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The self-association of flavin mononucleotide (FMN^{2-}) as determined by ^{1}H NMR shift measurements

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Abstract

The concentration dependence of the ^{1}H NMR chemical upfield shifts of the protons H6, H9, H7α, and H8α of the 7,8-dimethylisoalloxazine residue of flavin mononucleotide (FMN^{2-}) has been measured and the self-stacking tendency of FMN^{2-} was quantified with the isodesmic model of indefinite non-cooperative self-association. The stacking tendency of FMN^{2-} is considerable and described in the concentration range of 0.0025–0.1 M with the indicated model by $K = 27 \pm 15\ M^{-1}$ (25°C; $I = 0.1$–0.3 M). This result is compared with related ones from the literature. The caveats regarding the self-stacking properties of FMN^{2-} and their dependence on the concentration are discussed. © 1997 Elsevier Science B.V.

Keywords: Self-association constants; Concentration effects on self-stacking; Isoalloxazine residue; Isodesmic model for indefinite non-cooperative self-association; Self-stacking

Abbreviations and Definitions: I, ionic strength of a solution; M^{2+}, general divalent metal ion. Species which are given in the text without a charge either do not carry one or represent the species in general (i.e. independent from their protonation degree); which of the two versions applies is always clear from the context. The expression 'protonation' is used throughout this study for the addition of H^{+} or D^{+} ($=^{2}H^{+}$) to a basic site, i.e. independent of the kind of hydrogen isotope. However, which isotope is considered in a given equilibrium is always clearly defined.

1. Introduction

Flavoenzymes, often being metal ion-dependent, catalyze redox reactions [1,2] via the 7,8-dimethylisoalloxazine (dmia) residue; in fact, they are often at the crossroads of such events [3] and consequently intensively studied (see, e.g. the reviews [2–4]). One of the flavo-coenzymes which occurs in a large number of proteins, is flavin mononucleotide (FMN^{2-}), also known as riboflavin 5′-phosphate. FMN is composed of the 7,8-dimethylisoalloxazine (i.e., the flavin) ring system, which is bound via N10 to the methylene group of the sugar-related ribit giving 7,8-dimethyl-10-ribityl-isoalloxazine, also known as riboflavin or vitamin B_2 [1], which carries at its 5′ position a phosphate monoester residue (see Fig. 1).

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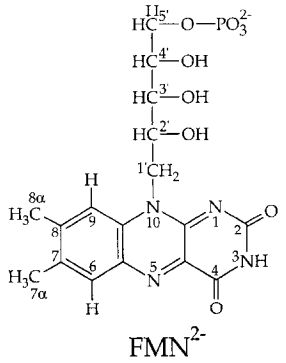

Fig. 1. Chemical structure of flavin mononucleotide (= FMN^{2-} = riboflavin 5′-phosphate).

Considering the flat nature [5] of the isoalloxazine ring system it is not surprising that the flavin moiety and aromatic residues of substrates or proteins undergo charge-transfer or stacking interactions [2,6]. Furthermore, the tendency of FMN [7,8] and of related compounds [9–11] to self-associate is also known. The latter phenomenon needed careful attention in our studies devoted to the stability and solution structure of binary M(FMN) complexes [12]. Of course, the same is even more true in studies on ternary or mixed-ligand M(Arm)(FMN) complexes, where Arm represents a heteroaromatic nitrogen base, like 1,10-phenanthroline [13], where intramolecular ligand–ligand stacking is expected [14].

For studies of metal ion complexes of the mentioned kind one has to ascertain that under the experimental conditions employed FMN^{2-} is present in its monomeric state. As the available constants for the self-association of FMN vary widely [7,8], we decided to study ourselves the self-stacking tendency of FMN^{2-} by ^{1}H NMR shift measurements; a method we had used before in connection with the self-stacking of nucleotides [10,11,15,16]. Indeed, the results show that the self-stacking tendency of FMN^{2-} is remarkable.

2. Experimental

2.1. Materials

The monosodium salt of riboflavin 5′-phosphate (FMN; pure) was from Serva Feinbiochemica GmbH, Heidelberg, Germany. Tetramethylammonium nitrate was from Fluka AG, Buchs, Switzerland. D_2O (99.8% D), NaOD (99.9% D), and DNO_3 (99% D) were from Ciba–Geigy AG, Basel, Switzerland. The buffers used for pH calibration (pH 4.64, 7.00, and 9.00; based on the NBS scale, now NIST) were from Metrohm AG, Herisau, Switzerland.

2.2. Apparatus and measurements

The ^{1}H NMR spectra of FMN were recorded with a Varian VXR 400 spectrometer (399.96 MHz) at 25°C in D_2O using the center peak of the tetraethylammonium ion triplet as internal reference (2.5 mM) (for details and justification see [15,16]). However, all measured chemical shifts were converted to the sodium 3-(trimethylsilyl)propane-1-sulfonate reference by adding 3.174 ppm ([11,17]; see also [18]). The pD of the solutions was measured with a Metrohm EA 125 glass electrode connected with a Metrohm 654 digital pH meter (Metrohm AG, Herisau, Switzerland); the final pD of the D_2O solutions was obtained by adding 0.40 to the pH meter reading [19,20].

Two sets of experiments were carried out, one at pD 8.60 and the other at 9.58 and for each concentration employed two independent measurements were made. The FMN concentration varied between 0.0025 M and 0.2 M (see Fig. 2, vide infra). The pD of each solution was adjusted by dotting with a glass rod using concentrated NaOD (or DNO_3) to the desired value. The NMR spectrum was immediately recorded thereafter, which took about 30 minutes, and then the pD of the solutions was measured again; the changes were within pD $\pm$ 0.05. The ionic strength (I) of the solutions was adjusted with $NaNO_3$ to 0.1 M when necessary; the ionic strength at [FMN] = 0.1 and 0.2 M corresponds to $I = 0.3$ and 0.6 M, respectively.

The assignment of the methyl protons of FMN in aqueous solution is unequivocal [21,22] whereas that of the aromatic protons H6 and H9 is somewhat ambiguous [22]. The assignments of Kainosho and Kyogoku [22] contrast with those of Sarma et al. [23] and Kotowycz et al. [24]; we followed the arguments of Kainosho and Kyogoku [22] and used their assignments, which means that the order of the peaks in relatively concentrated aqueous solutions is inverted

compared with those of lumiflavin [25] and riboflavin [26] in trifluoroacetic acid in which the intermolecular association of the bases is diminished [22]. At high temperature [24] or at infinite dilution the two peaks become practically identical [22,23], an observation which we also made (see Table 1, vide infra). However, it needs to be emphasized that the result regarding the self-association constant of FMN^{2-} is independent of any peak assignment (see also Section 3.2).

All experimental data were analyzed by using an IBM compatible desk computer (with a 80486 processor) connected to a Hewlett-Packard 7475 A plotter and a Brother M 1509 printer by using a Newton–Gauss nonlinear least-squares method. The results were calculated with a curve-fit program based on the isodesmic model of indefinite non-cooperative self-association (see Eqs. (1)–(3), vide infra).

3. Results and discussion

3.1. Evaluation procedure and conditions for the ^1H NMR shift experiments

As previously shown [10,11,15–17,20,27], ^1H NMR shift measurements are ideal to characterize the self association of nucleosides and their derivatives (N): upfield shifts of the resonances of nucleobase protons observed with increasing concentration of N confirm stack formation and allow a quantitative evaluation of the extent by employing the isodesmic model for indefinite non-cooperative self-association [15,28]. This model is based on the assumption that, e.g., for a nucleoside or derivative the equilibrium constants (Eq. (1)) for the Equilibria (2) are all equal:

$$K = [N_{n+1}]/([N_n][N]) \quad (1)$$

$$N_n + N \rightleftharpoons N_{n+1} \quad (2)$$

Expression (3) gives the relationship between the observed chemical shift (δ_{obs}) and a solution of the total concentration [N]:

$$\delta_{obs} = \delta_\infty + \frac{(\delta_\infty - \delta_0)\left[1 - (4K[N] + 1)^{0.5}\right]}{2K[N]} \quad (3)$$

In Eq. (3) δ_0 represents the shift at infinite dilution (free monomeric N) and δ_∞ the shift of a molecule in an infinitely long stack, while K is the association constant as defined in Eq. (1) (for further details see, e.g., [11b,20]; for a critical evaluation of the isodesmic model see [29]).

As the model described had been successfully applied previously to a heat of dilution study of the self-association of FMN [7] and because it is also known that upon self-association of FMN the expected upfield shifts are actually observed [8], we decided to carry out ^1H NMR shift measurements with FMN^{2-} and to apply Eqs. (1)–(3). Such measurements need to be made in D_2O and for well defined conditions it is necessary to take the acidity constants of $H(FMN)^-$, as defined below, into account:

$$H(FMN)^- \rightleftharpoons FMN^{2-} + H^+ \quad (4a)$$

$$K^H_{H(FMN)} = [FMN^{2-}][H^+]/[H(FMN)^-] \quad (4b)$$

$$FMN^{2-} \rightleftharpoons (FMN\text{–}H)^{3-} + H^+ \quad (5a)$$

$$K^H_{FMN} = [(FMN - H)^{3-}][H^+]/[FMN^{2-}] \quad (5b)$$

The corresponding acidity constants have recently been measured [12] by potentiometric pH titrations in aqueous solution (25°C; $I = 0.1$ M, $NaNO_3$): $pK^H = 6.18 \pm 0.1$ and $pK^H = 10.08 \pm 0.05$. The first of these two constants refers to the release of the proton from the monoprotonated phosphate residue in $H(FMN)^-$ whereas the second one is due to the deprotonation of the H(N3) site (see Fig. 1) of the flavin residue in FMN^{2-} [12]. The third value, $pK^H_{H_2(FMN)} = 0.7 \pm 0.5$, also determined recently [12], quantifies the release of the first proton from the phosphoric acid residue in $H_2(FMN)$; as this value is so low, i.e. at pH > 3 only $H(FMN)^-$ exists, it is without consequences for the present study and not considered further.

The acidity constants given above are valid for monomeric FMN species and refer to water as solvent. They can be transformed with the relation given in Eq. (6) [30],

$$pK_{a/D_2O} = 1.015pK_{a/H_2O} + 0.45 \quad (6)$$

which proved to give excellent results [11c,27,31], into the corresponding acidity constants valid now for D_2O as solvent. Application of Eq. (6) gives $pK^D_{D(FMN)} = 6.72$ and $pK^D_{FMN} = 10.68$ ($I = 0.1$ M;

25°C). These results show that the buffer regions are practically not overlapping and that FMN^{2-} reaches its maximum concentration in D_2O at pD 8.70.

3.2. Results regarding the self-association of FMN^{2-}

The ^1H NMR shift experiments carried out with FMN^{2-} in D_2O at pD 8.60 are shown in Fig. 2: the variation of the upfield shifts for the aromatic protons H6 and H9 as well as for the methyl protons H7α and H8α (see Fig. 1) as a function of the FMN^{2-} concentration is clearly seen. The 'curvature' of the series of the experimental data points immediately demonstrates without any mathematical

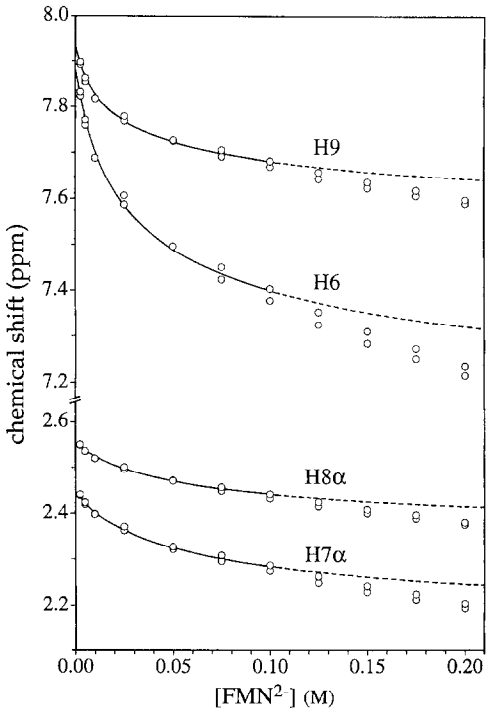

Fig. 2. Variations of the chemical shifts, δ, of H9, H6, H8α and H7α (from top to bottom) of FMN^{2-} (see Fig. 1) with increasing concentrations of FMN^{2-} in D_2O solutions at pD 8.60. The spectra were measured on a Varian VXR 400 spectrometer at 399.96 MHz, relative to internal $(CH_3)_4N^+/NO_3^-$ and converted to values relative to sodium 3-(trimethylsilyl)propane-1-sulfonate by adding 3.174 ppm. The curves shown are the computer-calculated best fit of the experimental data in the $[FMN^{2-}]$ range of 0.0025 to 0.10 M (I = 0.1–0.3 M; 25°C), calculated with K_{av} = 27 M^{-1} of Table 1 (see also Section 3.2), using the indefinite non-cooperative stacking model (Eqs. (1)–(3)); the resulting shifts are listed in Table 1.

evaluation that self-stacking occurs. Application of the isodesmic model for an indefinite non-cooperative self-association (Eqs. (1)–(3)) is somewhat hampered by two observations: (i) The evaluation of the chemical shifts of the aromatic and methyl protons leads to somewhat different values for the association constant according to Equilibrium (2). (ii) If one evaluates the whole concentration range measured, i.e. $[FMN^{2-}]$ = 0.0025–0.20 M, and deletes then the measured data at 0.2 M, next at 0.175 M, etc., the association constant increases systematically; this observation is made, despite the fact that the computer-calculated best fit of the experimental data is always very satisfactory.

The difficulty (ii) resolves itself to a large part because within the concentration range $[FMN^{2-}]$ = 0.0025–0.10 M relatively constant values for the association constants are observed (but see also Section 3.3). This means K still increases somewhat but the error limits of the various results for K are overlapping. Therefore, we used the mentioned concentration range for our final evaluation and ignored the data points at $[FMN^{2-}] > 0.1$ M; from Fig. 2 it is evident that these data points fall now below the calculated curves (which are computer-drawn with K_{av}; see below). The results obtained from this evaluation for the experiments at pD 8.60 are summarized in the upper part of Table 1.

The value given in Table 1 for the association constant (Eq. (1)) of the self-association of FMN^{2-} according to Equilibrium (2), i.e. $K_{av} = 27 \pm 15\ M^{-1}$, carries a relatively large error (for details see Table 1) which is evidently due to the poor agreement of the individual results obtained for the aromatic and methyl protons. On the other hand it needs to be emphasized that the computer-calculated best fit of the experimental data with a non-linear least-squares algorithm based on Eq. (3) and employing the result $K_{av} = 27\ M^{-1}$ is very satisfactory for the FMN concentration range between 0.0025 and 0.10 M for *all* protons, i.e. H6, H9, H7α, and H8α, as may be seen from the computer-drawn curves in Fig. 2. This result, together with the following one, then takes care of the problem mentioned above under point (i).

As the 'curvature' of the series of the experimental data points, i.e. the extent of the upfield shifts, for the protons H6 and H9 differs from that for H7α and

Table 1
Chemical shifts (ppm) of the protons of the flavin residue of monomeric (δ_0) and self-stacked (δ_∞) FMN^{2-} in D_2O at pD 8.60 and 9.58, together with the corresponding upfield shifts ($\Delta\delta = \delta_0 - \delta_\infty$) and the association constants, K (M^{-1}; Eq. (1)), calculated for the individual protons, resulting in the average constants K_{av} (M^{-1}; Eq. (1)) (25°C; I = 0.1 M ($NaNO_3$) to 0.3 M)[a]

pD	H	δ_0 (ppm)	δ_∞ (ppm)	$\Delta\delta$ (ppm)	K (M^{-1})	K_{av} (M^{-1})
8.60[b]	H6	7.863 ± 0.051	7.01 ± 0.17	0.85 ± 0.18	36.27 ± 8.13	27 ± 15
	H9	7.908 ± 0.027	7.49 ± 0.09	0.42 ± 0.09	48.96 ± 12.14	
	H7α	2.458 ± 0.019	2.15 ± 0.07	0.31 ± 0.07	17.23 ± 4.36	
	H8α	2.562 ± 0.014	2.34 ± 0.05	0.22 ± 0.05	15.93 ± 4.12	
9.58[c]	H6	7.814 ± 0.043	6.88 ± 0.24	0.93 ± 0.24	20.26 ± 6.43	15 ± 8
	H9	7.872 ± 0.024	7.39 ± 0.13	0.48 ± 0.13	22.20 ± 8.61	
	H7α	2.442 ± 0.017	2.09 ± 0.09	0.35 ± 0.10	7.72 ± 3.34	
	H8α	2.548 ± 0.013	2.30 ± 0.07	0.25 ± 0.07	9.11 ± 4.27	

[a] The range of error given with the values for K of the individual protons corresponds to the standard deviation (1σ). K_{av} is the weighted mean of the individual results calculated via log K; the range of error given here is *twice* the standard error (2σ); this is also true for the values of δ_0, δ_∞ and $\Delta\delta$ which were calculated with K_{av}.
[b] Evaluation of the experimental series shown in Fig. 2.
[c] At pD 9.58 about 7.5% of the FMN^{2-} species have lost their proton from the H(N3) site (see Fig. 1) and are thus present as $(FMN\text{-}H)^{3-}$ (see Section 3.2).

H8α (see Fig. 2), it is possible to evaluate also the shift differences, $\Delta\delta^*_{H6-H9} = \delta(H6) - \delta(H9)$ and $\Delta\delta^*_{H7\alpha-H8\alpha} = \delta(H7\alpha) - \delta(H8\alpha)$, in dependence on [FMN]. The association constants obtained are $K_{H6-H9} = 27.45 \pm 5.60$ (1σ) and $K_{H7\alpha-H8\alpha} = 20.06 \pm 7.61$ (1σ), respectively. Similar evaluations can also be made for other shift differences, but these $\Delta\delta^*$ values vary usually less in dependence on [FMN] and consequently less reliable results are obtained. Overall, the evaluation of the $\Delta\delta^*$ values is very satisfying; both given results are identical within their error limits with $K_{av} = 27 \pm 15\,M^{-1}$ of Table 1. In fact, if the weighted mean (via log K) is recalculated by using the four individual values of Table 1 plus the two values given above, one obtains $K^*_{av} = 27 \pm 10\,M^{-1}$ (2σ), which confirms the result of Table 1.

At this point one should add that the extent of the upfield shift, $\Delta\delta = \delta_0 - \delta_\infty$, for the various protons, especially the aromatic ones, is higher than expected for the shift due to a single adjacent molecule, as in the dimer. This confirms the assumption initially made (Eqs. (1)–(3)) that stacking proceeds beyond the dimer stage; a conclusion reached also already earlier for nucleotides [11,15,28c,32].

The results of a further set of experiments at pD 9.58 are listed in the lower part of Table 1. The values for δ_0, δ_∞, and $\Delta\delta$ are within their error limits identical with those obtained at pD 8.60, but the association constant, $K_{av} = 15 \pm 8\,M^{-1}$, appears to be somewhat smaller (though within the error limits of 2σ it is still identical with $K_{av} = 27 \pm 15\,M^{-1}$). Indeed, if the evaluations of $\Delta\delta^*_{H6-H9}$ and $\Delta\delta^*_{H7\alpha-H8\alpha}$ are also considered in the way described above and included into the results, then for pD 9.58 $K^*_{av} = 13 \pm 8\,M^{-1}$ (2σ) is obtained; a result identical within the error limits with the one given in the lower part of Table 1, but still more different from $K^*_{av} = 27 \pm 10\,M^{-1}$ valid at pD 8.60. What is the reason for this trend? Recalling the acidity constant $pK^D_{FMN} = 10.68$ given in Section 3.1, it is evident that at pD 9.58 already about 7.5% of the total FMN are deprotonated at N3 and exist as $(FMN\text{-}H)^{3-}$; i.e., in these species the flavin ring carries now a negative charge and this should give rise to repulsion within a stack—and this it does, as the trend in the above results indicates. Hence, we may generally conclude that deprotonation of the isalloxazine residue diminishes self-stacking of FMN^{2-}.

3.3. A caveat regarding the self-association of FMN

That the charge of the species which undergo stacking does affect the stability of a stack is evident from the result given at the end of the last section and it is also confirmed by the heat of dilution

studies of Medina de Gonzalez and Langerman [7]: These authors applied the isodesmic model and obtained at $I = 0.5$ M (25°C) in aqueous solution at pH 4.96, 7.00, and 8.98 the association constants $K = 339$, 410, and 187 M^{-1}, respectively. Considering the acidity constants given in Section 3.1 for $H(FMN)^-$ in aqueous solution this means that their association constants hold for the self-stacking of $H(FMN)^-$ (present to about 95% at pH 4.96), FMN^{2-} (present to 87% at pH 7.00) and for a mixture of about 92.5% FMN^{2-} with 7.5% $(FMN-H)^{3-}$ at pH 8.98, respectively; though the partial charge neutralization at the remote phosphate moiety does not become manifest, some deprotonation of the H(N3) site again immediately diminishes the self-stacking considerably.

However, all given association constants of Medina de Gonzalez and Langerman [7] are significantly larger than our value of $K_{av} = 27 \pm 15\ M^{-1}$ (I = 0.1 M; 25°C; Table 1) which is probably best compared with $K = 365\ M^{-1}$ ($I = 0.12$ M; 25°C) determined at pH 7.00 [7], if the experimental conditions are taken into account. The latter value is by a factor of more than 10 higher. Similarly, the constant given by Kharasch and Novak [8], $K = 140\ M^{-1}$, which appears to hold for the (2 monomer $\rightleftharpoons$ dimer) equilibrium, and which was determined by ^1H NMR shift measurements at 28°C in D_2O at pD 7.9 ($pH_{obs} = 7.5$ [8]) in 0.1 M phosphate buffer (i.e. $I \sim 0.25$ M based on $pK^{D}_{D_2PO_4} = 7.30$ as calculated [30] from $pK^{H}_{H_2PO_4} = 6.75$ [33]), seems also larger. As the evaluation process in [8] is equivocal, we enlarged fig. 2 of Ref. [8] with the experimental data and applied to these read-out data our evaluation method (Eqs. (1)–(3)) and obtained $K_{av} = 91 \pm 47$ (2σ); if one deletes the point due to the experiment with [FMN] = 0.001 M the result is $K_{av} = 78 \pm 34$ (2σ). Indeed, both these values are also larger than our constant.

The only reason for the discrepancies of these results can be discovered in the different concentration ranges of FMN applied in the experiments: in the heat of dilution study [7] the total FMN concentration varied from 0.0006 to 0.02 M and in the ^1H NMR shift experiments of [8] from 0.001 to 0.025 M. Hence, we carried out a further evaluation of our own NMR data for [FMN] = 0.0025 to 0.025 M (8 data points with 4 different concentrations; see Fig. 2) and obtained now a value for K_{av} which is within the error limits identical with the one we calculated from the data of Kharasch and Novak [8]. Consequently, the discrepancy between the NMR results is a matter of the concentration range employed and as far as heat of dilution studies are concerned, it has been concluded recently [29] that they have the tendency to give too large association constants.

It may be added that experimental difficulties [34] as well as differences due to the evaluation method [9c] used have been encountered before in attempts to quantify stacking associates (see also [29]). In any case, that self-stacking is quite pronounced with FMN^{2-} is apparent from Fig. 3, which was constructed with $K_{av} = 27\ M^{-1}$, where the formation degree of various stacked species in dependence on concentration is seen. In a solution with a total FMN^{2-} concentration of 0.05 M only about 30% is actually present in the monomeric form.

To conclude, considering all the indicated difficulties one has to admit that the value for the association constant of FMN^{2-} is poorly defined; depending on the FMN concentration considered, the association constant K according to Eq. (1) is most likely somewhere between 20 and 100 M^{-1}. At this point it may be added that application of the various models (including the "attenuated association constant" (AK) model) discussed and summarized in

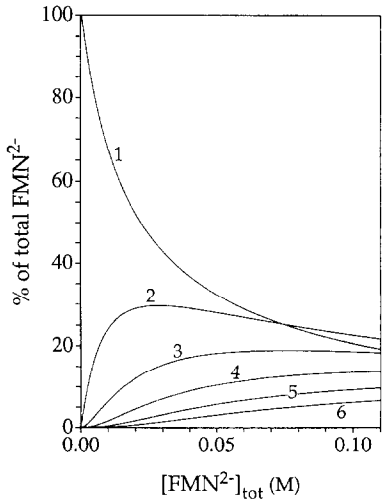

Fig. 3. Variation of the proportions of FMN^{2-} present in the monomer (1), dimer (2), trimer (3), tetramer (4), pentamer (5), and hexamer (6) in D_2O solutions at pD 8.60 as a function of the total concentration of FMN^{2-}; calculated with $K_{av} = 27\ M^{-1}$ (Table 1) for 25°C and $I = 0.1$ M ($NaNO_3$) to 0.3 M.

Ref. [29] do not resolve the indicated difficulty, i.e., that the result for K depends somewhat on the concentration range used for the evaluation.

However, the above mentioned result that K is between 20 and $100\,M^{-1}$ (Eq. (1)) still allows the conclusion that FMN^{2-} self-stacks much better than, e.g., 5′-AMP^{2-} ($K = 2.1 \pm 0.3\,M^{-1}$) [20,27]. Deletion of the phosphate group in 5′-AMP^{2-} gives the uncharged adenosine, a molecule that stacks considerably better ($K = 15 \pm 3\,M^{-1}$) [27], though not as well yet as the flat 1,10-phenanthroline ($K = 31.1 \pm 3.4\,M^{-1}$) [17]. Finally, why is the self-association of FMN^{2-} so difficult to quantify? The evident explanation is that with an increasing size of the associates the individual molecules in the stacks change their orientation and consequently also the intensity of their interactions; e.g., one could imagine that at low concentrations the flat flavin moieties are on top of each other with a maximum overlap of the rings, while at higher concentrations a part of the flavin residues may rotate into a more perpendicular orientation toward each other forming cross-like stacks. This assumption would also explain why the methyl protons sense a smaller extent of self-association than the protons at the aromatic ring. The latter will always be closer to the next neighbour(s) independent of the orientation of the flavin moieties toward each other.

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