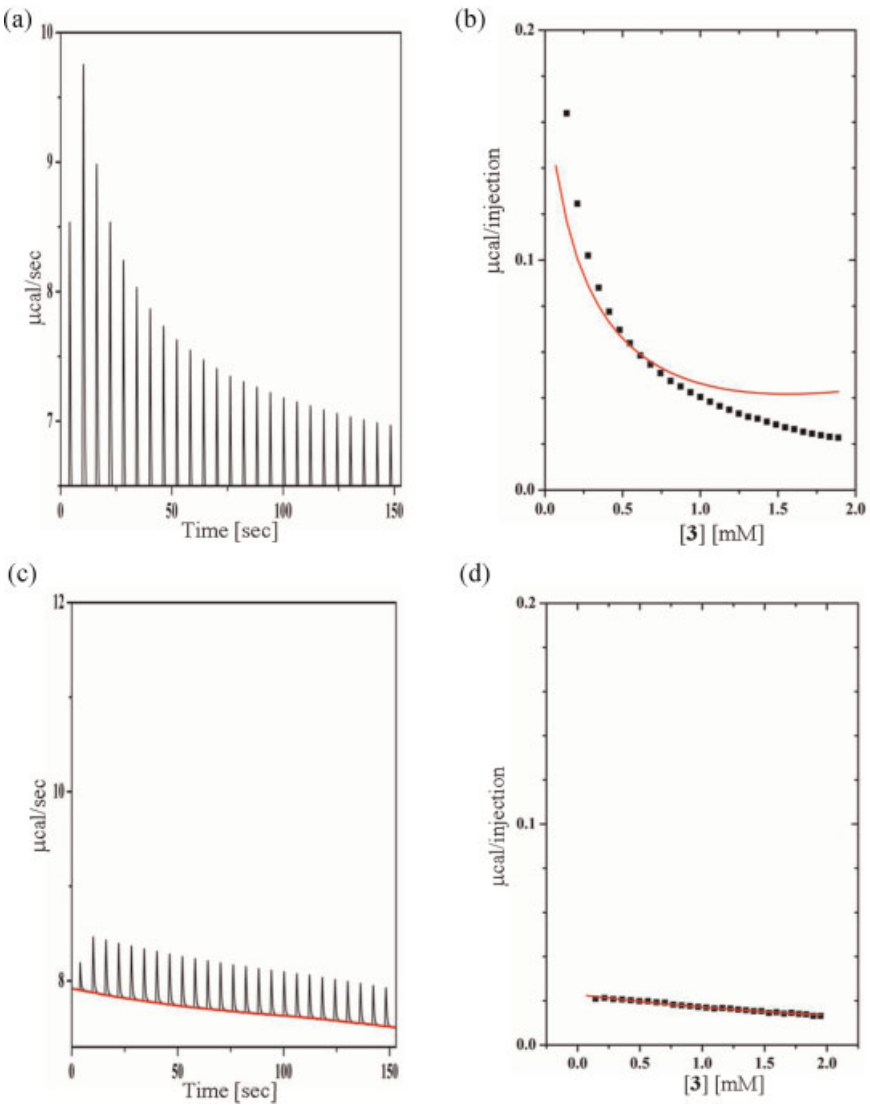


Figure 1. Plot of the heat pulses measured during the dilution of NAD^+ **2**: 30 × 10 µl portions of a solution of NAD^+ ($[NAD^+] = 10$ µM) (a) in H_2O, (c) in aqueous buffer were added to H_2O or buffer (each 1.4211 ml). (b) and (d) concentration dependence of the heat development (in µcal) per injection. The solid line in (b) represents the curve calculated with the assumption that a potential self-assembled dimer $(NAD^+)_2$ is formed. The missing fit of the calculation with the experimental data indicates a stoichiometry of the self-aggregate higher than that of a dimer

assumed formation of a self-assembled dimer $(NAD^+)_2$ failed (Figure 1(b)). This indicates that protonated NAD^+ forms a higher self-aggregate in water.[13] In buffered aqueous solution at pH = 7.2 no heat is evolved during the dilution of NAD^+ (Figure 1(c) and (d)) showing that deprotonated NAD^+ **2** exists as monomer under these conditions. Similar results by the use of ITC were obtained for AMP **5**. In pure water it forms an aggregate similar to NAD^+ whereas in buffered solution it exists as monomer. In the case of NMN **4**, no experimental evidence has been found by ITC for self-association under both conditions suggesting that NMN exists in its monomeric form in pure water, as well. (The experimental data of the ITC measurements of AMP and NMN are shown in the Supporting Information).

In pure water, the phosphonate-substituted clip **1b** reacts almost neutral (pH = 7.2), whereas the hydrogenphosphate-substituted clip **1c** reacts slightly acidic (pH = 4.5, [**1c**] = 3.77 mM) and the phosphate clip **1d** slightly basic (pH = 8.5, [**1d**] = 5.30 mM). In buffered solution at pH = 7.2 or 7.6 **1c** is partially deprotonated and **1d** partially protonated leading to one and the same compound independently from the starting material. The notation 'clip **1d**' in Tables 1–4 means that **1d** was used as starting material for the studies in buffer. The ^1H NMR spectra of the molecular clips **1b–d** (shown in the Supporting Information) are slightly concentration-dependent in D_2O and in buffered aqueous solution as well. From this finding, we extrapolated that these compounds form relatively weak self-assembled dimers (25 °C: K_{dim} $[M^{-1}] \leq 30$ (**1b** in buffer), 55 ± 8 (**1c** in D_2O), and 140 ± 60 (**1d** in buffer). Thus, the molecular clips **1b–d** having naphthalene sidewalls largely exist as monomers in dilute aqueous solution contrary to the corresponding phosphonate-substituted clip having anthracene sidewalls that forms a highly stable self-assembled dimer in water or buffer (25 °C: K_{dim} $[M^{-1}] = 1.6 \times 10^5$ or 2.3×10^5).[14]

NMR measurements: host–guest binding

Due to the magnetic anisotropy of the clip arene-units ^1H NMR spectroscopy represents a very sensitive probe for uncovering the complexation mode of guest molecules. The binding of the guest molecule inside the clip cavities can be easily detected by pronounced up-field shifts of the guest signals in the ^1H NMR spectrum of a host–guest mixture.[3] The binding of the already mentioned guest molecules by the molecular clips **1b,d** substituted by lithium methanephosphonate or lithium phosphate groups at the central benzene spacer-unit were studied in buffered and in pure aqueous solution by ^1H NMR spectroscopy. The maximum complexation-induced ^1H NMR shifts of the guest protons, $\Delta\delta_{max}$ ($\Delta\delta_{max} = \delta_0 - \delta_{HG}$), and the association constants, K_a, were determined by the methods of ^1H NMR titration, from the dependence of the observed complexation-induced ^1H NMR shifts, $\Delta\delta_{obs}$ ($\Delta\delta_{obs} = \delta_0 - \delta_{obs}$), of the guest protons either on dilution of the host concentration at constant guest concentration or on dilution of a solution containing both host and guest in *ca.* 1:1 ratio.[15]

Host–guest complexes with NAD^+ 2

Large $\Delta\delta_{max}$ values of the guest protons were found in the ^1H NMR titration experiments for the complex formation of NAD^+ **2** with the clips **1b** and **1d** in buffered aqueous solution at pH = 7.2 (Table 1). In pure water under slightly acidic condition, however, only small $\Delta\delta_{max}$ values of the corresponding NAD^+ protons were observed in the complexes of clips **1b** and **1c** (Table 1) analogously to those found for the NAD^+ complex of clip **1a**.[5] These findings allow the following conclusions: in buffered solution the nicotinamide ring of NAD^+ **2** is bound inside the cavity of clip **1b** or **1d** comparable to the corresponding complexes of NMNA **3** (*vide infra*). The relatively large $\Delta\delta_{max}$ values also observed for the adenine protons of **2** (Table 1) indicate that the adenine unit may be also positioned inside the clip cavity. A possible explanation for the observed shifts could be the existence of a shuttle process in a (1:1) complex occurring from the nicotinamide ring to the adenine unit, presumably via mutual dissociation/association, fast with respect to the "NMR timescale," so that all NMR signals are averaged.[16,17] Further insight into these intriguing complex structures comes from quantum-chemical ^1H NMR shift calculations (*vide infra*). The small $\Delta\delta_{max}$ values observed for all NAD^+ protons in the complexes of **1b** and **1c** in pure water clearly indicate that in these cases neither the nicotinamide ring nor the adenine unit is bound inside the clip cavity comparable to the reported NAD^+ complex of **1a**.[5]

On excitation with ultraviolet light at 280 nm the molecular clips **1b** and **1d** show a relatively strong fluorescence band at 340 nm which can be assigned to the emission of the naphthalene sidewalls (Figure 2).[18] The observation, that this fluorescence band is quenched by successive addition of NAD^+ **2** to a solution of **1b** or **1d**, indicates an electronic interaction of the clip naphthalene sidewalls with the NAD^+ nicotinamide ring and/or adenine unit. This finding was used to determine the binding

Table 1. Maximum complexation-induced ^1H NMR shifts ($\Delta\delta_{max}$) determined for the binding of NAD^+ **2** with the clips **1b** and **1d** in buffered aqueous solution at pH = 7.2 or with **1b** and **1c** in D_2O by ^1H NMR titration at 25 °C

Host	Solvent	$\Delta\delta_{max}$ (ppm) (guest)							
		N-2-H	N-4-H	N-5-H	N-6-H	N-1′-H	A-2-H	A-8-H	A-1′-H
1b	Buffer	1.20	2.88	3.17	1.54	0.28	0.52	1.61	0.59
1d	Buffer	1.22	2.76	3.18	1.51	0.22	0.41	0.90	0.51
1d	Buffer[a]	1.41	3.23	3.69	1.75	0.32	0.57	1.68	0.60
1b	D_2O	0.39	0.73	0.78	0.44	0.32	—	0.34	0.23
1c	D_2O	0.49	0.90	—	0.69	0.23	0.45	—	0.51

[a] Titration with constant guest concentration.

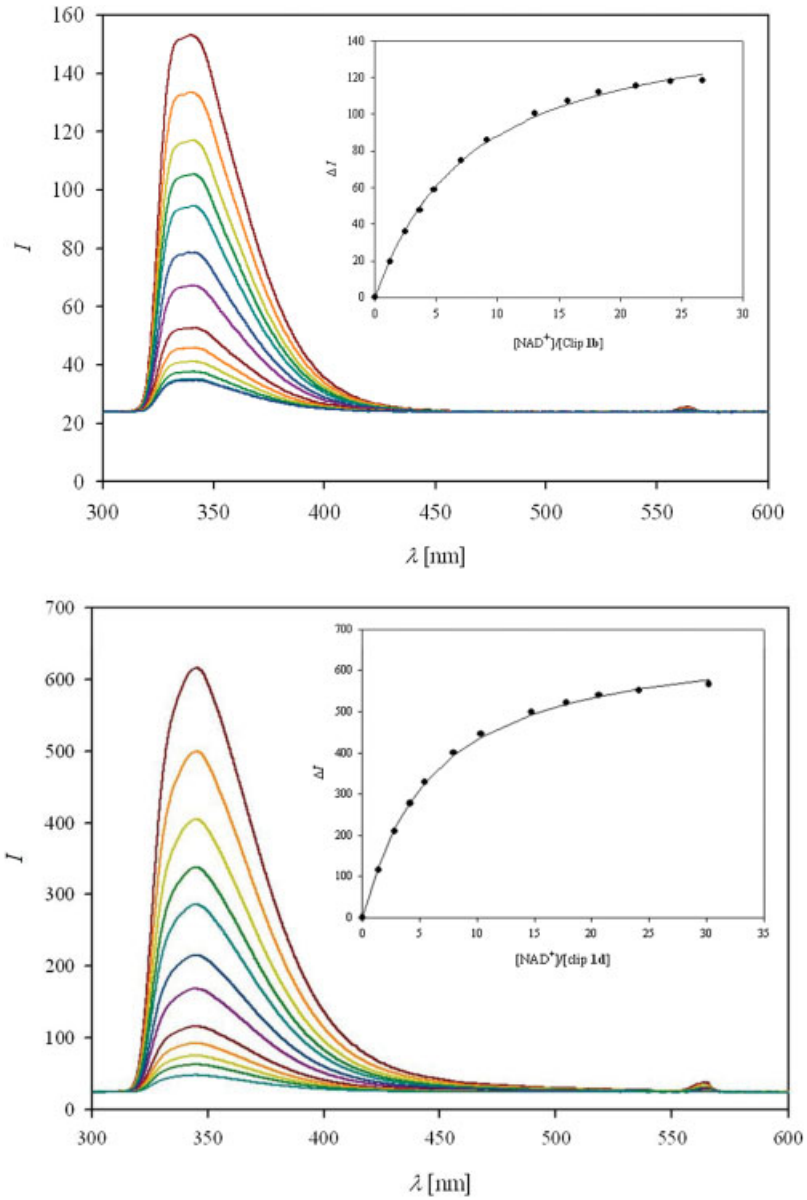


Figure 2. Changes in the fluorescence spectra of a solution of clip **1b** (top: 2.88×10^{-5} M) and **1d** (bottom: 2.63×10^{-5} M), respectively upon titration with NAD^+ **2** in buffered aqueous solution at pH = 7.6. The insets show the titration curves obtained by plotting the differences in the emission intensities $\Delta I = I_0 - I$ at 340 nm as function of the equivalents of NAD^+ **2**. The solid lines show the fitting based on the formation of 1:1 complexes

constants of the complex formation between clips **1b,d** and NAD^+ **2** by fluorometric titration independently to the NMR measurements. The binding constant determined for the formation of complex **2•1b** by the NMR titration experiment is in good agreement with that obtained by fluorometric titration, whereas in the case of complex **2•1d** the K_a value derived from the fluorometric titration is significantly larger than that determined from NMR measurement (Table 2). The difference between these two K_a values may be explained to be a result of the weak self-association of **1d** which is only important at higher concentrations of **1d** as used in the ^{1}H NMR titration.

Interesting information about the dynamic nature of these host–guest complexes comes from the chemical ^{1}H NMR shift nonequivalence in the clip's highly symmetrical naphthalene sidewalls induced by the binding of NAD^+ **2**. In the complexes **2•1b,d**, a remarkable splitting is observed for the ^{1}H NMR signals of the naphthalene protons of **1b,d**, which are equivalent in the free clips (Figure 3(a)). In the complexed clips, the naphthalene protons H^a and H^f show two separate singlets and the protons H^b–H^e an ABCD spectrum instead of one singlet and an A_2B_2 spectrum, respectively, as found in the free clips. The spectrum of the protons H^b–H^e calculated for an ABCD spin system is in good accord with the experimental data. Evidently, complex dissociation and association as well as conformational equilibration between the different complex structures proceed rapidly with respect to the "NMR time scale."[16,17] These dynamic processes lead to an averaging of the NMR signals of the complex and make the corresponding protons at the two naphthalene sidewalls of complexed **1d** equivalent to each other, whereas the front- and backside protons of each naphthalene sidewall remain none-

Table 2. Association constants, K_a, and free association enthalpies, ΔG, determined for the binding of NAD$^+$ **2** with clips **1b** and **1d** in buffered aqueous solution at pH = 7.2 or 7.6 by ^{1}HNMR or fluorometric titration (FT) at 25 °C

Host	Solvent	K_a (M^{-1})	ΔG (kcal/mol)	Method
1b	Buffer (pH = 7.2)	4000 ± 330	−4.9	^{1}H NMR
	Buffer (pH = 7.6)	4900 ± 200	−5.0	FT
1d	Buffer (pH = 7.2)	4800 ± 1300	−5.0	^{1}H NMR
	Buffer (pH = 7.6)	7100 ± 250	−5.3	FT

quivalent. This finding can be explained with a chirality transfer of the chiral guest NAD$^+$ **2** to the achiral clip **1d** as depicted in Figure 3(b) for one complex structure including the nicotinamide ring inside the clip cavity.

Host–guest complexes with NMNA 3, NMN 4, AMP 5, and NADH 6

Large binding constants, K_a, and $\Delta\delta_{max}$ values for the ^{1}H NMR signals assigned to the protons at the nicotinamide ring were found for the complex formation between NMNA **3** as guest molecule and the molecular clips **1b,d** as host molecule (Table 3). The $\Delta\delta_{max}$ values are similar to the values for the corresponding protons of the nicotinamide part in the host–guest complexes with NAD$^+$ **2** (Table 1). These data suggest the nicotinamide ring of NMNA **3** to be positioned inside the cavity of clip **1b** or **1d** comparable to the recently reported findings for clip **1a** and NMNA **3**.[5] This assumption is confirmed by *ab initio* calculations of the ^{1}H NMR chemical shifts of the guest protons (*vide infra*).

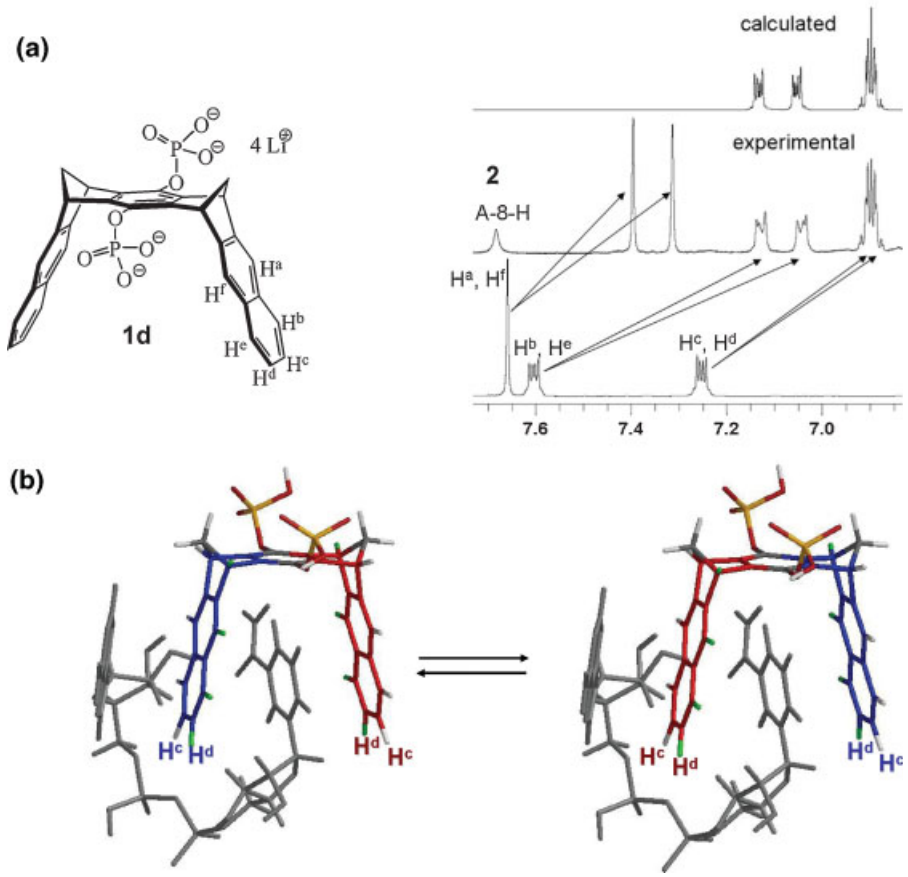


Figure 3. (a) Comparison of the ^{1}H NMR ABCD spin system calculated by WinDaisy (Bruker, top) with the experimental ^{1}H NMR spectra of the protons $H^a - H^f$ of clip **1d** in the NAD$^+$ complex **2•1d** (middle) and in pure clip **1d** (bottom) in D_2O. (b) A formal 180° rotation around the C_2 axis through the central benzene spacer-unit of the clip molecule leads to a pair-wise exchange of the host protons (e.g. H^c and H^d) at the naphthalene sidewall marked in blue with those of the other one marked in red as it is shown for one complex structure including the nicotinamide ring inside the clip cavity (the structure for complex **2•1** (R = $OP(OH)O_2^-$) was calculated by force field (MacroModel, Monte-Carlo conformer search, AMBER*/H_2O)).[16] Similar conclusions can be drawn for other complex structures, e.g. for those including the adenine system inside the clip cavity. In any case, the corresponding host protons at one and the same naphthalene sidewall remain nonequivalent

Table 3. Association constants, K_a, free association enthalpies, ΔG, and maximum complexation-induced ^{1}H NMR shifts, $\Delta\delta_{max}$, determined for binding of NMNA **3** with the clips **1b,d** in buffered aqueous solution at pH = 7.2 by ^{1}HNMR titration at 25 °C

Host	K_a (M^{-1})	ΔG (kcal/mol)	$\Delta\delta_{max}$(ppm) (guest)				
			2-H	4-H	5-H	6-H	N–CH_3
1b	11 200 ± 1100	−5.5	1.61	3.47	3.07	2.25	0.99
1d	33 800 ± 1700	−6.2	1.81	3.69	3.32	2.62	0.99

The comparison of NAD^+ **2** with the nucleotides NMN **4** and AMP **5** (the two fragments formally obtained by hydrolysis of the diphosphate linkage of NAD^+ **2**) allows us to determine the specific contributions of the nicotinamide and adenine unit toward the structure and stability of the NAD^+ complexes. This is especially important in light of the question, if both heterocycles are accommodated inside the clip cavity as assumed from $\Delta\delta_{max}$ values observed for the NAD^+ complexes. Both nucleotides form complexes with the clips **1b** and **1d** in aqueous buffer. All complexes **4•1b,d** and **5•1b,d** display large complexation-induced ^{1}H NMR shifts, $\Delta\delta_{max}$, of the guest protons similar to those found for the corresponding protons in the NAD^+ complexes **2•1b** and **2•1d** (Tables 1 and 4). Inclusion of each heterocycle in the clip cavity strongly supports the above postulate that also in the NAD^+ complexes both ring systems are positioned inside the clip cavity. Our quantum-chemical studies of complexation induced ^{1}H NMR shifts (described later in this work) further show that the structure, where the nicotinamide part of the NAD^+ is positioned inside the cavity yields chemical shifts in better agreement with the experimental results, so that it seems to be preferred.

NADH **6**, the product of enzymatic NAD^+ reduction, forms complexes with **1b** and **1d** in buffered aqueous solution, too. The large $\Delta\delta_{max}$ values observed for the adenine guest protons and the negligibly small values found for the protons assigned to the dihydronicotinamide ring of **6** allow the conclusion that only the adenine ring is included into the clip cavity. This fact coincides with the electron-rich character of the enamine structure of the dihydronicotinamide ring in NADH as opposed to the extremely electron-poor pyridinium cation of NAD^+, as illustrated in electron potential surface (EPS) calculations.[5] A molecular clip such as **1b** or **1d** is thus able to distinguish between both oxidation states of NAD^+/NADH. This is important for competition experiments with enzymes – the clip is not prone to undergo product inhibition with NADH, the product of enzymatic oxidation.

Another interesting observation comes from the comparison of the binding constants determined for the formation of the host–guest complexes of NAD^+ **2**, NMN **4**, AMP **5**, and NADH **6** with clips **1b,d**: the constants determined for the complexes of NMN **4**, AMP **5**, and NADH **6** are significantly smaller than those found for the NAD^+ complexes. This finding indicates that the multiple aromatic $\pi-\pi$ and cation$-\pi$ host–guest interactions in the NAD^+ complexes have a synergetic effect on the complex stability.

Quantum-chemical calculations of ^{1}H NMR shifts and binding energies

In order to gain further insight into possible binding modes of the host–guest complexes studied in this work, all host–guest complexes with NAD^+, NMNA, NMN, AMP, and NADH as guest molecules have been studied by quantum-chemical *ab initio* calculations of ^{1}H NMR shieldings based on force-field optimized

Table 4. Association constants, K_a, free association enthalpies, ΔG, and maximum complexation-induced ^{1}H NMR shifts, $\Delta\delta_{max}$, determined for binding of NMN **4**, AMP **5**, and NADH **6** with **1b** and **1d** in buffered aqueous solution at pH = 7.2 by ^{1}H NMR titration at 25 °C

Host	K_a (M^{-1})	ΔG (kcal/mol)	$\Delta\delta_{max}$ (guest) (ppm)					
NMN			2-H	4-H	5-H	6-H	1′-H	4′-H
1b	550 ± 45	−3.7	1.28	2.88	3.49	1.75	0.62	0.07
1d	1250 ± 70	−4.2	1.50	3.20	4.04	1.97	0.61	0.09
AMP			2-H	8-H	1′-H	3′-H		
1b	910 ± 85	−4.0	0.38	1.51	0.27	0.03		
1d	680 ± 60	−3.9	0.74	3.14	0.48	0.08		
NADH			N-2-H	N-4-H	N-1′-H	A-2-H	A-8-H	A-1′-H
1b	800 ± 60	−4.0	0.22	-	0.24	1.26	0.49	0.29
1d	400 ± 25	−3.5	0.12	0.2/0.3	0.10	0.90	3.14	0.53

structures (refer to "Computational Details" section and Supporting Information). We focus here on the complexation-induced ^{1}H NMR chemical shifts for the guest molecule calculated as the difference between the shieldings of the free and bound guest. Due to the flexibility of the present host–guest complexes a multitude of possible structures need to be studied, so that for each system various phosphate- or phosphonate-substituted structures have been calculated with a minimum of six structures for each host–guest complex. For the free NAD$^+$ and NADH guests, three possible structures were investigated, whereas for NMNA, NMN, and AMP one free guest structure was considered. Since all the *ab initio* calculations have been performed in the gas phase, we focus in the following discussion only on hydrogen atoms located at aromatic or sugar rings not prone to hydrogen bonding. For each host–guest complex the proton chemical shieldings of at least one of the investigated structures agree within 1.4 ppm with the experimental results obtained in buffered aqueous solution. This agreement can be considered sufficient to assign the experimentally observed, large complexation-induced shieldings of up to 4 ppm, so that the structure of the host–guest complexes can be understood. Here, the error bars of the calculations arising due to uncertainties within the force-field optimized structures, the chosen quantum-chemical approximation for calculating NMR chemical shifts, and the neglect of solvent effects can be estimated to be typically in the order of 1 ppm for the considered protons.[5,21]

A comparison between the experimental and calculated $\Delta\delta_{max}$ data for the host–guest complexes of phosphonate and phosphate clip **1b** and **1d** with all guest molecules **2–6** is shown in Table 5. From the calculations only the data are listed which give the best fit with the experimental values. The observed differences between the experimental and calculated data result from the difficulties mentioned above and the fact that each host–guest complex certainly consists of several conformers which are in a dynamic equilibrium and contribute to its overall structure so that the experimental chemical ^{1}H NMR shifts are averaged values. More detailed data are presented in the Supporting Information, while we focus in the following on the most important aspects for the NAD$^+$ host–guest complex (as shown in Figure 4).

The two fundamentally different possibilities of binding NAD$^+$ either by the adenine or the nicotinamide unit can be distinguished by comparing quantum-chemically computed and experimental NMR data: here, we considered six structures where nicotinamide is bound inside the clip cavity and three structures with the adenine part inside the cavity (of these nine structures, one nicotinamide and one adenine bound structure were taken from Reference [5]; the other seven structures were calculated in this work, refer to Supporting Information for details).

The nicotinamide-bound structures clearly show a better agreement with the experiment than the ones with adenine

Table 5. Comparison of the experimental complexation-induced ^{1}H NMR shifts, $\Delta\delta_{max}$, of the guest protons in buffered aqueous solution with those calculated by *ab initio* methods

Complex	N-2-H	N-4-H	N-5-H	N-6-H	N-1′-H	A-2-H	A-8-H	A-1′-H
NAD$^+$ **2•1b**	1.2	2.9	3.2	1.5	0.3	0.5	1.6	0.6
NAD-Nic[a]	2.0	3.7	3.3	1.8	1.0	0.5	1.0	0.4
NAD-Ade[a]	1.6	2.2	0.7	0.8	0.9	2.6	1.4	0.5
NAD$^+$ **2•1d**	1.2	2.8	3.2	1.5	0.2	0.4	0.9	0.5
NAD′-Nic[a]	2.2	3.8	3.6	1.5	1.6	0.9	0.4	1.0
NAD′-Ade[a]	1.5	1.4	0.3	−0.1	0.6	2.9	1.1	0.9
NMNA **3•1b**	1.6	3.5	3.1	2.3	1.0	—	—	—
NMNA6[a]	1.7	3.4	3.3	3.0	1.5	—	—	—
NMNA **3•1d**	1.8	3.7	3.3	2.6	1.0	—	—	—
NMNA2[a]	2.1	3.6	3.0	1.4	0.9	—	—	—
NMN **4•1b**	1.3	2.9	3.5	1.8	0.6	—	—	—
NMN6[a]	1.6	3.1	3.0	1.8	0.6	—	—	—
NMN **4•1d**	1.5	3.2	4.0	2.0	0.6	—	—	—
NMN1[a]	1.6	5.2	2.6	2.5	1.1	—	—	—
AMP **5•1b**	—	—	—	—	—	0.4	1.5	0.3
AMP9[a]	—	—	—	—	—	2.7	1.5	0.8
AMP **5•1d**	—	—	—	—	—	0.7	3.1	0.5
AMP2[a]	—	—	—	—	—	0.9	2.7	1.9
NADH **6•1b**	0.2	–	–	–	0.2	1.3	0.5	0.3
NADH8[a]	−0.5	−0.7	−1.1	−1.5	−0.2	2.4	1.0	1.0
NADH **6•1d**	0.1	0.2/0.3	—	—	0.1	0.9	3.1	0.5
NADH3[a]	1.6	−0.5	−0.5	0.0	0.5	1.0	4.4	1.6

[a] Calculated structure (as shown in Figure 4 or Supporting Information: Table QC-1 – QC-9; NAD-Nic = NAD+5, NAD-Ade = NAD+9, NAD′-Nic = NAD+3, NAD′-Ade = NAD+7, compared to G3; NADH8 is compared to free guest G2 and NADH3 to G3).

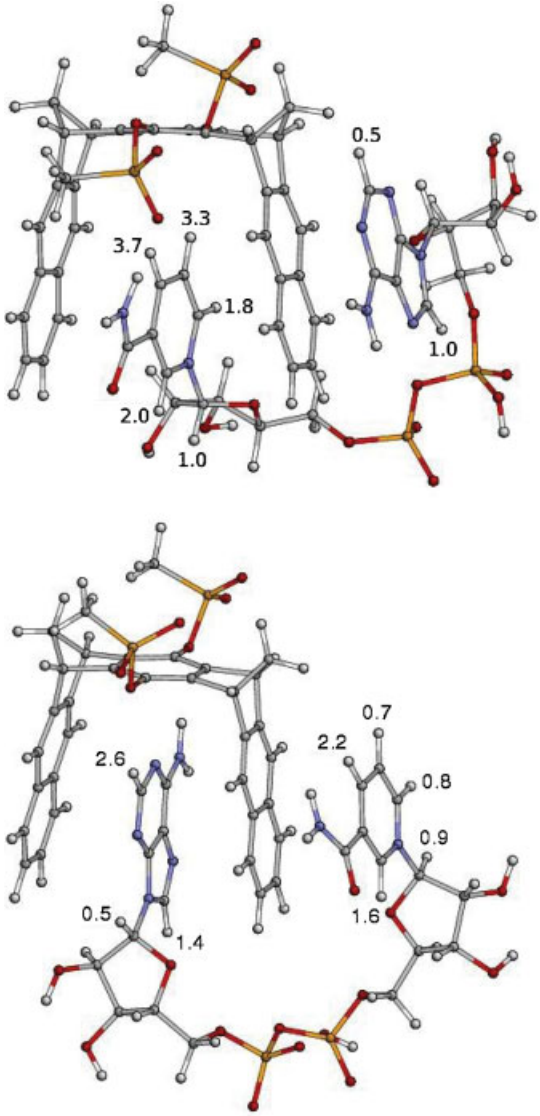


Figure 4. AMBER*/H_2O structures NAD-Nic (top) and NAD-Ade (bottom) of complex **2•1** (R = $OP(Me)O_2^-$) and the complexation-induced ^1H NMR shifts ($\Delta\delta_{max}$) [ppm] computed at the GIAO-HF/SVP level as differences of the chemical ^1H NMR shifts (δ) for free and complexed guest protons. The listed data are the calculated $\Delta\delta_{max}$ values in ppm. The displayed structures NAD-Nic (nicotinamide binding) and NAD-Ade (adenine binding) are the ones for which the calculated $\Delta\delta_{max}$ values show the smallest deviations to the experimental data (in buffered aqueous solution). Structure NAD-Ade was taken from Reference [5].

inside the clips' cavity: for the nicotinamide-bound complexes the best agreement is observed for the NAD-Nic structure displayed in Figure 4 with maximum deviations for the calculated ^1H NMR shifts of nicotinamide of 0.8 ppm, while the chemical shifts of its adenine unit outside the cavity agree within 0.6 ppm. Complex-induced shieldings of other nicotinamide-bound structures deviate stronger by 1.4–3.9 ppm for their nicotinamide unit and 0.5–1.1 ppm for their adenine unit.

In contrast, the alternative binding modes via adenine show maximum deviations of 2.1–2.6 ppm in comparison to the experiment for their complexed unit, while deviations of 2.5–3.7 ppm are computed for their nicotinamide part outside the cavity.

The good agreement of the quantum-chemical and experimental results for the nicotinamide-bound complex provides strong evidence that it is the most prominent structure in the complexation process, while complexation of the adenine part of NAD^+ is less dominant. At the same stage it is important to stress, that the flexibility of the host–guest complexes is rather large and a dynamic process between various binding motifs is expected.

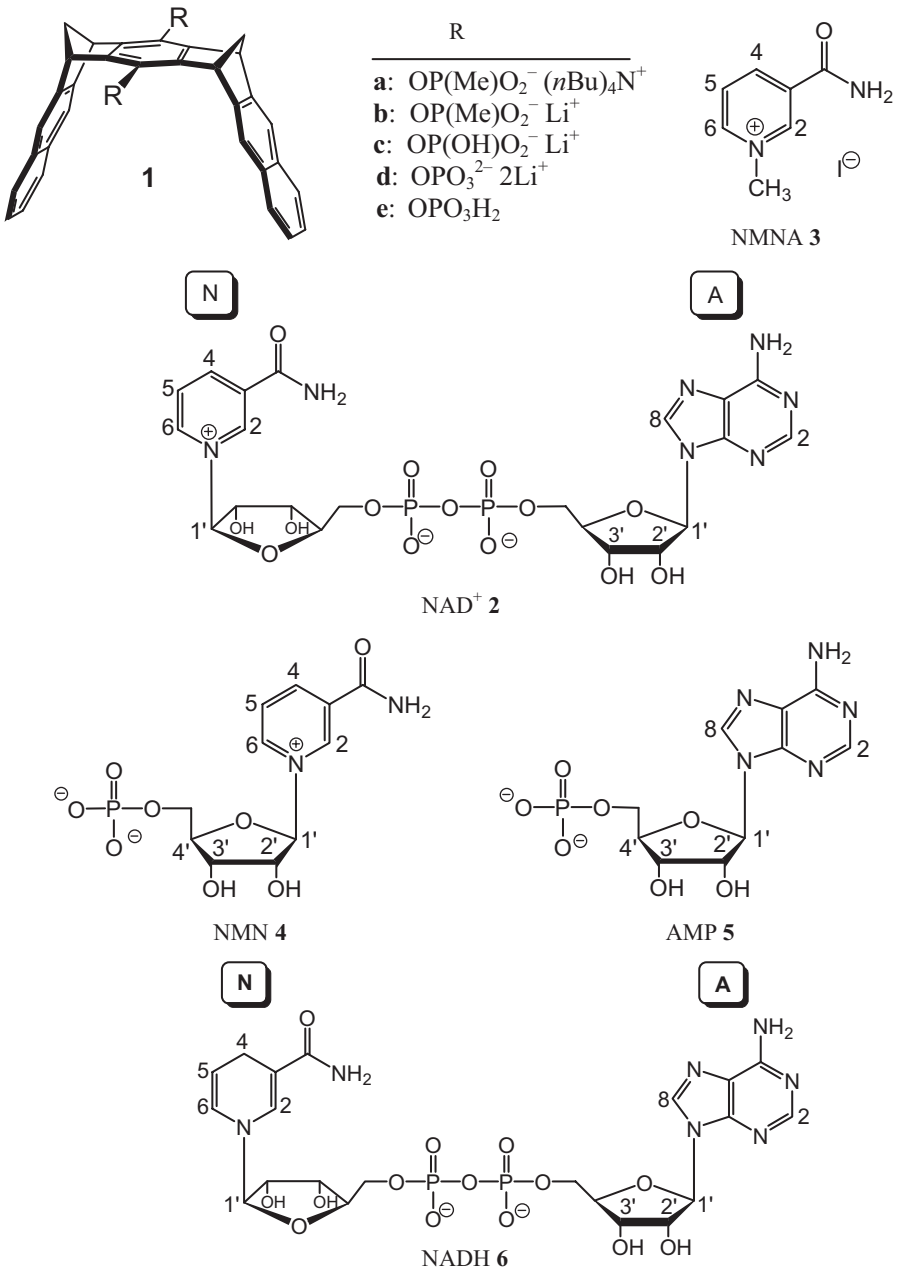
Binding energies

Besides the results derived above by comparing computed and experimental complexation-induced chemical shieldings, there are also energetic indications for the preferred binding via the nicotinamide unit of NAD^+. While we have published already interaction energies for the example of two possible structures denoted in Reference [5] as NAD-1 and NAD-2 without counterpoise correction, we provide in our present work also interaction energies computed at the most reliable RI-MP2/SVP level with counterpoise correction (as shown in Table 6). The counterpoise corrected data indicate that the interaction energy within the nicotinamide bound structure NAD-1 is stronger by 3 to 27 kcal/mol as compared to the one for adenine binding (NAD-2) depending on the individual charges of the complex components. As expected, the counterpoise correction slightly reduces the interaction energies, however, the relative energies of nicotinamide *versus* adenine binding are only influenced by 4 kcal/mol at most. A similar behavior of basis-set superposition effects is also found for the host–guest interaction within a molecular tweezer binding dicyano-benzene: the total interaction energy as computed at the RI-MP2/SVP level is reduced from 39 to 25 kcal/mol, however, the additivity of interaction energies is preserved upon counterpoise correction; one part of the tweezer binds the guest by 8kcal/mol and the other two parts by 7 kcal/mol which adds up to 22 kcal/mol. This means that 88% of the binding energy results from the aromatic parts of the clip, while without counterpoise correction it is fairly similar with a contribution of 95%.

Table 6. Host–guest interaction energies (*E*int) in kcal/mol within NAD-1 and NAD-2 structures[5] as obtained at the RI-MP2/SVP level both without and with counterpoise corrections (cp-corr) For NAD-2 the differences of the interaction energies ($\Delta E = E_{int}$ (NAD-1) − *E*int (NAD-2)) are given

Charge		NAD-1		Difference (NAD-1 − NAD-2)	
Clip	NAD^+	E_{int}	E_{int} (cp-corr)	ΔE_{int}	ΔE_{int} (cp-corr)
−2	+1[a]	−152	−136	—	—
−2	0[a]	−46	−30	−9	−13
0	0[a]	−34	−18	+1	−3
0	+1	−79	−48	−8	−8
0	0	−78	−47	−7	−8
−2	+1	−171	−139	−27	−27

[a] Only the part of the guest molecule bound inside the clip is considered.



Scheme 1. Structures of molecular clips **1a–e** as well as NAD^+ **2**, NMNA **3**, NMN **4**, AMP **5**, NADH **6** (N = nicotinamide; A = adenine) with their respective numbering schemes

CONCLUSION

NAD^+ **2** forms host–guest complexes with the molecular clips **1b–d** displaying large complexation-induced ^1H NMR shifts, $\Delta\delta_{max}$, of the guest signals assigned to the nicotinamide as well as adenine protons in buffered aqueous solution at pH = 7.2. In contrast, NMR shifts of the corresponding complexes remain small in pure water. We conclude that in buffer at neutral pH value, NAD^+ **2** forms host–guest complexes with clips **1b–d**, where either the nicotinamide or the adenine part can be bound inside the clip cavity, whereas in the complexes dissolved in pure water under slightly acidic conditions both heterocyclic units are located outside the clip cavity. According to ITC measurements the acidic aqueous solution contains aggregated NAD^+ **2** molecules which evidently do not dissociate in the presence of 1 mol equivalent of clip **1a**, **1b**, or **1c** and, hence, cannot be accommodated inside the clip cavity. In the formal 1:1 complexes observed by the NMR dilution experiments, we assume that clip molecules stick to the outer surface of the NAD^+ aggregate. Interestingly, this assumption is supported by the finding that in pure water the complexation-induced ^1H NMR shifts of the NAD^+ guest signals increase by addition of excess clip **1b** or **1c** approaching the $\Delta\delta_{max}$ values observed in buffered aqueous solution. The excess of host, evidently, affects the dissociation of the NAD^+ aggregate by clipping one of the heterocyclic units. The true structural switch between aggregated and monomeric NAD^+ is, however, triggered by deprotonation. In water under slightly acidic conditions, the NAD^+ molecule exists in its zwitterionic form presumably protonated at nitrogen atom N-1 of the adenine unit.[12] Thus, it forms the higher aggregate detected

by the ITC measurements most likely resulting from either hydrophobic or electrostatic interactions.[23–26] In buffered solution at pH = 7.2, however, the overall neutral NAD^+ molecules are deprotonated and, hence, become negatively charged. The negative overall charge destabilizes existing aggregates leading to efficient monomerization of the cofactor.

Monomeric NAD^+ forms clean (1:1) complexes with the clips **1b** and **1d** including either the nicotinamide or the adenine unit inside the clip cavity. If both heterocyclic units are included, the clip has to shuttle in the (1:1) complex from one to the other unit rapidly on the "NMR time scale" in order to explain the observed complexation-induced ^{1}H NMR up-field shifts of the signals assigned to the nicotinamide and adenine guest protons. As an additional rapid process, a formal 180° rotation of the clip molecule around the guest unit was detected by the complexation-induced ^{1}H NMR shifts of the host protons resulting from a chirality transfer of the chiral guest to the achiral host (Figure 3). These two dynamic processes must occur via mutual complex dissociation and association and not by "intramolecular" conformational isomerizations, as they were found for the guest rotation and shuttle processes inside the clip or tweezer cavity of other host–guest systems.[16,17] Our conclusion, that both units of NAD^+ **2** are bound inside the clip cavity, is supported by the observation that NMNA **3** and the nucleotides NMN **4** and AMP **5** form complexes with the clips **1b** and **1d** showing large $\Delta\delta_{max}$ values for the guest ^{1}H NMR signals assigned to the nicotinamide or adenine protons comparable to those found for the corresponding protons of the NAD^+ complexes. Intriguingly, quantum-chemically calculated complexation-induced ^{1}H NMR shifts for the nicotinamide bound NAD^+ complex show a better agreement with the experimentally observed shieldings than the ones calculated for the adenine bound NAD^+ complex.

Importantly, complexes of NMN **4**, AMP **5**, and NADH **6** are significantly less stable than those of NAD^+ **2**, indicating that multiple aromatic $\pi-\pi$ and cation$-\pi$ host–guest interactions occurring in the NAD^+ host–guest complexes have a synergetic effect on the complex stability. The clips' (**1b,d**) fluorescence emission is almost entirely quenched by complex formation with NAD^+ – an excellent starting point for the development of chemical sensors.

The inclusion of NAD^+ and other enzyme cofactors at their active sites by molecular clips is a prerequisite for the systematic investigation of their effects on enzymatic cofactor-mediated processes. Applying the optimum conditions for efficient NAD^+ inclusion inside the clip cavity detailed in this work, we recently proceeded to experiments with cofactor-dependent enzymes. Indeed, the enzymatic oxidation of alcohol catalyzed by AD[10] using NAD^+ as cofactor is inhibited by the addition of clip **1b** or **1d**. Detailed mechanistic investigations point to a typical behavior of both clips as competitive inhibitors (Lineweaver–Burk plots, inhibition reversal by cofactor addition).[10] We conclude that also under physiological conditions the molecular clips are able to capture NAD^+ and deplete the cofactor concentration inside the Rossman fold.

EXPERIMENTAL SECTION

General experimental details

IR: Bio-Rad FTS 135. UV: J+M Tidas FG Cosytec RS 422. ^{1}H-NMR, ^{13}C-NMR, DEPT H,H-COSY, C,H-COSY, NOESY, HMQC, HMBC, ^{1}H-NMR titration experiments: DRX 500. The undeuterated amount of the solvent was used as an internal standard. The ^{1}H and ^{13}C NMR signals were assigned by the 2D experiments mentioned above. Positions of the protons of the methylene bridges are indicated by the letters *i* (*innen*, toward the center of the molecule) and *a* (*aussen*, away from the center of the molecule). MS: Fison Instruments VG ProSpec 3000 (70 eV). All melting points are uncorrected. Column chromatography: silica gel 0.063–0.2 mm. All solvents were distilled prior to use. The aqueous phosphate buffer at pH = 7.2 (used for the ^{1}H NMR titration experiments) was prepared by dissolving NaOH (1.06 mmol) and KH_2PO_4 (1.33 mmol) in 20 ml of D_2O and that at pH = 7.6 (used for the fluorometric titration experiments) by the addition of NaOH (10%) to a solution containing Na_2HPO_4 (100 mM), NaH_2PO_4 (100 mM), $MgCl_2$ (7 mM), and ethylenediamine tetraacetic acid, EDTA (0,1 mM) until the desired pH value of 7.6 has been achieved.

The molecular clips **1b** and **1d** were synthesized and characterized as previously described.[6]

Dilithium-(6α, 8α, 15α, 17α)-6, 8, 15, 17-tetrahydro-6:17, 8:15-dimethanoheptacenyl-7,16-bisphosphate 1c

$LiOH \cdot H_2O$ (9.6 mg, 228.9 μmol) is added to the stirred solution of phosphoric acid **1e** (68.7 mg, 114.9 μmol) in 5 ml of methanol at room temperature. After stirring of the clear solution for 30 min, methanol is evaporated *in vacuo*. The residue is dried several hours *in vacuo* to give 70 mg of **1c** as beige solid in quantitative yield. Mp = 223 °C (decomposition); ^{1}H NMR (500 MHz, D_2O): δ = 2.41 (d, 2 H, ^2J (19-H^a, 19-H^i) = 8.2 Hz, 19-H^a, 20-H^a), 2.69 (d, 2 H, 19-H^i, 20-H^i), 4.73 (s, 4 H, 6-H, 8-H, 15-H, 17-H), 6.87 (m, 4 H, 2-H, 3-H, 11-H, 12-H), 6.97 (m, 4 H, 1-H, 4-H, 10-H 13-H), 7.30 (s, 4 H, 5-H, 9-H, 14-H, 18-H); ^{13}C NMR (125.7 MHz, D_2O): δ = 48.40 (s, CH_2, C-19, C-20), 64.46 (s, CH, C-6, C-8, C-15, C-17), 120.23 (s, CH, C-5, C-9, C-14, C-18), 125.32 (s, CH, C-2, C-3, C-11, C-12), 127.66 (s, CH, C-1, C-4, C-10, C-13), 131.88 (s, C-4a, C-9a, C-13a, C-18a), 141.95 (s, C-6a, C-7a, C-15a, C-16a), 147.55 (s, C-5a, C-8a, C-14a, C-17a) the signal of the carbons C-7 and C-16 is not observed; ^{31}P NMR (202 MHz, D_2O): δ = −3.01 (s, 2 P, OP(O)(OH)OLi).

The association constants K_a and the complexation-induced chemical ^{1}H NMR shifts, $\Delta\delta_{max}$, were determined by ^{1}H NMR titration as described in References [5,6] Host H and guest G are in equilibrium with the 1:1-complex HG. The association constant K_a is then defined by Eqn (1). $[H]_0$ and $[G]_0$ are the starting concentrations of host and guest, respectively.

$$K_a = \frac{[HG]}{[H] \cdot [G]} = \frac{[HG]}{([H]_0 - [HG]) \cdot ([G]_0 - [HG])} \quad (1)$$

The observed chemical shift δ_{obs} of the guest proton in the ^{1}H NMR spectrum (Bruker instrument DRX 500, 500 MHz, 25 °C) of a host and guest mixture is an averaged value between free (δ_0) and complexed guest (δ_{HG}), in the case of the exchange between free and complexed guest being fast with respect to the NMR time scale (Eqn (2)). Combination of Eqns (1) and (2) and the use of differences in chemical shift ($\Delta\delta = \delta_0 - \delta_{obs}$; $\Delta\delta_{max} = \delta_0 - \delta_{HG}$) leads to Eqns (3) and (4), respectively.

$$\delta_{obs} = \frac{[G]}{[G] + [HG]} \cdot \delta_0 + \frac{[HG]}{[G] + [HG]} \cdot \delta_{HG} \quad (2)$$

$$\Delta\delta = \frac{\Delta\delta_{max}}{[G]_0}\cdot\left(\frac{1}{2}\left([H]_0+[G]_0+\frac{1}{K_a}\right)-\sqrt{\frac{1}{4}\cdot\left([H]_0+[G]_0+\frac{1}{K_a}\right)^2-[H]_0\cdot[G]_0}\right) \quad (3)$$

$$\Delta\delta = \frac{\Delta\delta_{max}}{2}\left(K+1+\frac{K}{[H]_0K_a}-\sqrt{K^2+\frac{2K^2}{[H]_0K_a}-2K+\frac{K^2}{[H]_0^2K_a^2}+\frac{2K}{[H]_0K_a}+1}\right) \quad \text{with } K=\frac{[H]_0}{[G]_0} \quad (4)$$

Two types of titration experiments were performed: (i) in the case of $K_a \leq 4000\ M^{-1}$, the total guest concentration $[G]_0$ was kept constant, whereas the total host concentration $[H]_0$ was varied (Eqn (3)); (ii) in the case of $K_a \geq 4000\ M^{-1}$, dilution titrations were performed by the use of solutions containing equimolar amounts of host and guest with concentrations in the range between 0.5 and 4 mM (Eqn (4)). In both experiments, $\Delta\delta$ was determined as the difference of the chemical shifts of the proton of pure guest (δ_0) and of the guest in the various host–guest mixtures (δ_{obs}). The dependence of δ_{obs} on the host concentrations afforded the data pairs – $\Delta\delta$ and $[H]_0$. Fitting of these data to the 1:1 binding isotherm (Eqns (3) and (4), respectively) by iterative methods using the computer program TableCurve 2D, version 5.0 delivered the parameters K_a and $\Delta\delta_{max}$. In the case of mixed (1:1) and (1:2) or (2:1) complex stoichiometries the data pairs – δ_{obs} and $[H]_0$ – were used for the fitting of the binding isotherm in the computer program HOSTEST *version* 5.60.[15] Since the guest molecules (used in this work) possess more than one kind of nonequivalent protons, K_a and $\Delta\delta_{max}$ were determined for the guest protons which can be unambiguously assigned over the total range of concentration and show substantial complexation-induced shifts, $\Delta\delta$. In these cases the average of the K_a values (determined independently) is given in Tables 2–4. If the guest proton signals cannot be unambiguously assigned at each concentration because they are broadened or superimposed by other NMR signals, the $\Delta\delta_{max}$ values of these guest protons were calculated by the use of Eqn (5). The experimental error was estimated to be small for the $\Delta\delta_{max}$ values (<5%), but large for the binding constants K_a (≤20%), particularly in the case of the highly stable complexes for which these data have to be determined by NMR dilution titrations as described above.

$$\Delta\delta_{n,max} = \Delta\delta_n\frac{\Delta\delta_{1,max}}{\Delta\delta_1} \quad (5)$$

Job-plot analysis

Equimolar solutions of host and guest compound (each approximately 10 mmol/10 ml) were prepared and mixed in various portions, so that the sum of the portions is constant for each mixture. ^{1}H NMR spectra of the mixtures were recorded, and the chemical shifts were analyzed by Job's method modified for NMR spectroscopy.

Fluorescence measurements

The fluorescence spectra were measured in Jasco spectrofluorometer FP-6500 at 25 °C. The fluorometric titration with NAD^+ **2** as guest molecule was conducted by keeping the concentration of clip **1b** or **1c** as host molecule constant and varying of the NAD^+ concentration. Each sample was excited at 280 nm and the fluorescence intensity was monitored at 340 nm. The association constants, K_a, were determined from the fitting of the data pairs – ΔI_{obs} and $[G]_0/[H]_0$ – to the binding isotherm given in Eqn (6):

$$I = I_0+\frac{\Delta I}{[G]_0}\left(\frac{[H]_0+[G]_0+1/K_a}{2}-\sqrt{\frac{([H]_0+[G]_0+1/K_a)^2}{4}-[H]_0[G]_0}\right) \quad (6)$$

Isothermal titration calorimetry (ITC) measurements

ITC measurements were carried out by the use of a Microcal Calorimeter at 25.0 °C (VP-ITC Microcalorimeter; MicroCal).

Computational details

The *ab initio* calculations have been carried out with a development version of the Q-Chem program package.[27] NMR chemical shifts were calculated using the GIAO-HF Scheme[28–30] with the SVP basis set,[31] employing the linear-scaling density matrix-based GIAO-HF method (D-GIAO-HF) described in References [21,32,33]. Relative chemical shifts were obtained by referring to a TMS molecule calculated at the same level of theory. Structures were optimized by force-field (Monte-Carlo conformer search employing structures calculated by AMBER*/H_2O, MacroModel),[19,20] which typically provides a good starting point for the calculation of NMR chemical shieldings in the present systems, as it was demonstrated in previous work on a simple model system.[34] All binding energies were calculated using the RI-MP2 method (refer to References [35,36] and references therein) with the SVP basis set.

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chloroform. An independent study with the lithium methanephosphonate **clip 1b** as host molecule and tetra-*n*-butylammonium bromide as guest molecule showed that 1b forms a (1:1) complex with the tetra-*n*-butylammonium cation ($K_a = 3800\ M^{-1}$).

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