

The Crystal Structure of a Flavin–Nicotinamide Biscoenzyme in Two Oxidation States. Models for Flavin–Nicotinamide Interactions

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Crystals of the fully oxidized propyl-linked flavin–nicotinamide biscoenzyme $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+ \cdot 7\text{H}_2\text{O}$, $\text{C}_{21}\text{H}_{34}\text{N}_6\text{O}_{10}$, have the space group $P2_1/c$ with unit-cell parameters $a = 7.740(3)$, $b = 23.800(12)$, $c = 13.958(5)$ Å and $\beta = 101.93(3)^\circ$ with four formula units per unit cell. Crystals of the biscoenzyme in which the flavin moiety is fully reduced, $(\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+) \text{NO}_3^- \cdot 4\text{H}_2\text{O}$, $\text{C}_{21}\text{H}_{30}\text{N}_7\text{O}_{10}$, are red in color and have the space group $P1$ with unit-cell parameters $a = 8.068(3)$, $b = 11.369(3)$, $c = 14.170(7)$ Å, $\alpha = 77.89(3)$, $\beta = 88.99(4)$ and $\gamma = 89.12(3)^\circ$ with two formula units per unit cell. Intensity data were measured with an automated diffractometer using Cu $K\alpha$ radiation. Both structures were solved by direct methods and were refined by least-squares procedures to final R factors of 0.064 based on 3318 observed reflections for $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+ \cdot 7\text{H}_2\text{O}$ and 0.069 based on 2963 observed reflections for $(\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+) \text{NO}_3^- \cdot 4\text{H}_2\text{O}$. In both structures the biscoenzymes are in extended conformations. The flavin residues stack in infinite columns of centrosymmetrically related molecules in which there are no interatomic contacts that are less than van der Waals distances. The reduced flavin moiety of $\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+$ has an unexpectedly small bending angle of 12.7° . The nicotinamide residues in both biscoenzyme structures extend alternately to opposite sides of the flavin stacks. In both structures the flavin and nicotinamide residues associate through distorted hydrogen-bonding interactions.

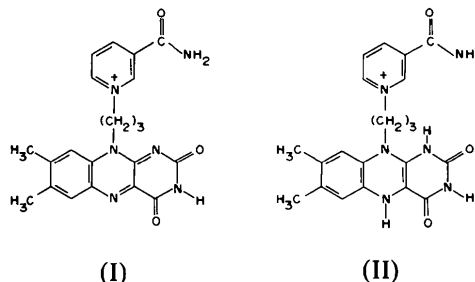
Introduction

The reduction of an enzyme-bound flavin by reduced nicotinamide adenine dinucleotide (NADH) is a primary step in a number of important biological oxidation–reduction processes such as electron transport. Interactions between flavin and nicotinamide species of differing oxidation states have been described in a number of flavoenzyme-catalyzed reactions (Massey & Ghisla, 1974).

The use of flavin–nicotinamide biscoenzyme model compounds has led to the characterization of flavin/nicotinamide interactions in solution as charge-transfer complexation (Proffitt, Ingraham & Blankenhorn, 1974). Furthermore, kinetic and spectral data have indicated that a kinetically important charge-transfer complex between reduced nicotinamide and oxidized flavin occurs prior to the reduction of flavins (Porter, Blankenhorn & Ingraham, 1973; Blankenhorn, 1975*a,b*, 1976*a,b*; Bruce, Main, Smith & Bruce, 1971). The geometry of this complex has been

proposed to be one in which the two-ring systems are face-to-face such that the nicotinamide C(4) atom is opposite the flavin C(5) atom (Blankenhorn, 1975*a*).

The present study was undertaken in order to visualize the associations between nicotinamide and flavin in their various oxidation states. In doing so we have elucidated the structures of two propyl-linked flavin–nicotinamide biscoenzymes. The first of these, $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$, in which both coenzyme moieties are fully oxidized, is generated by the ionization of the relatively acidic flavin N(3) proton from 10-[3-(3-carbamoyl-1-pyridinio)propyl]-7,8-dimethyliso-alloxazine [$\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ (I)]. The second biscoenzyme, 10-[3-(3-carbamoyl-1-pyridinio)propyl]-1,5-dihy-



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dro-7,8-dimethylisoalloxazine [$\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+$ (II)] is generated from $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+$ by the selective reduction of its flavin moiety. These compounds exhibit intermolecular associations that are of potential biological significance.

A preliminary account of this work has previously appeared (Porter, Bright & Voet, 1977).

Experimental

(i) $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+ \cdot 7\text{H}_2\text{O}$

($\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+$) Br^- was synthesized as has been previously described (Proffitt, Ingraham & Blankenhorn, 1974; Blankenhorn, 1975*a*). The product, which yielded a single fluorescent spot on TLC (solvent: 30% 1*M* ammonium acetate at pH 7 and 70% ethanol) had the same melting point and visible and ultraviolet spectra that had been reported previously for this compound (Blankenhorn, 1975*a*).

Needle-shaped yellow crystals were grown by the vapor equilibration of a 10 *mM* aqueous NH_3 solution with a 5 *mM* ($\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+$) Br^- solution. Chromatography of these crystals on TLC plates followed by staining with AgNO_3 failed to reveal the presence of bromide ion. Hence the crystals contain the zwitterionic species $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+$ which is derived from $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+$ by the ionization of the relatively acidic N(3) position ($\text{pK} \sim 10$) of the flavin.

A crystal measuring $0.16 \times 0.13 \times 0.62$ mm was sealed inside a thin-walled glass capillary tube to prevent the observed disintegration of the crystals upon exposure to air (presumably due to loss of solvent of crystallization). Preliminary oscillation and Weissenberg photographs of the crystal revealed that it had monoclinic lattice symmetry. The systematic absences of the $h0l$ reflections for l odd and the $0k0$ reflections for k odd indicate that the space group of the crystal is $P2_1/c$. All subsequent X-ray measurements were made

on a Picker FACS-I diffractometer equipped with a graphite monochromator and employing $\text{Cu } K\alpha$ radiation ($\lambda = 1.5418 \text{ \AA}$). The unit-cell parameters, as calculated from the angular positions of 12 reflections, are presented in Table 1.

The crystal density of 1.392 g cm^{-3} , as determined by flotation in a CCl_4 -cyclohexane mixture, is in excellent agreement with the stoichiometry of the asymmetric unit being $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+ \cdot 7\text{H}_2\text{O}$.

3806 unique X-ray reflections were measured to the limit $2\theta = 125^\circ$ with the θ - 2θ scan mode, a scan rate of 2° min^{-1} and a basic scan width of 1.75° . Stationary background counts of 20s duration each were taken at both limits of each scan. The three standard reflections that were monitored after every 50 reflections showed no significant decay over the course of the data-collection process.

(ii) $(\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+)\text{NO}_3^- \cdot 4\text{H}_2\text{O}$

A solution containing 50 μmol of ($\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+$) Br^- and 250 μmol of dithiothreitol in 5 ml of water was placed in a vial, adjusted to pH 8.0 with KOH and deoxygenated with 99.999% argon. The vial was then capped and placed in an anaerobic glove box where all further chemical manipulations were performed. As the flavin was selectively reduced by the dithiothreitol the solution changed color from the yellow characteristic of oxidized flavins to dark bluish-black. After a day, the zwitterionic species $\text{HFl}_{\text{red}}-\text{C}_3-\text{Nic}^+$ had precipitated as black spherical clumps of crystallites that were unsuitable for X-ray study. These were collected and dissolved, at about 50°C , in a minimal amount of 50 *mM* HNO_3 to form a dark-brown solution with pH 1.6. After several days, red plate-like crystals appeared. These crystals are stable in air for several minutes but longer exposures result in their disintegration and the return of the yellow color of oxidized flavins.

A crystal measuring $0.75 \times 0.08 \times 0.12$ mm was sealed inside a thin-walled glass capillary tube together with a small amount of 0.1 *M* $\text{Na}_2\text{S}_2\text{O}_4$ to act as an oxygen scavenger. Preliminary oscillation, Weissenberg and precession photographs revealed that the crystal had triclinic lattice symmetry. The statistical distribution of the subsequently measured diffraction data indicated the presence of a center of symmetry (Howells, Phillips & Rodgers, 1950) and hence the space group of the crystal was assumed to be $P\bar{1}$. This was eventually confirmed by the successful refinement of the structure.

All subsequent X-ray measurements were made on a Picker FACS-I diffractometer equipped with a graphite monochromator and employing $\text{Cu } K\alpha$ radiation. The unit-cell parameters, as determined from the angular positions of 12 reflections, are presented in Table 1. The crystal density of 1.405 g cm^{-3} , as determined by

Table 1. *Crystal data*

(1) $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+ \cdot 7\text{H}_2\text{O}$ $\text{C}_{21}\text{H}_{20}\text{N}_6\text{O}_3 \cdot 7\text{H}_2\text{O}$	Formula weight 530.6 Space group $P2_1/c$ $Z = 4$ $d_o = 1.392 \text{ g cm}^{-3}$ $d_c = 1.401$ $F(000) = 1128$
(2) $(\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+)\text{NO}_3^- \cdot 4\text{H}_2\text{O}$ $(\text{C}_{21}\text{H}_{22}\text{N}_6\text{O}_3)^+\text{NO}_3^- \cdot 4\text{H}_2\text{O}$	Formula weight 540.5 Space group $P\bar{1}$ $Z = 2$ $d_o = 1.405 \text{ g cm}^{-3}$ $d_c = 1.413$ $F(000) = 570$ $V = 1270.5 \text{ \AA}^3$
$a = 7.740 (3) \text{ \AA}$ $b = 23.800 (12)$ $c = 13.958 (5)$ $\beta = 101.93 (3)^\circ$ $V = 2515.7 \text{ \AA}^3$	
$a = 8.068 (3) \text{ \AA}$ $b = 11.369 (3)$ $c = 14.170 (7)$ $\alpha = 77.89 (3)^\circ$ $\beta = 88.99 (4)$ $\gamma = 89.12 (3)$	

flotation in a CCl_4 -cyclohexane mixture, is in excellent agreement with the contents of the asymmetric unit of the unit cell being $(\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+)\text{NO}_3^- \cdot 4\text{H}_2\text{O}$. 4076 unique X-ray reflections were measured with the same procedures and limits described above for the crystal of $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+ \cdot 7\text{H}_2\text{O}$.

The intensities of the $\bar{1}1\bar{4}$, 023 and $\bar{2}\bar{2}1$ reflections, which were monitored after every 50 reflections, dropped throughout the data collection process to reach final values of 83, 81 and 82%, respectively, of their initial values. The decay of the three standard reflections followed each other closely. It was therefore assumed that this decay was isotropic. Accordingly, the reflection intensities were corrected for radiation damage with a second-order polynomial that related the sequence in which the reflections were measured to the average remaining fraction of the initial intensities of the three standard reflections.

Structure determination and refinement

In both structures the measured intensities, I , were corrected for Lorentz and polarization effects, Lp . No absorption corrections were made. Standard deviations, $\sigma(I)$, were calculated according to counter statistics (Stout & Jensen, 1968) with an instrumental instability factor of 0.03 assumed for both structures. 488 reflections for $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ and 1113 reflections for $(\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+)\text{NO}_3^-$ had $I < 2.33\sigma(I)$ and were therefore considered to be unobserved.

Both structures were solved uneventfully with the direct-methods program *MULTAN* (Main, Woolfson & Germain, 1971). All thirty non-H atoms of the $\text{Fl}-\text{C}_3-\text{Nic}^+$ skeleton appeared as the highest peaks in the E maps based on those reflections with $|E| > 1.5$ for both structures.

Fourier maps based on the positions of the 30 $\text{Fl}-\text{C}_3-\text{Nic}^+$ non-H atoms revealed the presence of seven water O atoms in the case of $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ and four water O atoms and a NO_3^- ion in the case of $(\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+)\text{NO}_3^-$. (Throughout this report the solvent molecules in the $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ structure will be designated as water. However, we have not been able to completely rule out the possibility that some of them may be NH_3 because, as will be seen below, the H atoms about the solvent molecules could not all be located.)

Both structures were refined by full-matrix least-squares procedures in which the quantity minimized was $\sum w(|F_o| - |F_c|)^2$ where the relative weighting factor was $w = ILp/\sigma^2(I) = 1/\sigma^2(F_o)$. The atomic scattering factors for non-H atoms were taken from Cromer & Waber (1965) and those for H atoms were taken from Stewart, Davidson & Simpson (1965).

The variation of the scale factor, the atomic positions and the isotropic thermal parameters reduced the R factor from its initial value 0.564 to 0.135 in the case of $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ and from 0.351 to 0.165 for $\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+$. These were followed by similar refinements in which the temperature factors were refined anisotropically. The difference Fourier map at this stage for the $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ structure revealed the presence of all non-solvent H atoms and eight of the expected fourteen solvent H atoms. Similarly, the difference Fourier map for $(\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+)\text{NO}_3^-$ indicated the presence of all non-solvent H atoms except two each at the $\text{C}(7\alpha)$ and $\text{C}(8\alpha)$ methyl groups and two of the water H atoms.

In the final refinements, both of which were carried out in sections because of computer limitations, the temperature factors of the non-H atoms were varied anisotropically and those of the H atoms were varied isotropically. This reduced the R factor to its final value of

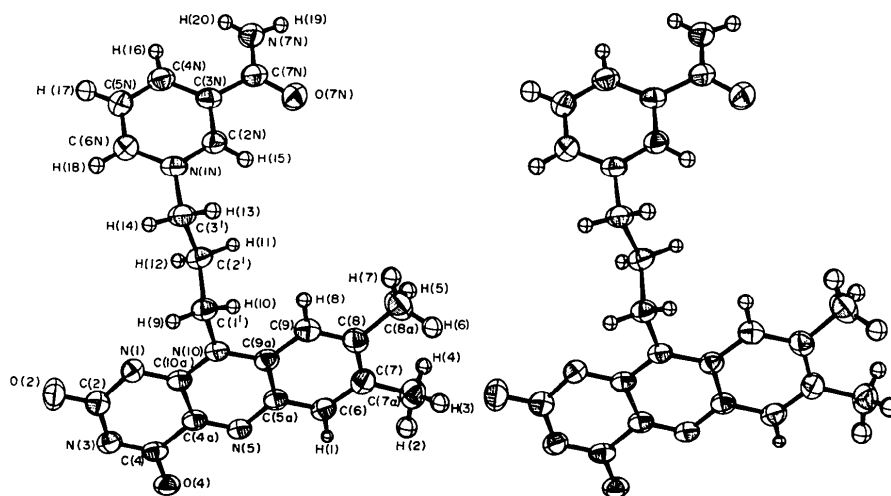


Fig. 1. A stereodrawing of the $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ zwitterion. Non-hydrogen atoms are shown as thermal ellipsoids at the 50% probability level. H atoms are drawn as spheres at the 25% probability level.

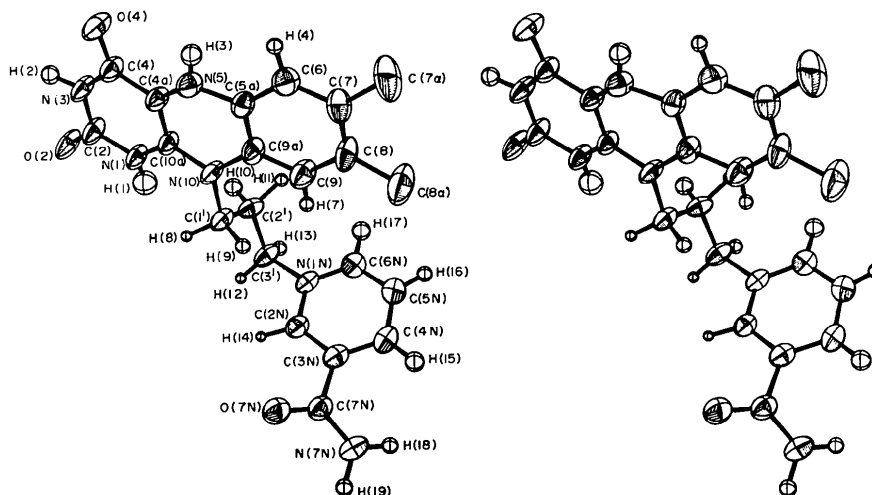


Fig. 2. A stereodrawing of the $\text{H}_2\text{F}_{1\text{red}}\text{-C}_3\text{-Nic}^+$ ion. Non-hydrogen atoms are shown as thermal ellipsoids at the 50% probability level. H atoms are drawn as spheres at the 25% probability level.

Table 2. *Positional parameters for $\text{F}_{1\text{ox}}\text{-C}_3\text{-Nic}^+ \cdot 7\text{H}_2\text{O}$*

The positional parameters are expressed as fractions of a unit-cell edge and are $\times 10^4$ for C, N, O, and $\times 10^3$ for H atoms. Standard deviations as determined from the variance-covariance matrix of the final cycle of least-squares refinement are given in parentheses and refer to the least significant digits of their corresponding parameters. The suffixes *N* and *W* refer to the nicotinamide moieties and to water molecules, respectively.

	<i>x</i>	<i>y</i>	<i>z</i>		<i>x</i>	<i>y</i>	<i>z</i>
N(1)	1157 (3)	3606 (1)	214 (1)	O(4 <i>W</i>)	8737 (5)	2095 (1)	1893 (2)
C(2)	71 (4)	3327 (1)	-554 (2)	O(5 <i>W</i>)	5527 (4)	1913 (1)	503 (2)
O(2)	-283 (3)	2829 (1)	-412 (2)	O(6 <i>W</i>)	874 (4)	2955 (1)	1941 (2)
N(3)	-608 (3)	3566 (1)	-1450 (2)	O(7 <i>W</i>)	4420 (4)	2656 (1)	2082 (2)
C(4)	-257 (3)	4098 (1)	-1625 (1)	H(1)	1768 (3)	1406 (1)	106 (2)
O(4)	-820 (3)	4334 (1)	-2430 (1)	H(2)	370 (6)	678 (2)	-70 (3)
C(4a)	900 (3)	4426 (1)	-837 (2)	H(3)	573 (5)	676 (2)	12 (3)
N(5)	1289 (3)	4944 (1)	-997 (1)	H(4)	401 (4)	693 (1)	48 (2)
C(5a)	2414 (3)	5223 (1)	-265 (2)	H(5)	514 (5)	646 (2)	217 (2)
C(6)	2841 (4)	5781 (1)	-430 (2)	H(6)	683 (6)	638 (2)	175 (3)
C(7)	3944 (4)	6091 (1)	265 (2)	H(7)	625 (5)	598 (2)	253 (3)
C(7a)	4363 (5)	6691 (1)	52 (3)	H(8)	465 (4)	515 (1)	196 (2)
C(8)	4653 (4)	5840 (1)	1180 (2)	H(9)	355 (4)	373 (1)	144 (2)
C(8a)	5778 (5)	6170 (2)	1981 (3)	H(10)	474 (4)	425 (1)	187 (2)
C(9)	4262 (4)	5286 (1)	1344 (2)	H(11)	269 (3)	460 (1)	277 (2)
C(9a)	3159 (3)	4967 (1)	633 (2)	H(12)	144 (4)	412 (1)	230 (2)
N(10)	2736 (3)	4409 (1)	769 (1)	H(13)	469 (4)	400 (1)	356 (2)
C(10a)	1583 (3)	4126 (1)	64 (2)	H(14)	342 (4)	345 (1)	319 (2)
C(1')	3571 (4)	4108 (1)	1673 (2)	H(15)	334 (4)	464 (1)	448 (2)
C(2')	2604 (4)	4202 (1)	2501 (1)	H(16)	36 (4)	404 (1)	627 (2)
C(3')	3487 (4)	3844 (1)	3366 (2)	H(17)	20 (5)	319 (2)	541 (3)
N(1 <i>N</i>)	2569 (3)	3895 (1)	4197 (1)	H(18)	161 (4)	311 (1)	409 (2)
C(2 <i>N</i>)	2687 (4)	4378 (1)	4693 (2)	H(19)	150 (4)	547 (1)	696 (2)
C(3 <i>N</i>)	1868 (4)	4452 (1)	5470 (2)	H(20)	76 (4)	488 (1)	691 (2)
C(4 <i>N</i>)	904 (4)	4004 (1)	5734 (2)	H(1 <i>W</i>)	780 (6)	263 (2)	397 (3)
C(5 <i>N</i>)	828 (4)	3506 (1)	5227 (2)	H(1' <i>W</i>)	630 (7)	275 (2)	314 (4)
C(6 <i>N</i>)	1646 (4)	3457 (1)	4448 (2)	H(2 <i>W</i>)	883 (8)	343 (2)	227 (4)
C(7 <i>N</i>)	2098 (4)	5018 (1)	5958 (2)	H(2' <i>W</i>)	775 (4)	343 (1)	304 (2)
N(7 <i>N</i>)	1362 (4)	5116 (1)	6713 (2)	H(3 <i>W</i>)	668 (4)	66 (1)	64 (2)
O(7 <i>N</i>)	2963 (3)	5381 (1)	5632 (2)	H(4 <i>W</i>)	779 (5)	223 (2)	210 (3)
O(1 <i>W</i>)	6927 (4)	2870 (1)	3773 (2)	H(5 <i>W</i>)	614 (6)	152 (2)	29 (3)
O(2 <i>W</i>)	8400 (4)	3683 (1)	2728 (2)	H(6 <i>W</i>)	4 (5)	266 (2)	181 (3)
O(3 <i>W</i>)	6258 (3)	802 (1)	1100 (2)				

0.064 based on 3318 observed reflections in the case of $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ and 0.069 based on 2963 observed reflections for $(\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+)\text{NO}_3^-$.^{*} The final parameter shifts were all less than their corresponding estimated standard deviations. The heights of the highest peaks in the final difference Fourier maps were 0.38 and 0.32 $\text{e} \text{ \AA}^{-3}$, respectively, for $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ and $(\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+)\text{NO}_3^-$. No additional H atoms were apparent in these maps.

The molecular structures

The molecular configurations of $\text{Fl}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ and $\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+$ are illustrated in Figs. 1 and 2, respectively, together with the atomic numbering

^{*} Lists of structure factors and thermal parameters have been deposited with the British Library Lending Division as Supplementary Publication No. SUP 33045 (43 pp.). Copies may be obtained through The Executive Secretary, International Union of Crystallography, 13 White Friars, Chester CH1 1NZ, England.

schemes used in this report. Standard coenzyme numbering systems have been adopted wherever practicable. Tables 2 and 3 contain the final atomic positional parameters of the two structures.^{*} The covalent distances and angles are presented in Figs. 3 and 4.

The covalent bonding parameters of the nicotinamide moieties are in agreement with the corresponding quantities reported for other nicotinamide-containing structures (Wright & King, 1954; Brändén, Lindqvist & Zeppezaur, 1968; Voet, 1973; Johnson, Maier & Paul, 1973; Johnson, Frank & Paul, 1973; Frank, Thayer & Paul, 1973; Freeman & Bugg, 1974; Herriott, Cammerman & Deranleau, 1974). Tables 4 and 5 indicate that the pyridinium rings in both structures are nearly planar. In both molecules the amide group is oriented such that the $\text{C}(7N) - \text{N}(7N)$ bond is *trans* to the $\text{N}(1N) - \text{C}(3')$ bond. Such

^{*} See previous footnote.

Table 3. *Positional parameters for $(\text{H}_2\text{Fl}_{\text{red}} - \text{C}_3 - \text{Nic}^+)\text{NO}_3^- \cdot 4\text{H}_2\text{O}$*

The positional parameters are expressed as fractions of a unit-cell edge and are $\times 10^4$ for C, N, O, and $\times 10^3$ for H atoms. Standard deviations as determined from the variance-covariance matrix of the final cycle of least-squares refinement are given in parentheses and refer to the least significant digits of their corresponding parameters. The suffixes *N*, *X* and *W* refer to the nicotinamide moiety, to the nitrate ion and to the water molecules, respectively.

	<i>x</i>	<i>y</i>	<i>z</i>		<i>x</i>	<i>y</i>	<i>z</i>
N(1)	6579 (5)	2768 (3)	4145 (2)	O(2 <i>X</i>)	11403 (4)	3390 (3)	399 (2)
C(2)	5958 (6)	1649 (3)	4492 (3)	O(3 <i>X</i>)	9117 (5)	2569 (4)	913 (3)
O(2)	5528 (4)	1000 (2)	3943 (2)	O(1 <i>W</i>)	5057 (5)	643 (3)	1956 (2)
N(3)	5792 (5)	1302 (3)	5463 (2)	O(2 <i>W</i>)	6371 (6)	3027 (3)	2145 (2)
C(4)	6243 (5)	1988 (3)	6124 (3)	O(3 <i>W</i>)	4204 (9)	4504 (6)	943 (4)
O(4)	6040 (4)	1573 (2)	7005 (2)	O(4 <i>W</i>)	3206 (5)	1307 (3)	373 (2)
C(4a)	6896 (5)	3120 (3)	5730 (3)	H(1)	660 (10)	305 (7)	347 (6)
N(5)	7534 (5)	3826 (3)	6352 (3)	H(2)	557 (6)	54 (4)	569 (4)
C(5a)	7708 (5)	5052 (3)	5972 (3)	H(3)	708 (7)	377 (5)	680 (4)
C(6)	7851 (6)	5888 (4)	6541 (3)	H(4)	767 (5)	563 (4)	714 (3)
C(7)	8223 (6)	7080 (4)	6181 (3)	H(5)	795 (7)	870 (5)	660 (4)
C(7 <i>n</i>)	8424 (11)	7934 (5)	6863 (5)	H(6)	944 (7)	875 (5)	421 (4)
C(8)	8475 (6)	7456 (4)	5194 (3)	H(7)	844 (5)	687 (3)	395 (3)
C(8 <i>n</i>)	8967 (11)	8732 (5)	4748 (5)	H(8)	904 (5)	550 (4)	328 (3)
C(9)	8268 (6)	6627 (4)	4600 (3)	H(9)	840 (4)	424 (3)	309 (2)
C(9a)	7906 (5)	5445 (3)	4969 (3)	H(10)	573 (6)	522 (4)	274 (3)
N(10)	7728 (4)	4595 (3)	4354 (2)	H(11)	608 (4)	626 (3)	314 (3)
C(10a)	7075 (5)	3500 (3)	4752 (3)	H(12)	759 (4)	566 (3)	142 (2)
C(1')	8042 (5)	4988 (3)	3317 (3)	H(13)	601 (6)	662 (4)	138 (3)
C(2')	6597 (5)	5666 (4)	2789 (3)	H(14)	1011 (4)	631 (3)	138 (2)
C(3')	7033 (5)	6234 (4)	1747 (3)	H(15)	1090 (6)	986 (5)	142 (4)
N(1 <i>N</i>)	8182 (4)	7270 (3)	1685 (2)	H(16)	821 (5)	1003 (4)	181 (3)
C(2 <i>N</i>)	9789 (5)	7122 (4)	1463 (3)	H(17)	639 (6)	828 (4)	200 (3)
C(3 <i>N</i>)	10864 (5)	8083 (3)	1353 (3)	H(18)	1310 (5)	952 (4)	41 (3)
C(4 <i>N</i>)	10277 (6)	9179 (4)	1477 (3)	H(19)	1454 (6)	856 (4)	32 (3)
C(5 <i>N</i>)	8618 (6)	9297 (4)	1733 (4)	H(1 <i>W</i>)	723 (7)	277 (5)	180 (4)
C(6 <i>N</i>)	7592 (6)	8333 (4)	1824 (3)	H(2 <i>W</i>)	566 (6)	352 (4)	187 (4)
C(7 <i>N</i>)	12636 (5)	7813 (4)	1100 (3)	H(2' <i>W</i>)	499 (8)	1109 (5)	215 (5)
N(7 <i>N</i>)	13465 (5)	8707 (4)	565 (3)	H(3 <i>W</i>)	344 (7)	445 (6)	87 (6)
O(7 <i>N</i>)	13172 (4)	6791 (3)	1380 (2)	H(4 <i>W</i>)	387 (6)	110 (4)	98 (4)
N <i>X</i>	10090 (5)	3400 (4)	884 (3)	H(4' <i>W</i>)	269 (6)	196 (4)	33 (4)
O(1 <i>X</i>)	9819 (5)	4212 (3)	1318 (3)				

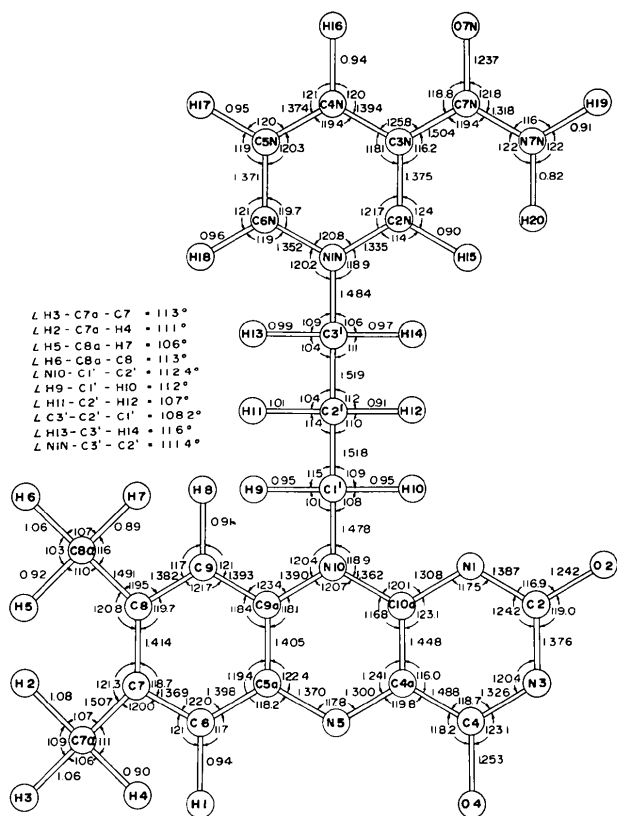


Fig. 3. The covalent distances (\AA) and angles ($^\circ$) of the $\text{FI}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ zwitterion. The average estimated standard deviations of these quantities are 0.003 \AA and 0.2° , respectively, for bonds not involving H atoms, and 0.03 \AA and 2° , respectively, for bonds involving H atoms.

orientations are found in the majority of known nicotinamide structures.

In $\text{FI}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$, the amide plane and the least-squares plane of the pyridinium ring form a dihedral angle of 1.1° . Hence, as can be seen in Table 4, these two groups are nearly coplanar as is the case in the majority of known nicotinamide structures in which the amide group is unsubstituted. In contrast, the amide group of the $\text{H}_2\text{FI}_{\text{red}} - \text{C}_3 - \text{Nic}^+$ ion is twisted about its $\text{C}(3N) - \text{C}(7N)$ bond such that the $\text{C}(2N) - \text{C}(3N) - \text{C}(7N) - \text{O}(7N)$ torsion angle is 29.4° . Similar non-planar nicotinamide conformations have been observed in the structures of nicotinamide (Wright & King, 1954), *N*-[3-(aden-9-yl)propyl]-3-carbamoylpyridinium bromide (Johnson, Maier & Paul, 1973) and the chloride and iodide salts of 1-methylnicotinamide (Freeman & Bugg, 1974).

The covalent bonding parameters of the flavin residue of $\text{FI}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ are largely in close agreement with the corresponding quantities of the 'idealized' lumiflavin molecule (Wang & Fritchie, 1973). The only significant exceptions to this are the expected distor-

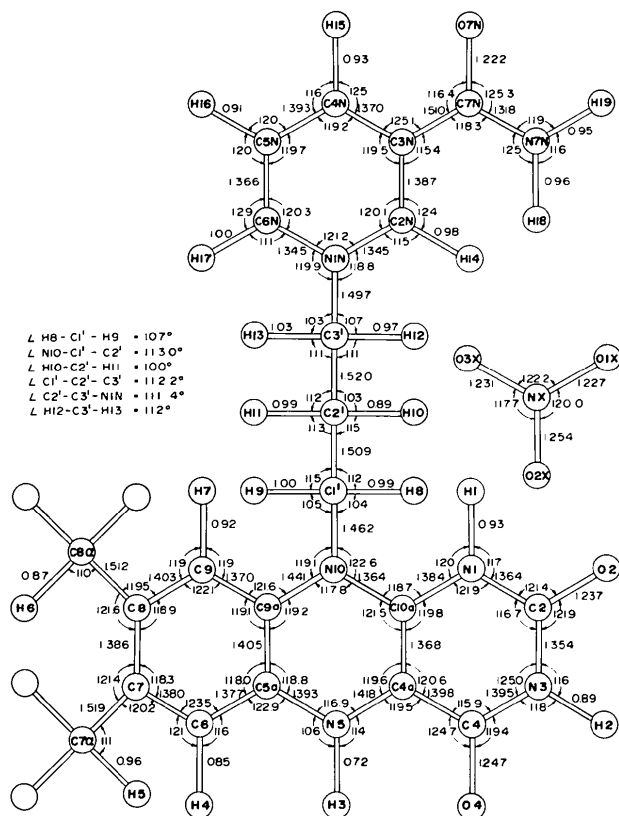


Fig. 4. The covalent distances (\AA) and angles ($^\circ$) of the $\text{H}_2\text{FI}_{\text{red}}^- - \text{C}_3 - \text{Nic}^+$ and nitrate ions. The average estimated standard deviations of these quantities are 0.005 \AA and 0.4° , respectively, for bonds not involving H atoms, and 0.05 \AA and 3° , respectively, for bonds involving H atoms. It should be noted that four of the six methyl H atoms of the flavin group were not located.

tions of the pyrimidinoid ring in the region of the ionized N(3) position. Here the bond parameters vary by as much as 0.04 \AA and 4° from the corresponding quantities found in neutral oxidized flavins. Similar alterations in bond parameters have been described in other heterocyclic ring systems in which a N atom has lost an exocyclic bond (Singh, 1965; Voet & Rich, 1970).

The flavin residue of $\text{FI}_{\text{ox}}^- - \text{C}_3 - \text{Nic}^+$ is nearly planar (Table 4) as has been observed for other oxidized flavin derivatives (Kierkegaard *et al.*, 1971). The dihedral angle between the least-squares planes of the benzenoid and the pyrimidinoid portions of the flavin group is 1.5° .

The covalent bonding parameters of the 1,5-dihydroflavin residue of $(\text{H}_2\text{FI}_{\text{red}} - \text{C}_3 - \text{Nic}^+) \text{NO}_3^-$ are in good agreement with the corresponding quantities in other known 1,5-dihydroflavin structures (Norrestam, Kierkegaard, Stensland & Törbjörnsson, 1969; Werner & Rönnquist, 1970; Leijonmarck & Werner, 1971; Norrestam & von Glehn, 1972).

The eight atoms of the pyrimidinoid group and the six atoms of the benzenoid ring of the $\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+$ flavin moiety are both highly planar (Table 5). The reduced flavin nucleus assumes the expected 'butterfly' conformation (Dudley, Ehrenberg, Hemmerich & Müller, 1964) with a 12.7° dihedral angle between the foregoing planes. The corresponding angles in the four previously reported 1,5-dihydroflavin structures range from 28.8 to 35.5° and average 32° (Norrestam, Kierkegaard, Stensland & Torbjörnsson, 1969; Werner & Rönnquist, 1970; Leijonmarck & Werner, 1971; Norrestam & von Glehn, 1972). These quantities are all more than twice the angle found in the present structure. The pyrazoid ring is in the usual boat conformation.

The propyl group of $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+$ is in the all *trans*, fully extended conformation. The least-squares planes through the nicotinamide and flavin moieties form a dihedral angle of 59.5° . The propyl group of the $\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+$ is folded such that its conformation about the $\text{C}(1')-\text{C}(2')$ bond is *trans* and that about the $\text{C}(2')-\text{C}(3')$ bond is *gauche*. The dihedral angle between the pyridinium ring and the entire 1,5-dihydroflavin residue is 73.4° . There are no unusual bond lengths or angles in either propyl group.

Table 4. Deviations (Å) of non-hydrogen atoms from the least-squares plane through the indicated atoms of $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+$

Equations of the least-squares planes

$$\begin{aligned} \text{Flavin group: } & -6.8403x + 7.3301y + 7.3626z = 1.9749 \text{ Å} \\ \text{Nicotinamide ring: } & 5.4863x - 7.8217y + 6.4762z = 1.0839 \text{ Å} \\ \text{Amide group: } & 5.5581x - 7.3995y + 6.4288z = 1.2859 \text{ Å} \end{aligned}$$

Atom	Flavin	Atom	Nicotinamide ring	Amide
N(1)	0.034	N(1N)	-0.004	-0.043*
C(2)	0.007	C(2N)	0.004	-0.016*
O(2)	-0.012	C(3N)	0.001	-0.025*
N(3)	-0.014	C(4N)	-0.008	0.061*
C(4)	0.007	C(5N)	0.009	0.064*
O(4)	-0.027	C(6N)	-0.003	0.068*
C(4a)	0.038	C(7N)	0.003*	0.000
N(5)	0.034	O(7N)	0.008*	0.000
C(5a)	0.008	N(7N)	-0.020*	0.000
C(6)	0.003	C(3')	0.001*	0.029*
C(7)	-0.013	R.m.s.		
C(7a)	-0.013	deviation	0.005	0.000
C(8)	-0.009			
C(8a)	0.048			
C(9)	-0.027			
C(9a)	-0.029			
N(10)	-0.048			
C(10a)	0.012			
C(1')	-0.177*			
R.m.s.				
deviation	0.026			

* Atoms not included in the least-squares fit.

The intermolecular interactions

(i) $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+ \cdot 7\text{H}_2\text{O}$

Fig. 5 is a stereodiagram illustrating the packing of the $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+ \cdot 7\text{H}_2\text{O}$ structure in its unit cell. It can be seen that the crystal structure largely consists of broad bands, endlessly extending parallel to the (010) plane, that contain the $\text{Fl}_{\text{ox}}-\text{C}_3-\text{Nic}^+$ zwitterions. Within these bands the flavin residues associate by forming infinite stacks of overlapping parallel ring molecules. The distances between the least-squares planes of the centrosymmetrically related, and therefore parallel, flavin residues are 3.460 and 3.385 Å for the two different flavin stacking interactions illustrated in Fig. 6(a) and (b) respectively. There is no interatomic contact in either interaction that is less than its corresponding van der Waals distance. Hence, these associations must be classified as normal stacking interactions.

The nicotinamide residues extend alternately to opposite sides of the flavin stacks. The nicotinamide groups within a given band of heterocycles are parallel on account of symmetry considerations. However, as

Table 5. Deviations (Å) of non-hydrogen atoms from the least-squares plane through the indicated atoms of $\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+$

Equations of the least-squares planes

$$\begin{aligned} \text{Pyrimidinoid ring: } & 7.3805x - 4.4068y - 0.4173z = 3.4749 \text{ Å} \\ \text{Benzenoid ring: } & 7.8569x - 2.0295y + 0.8745z = 5.4510 \text{ Å} \\ \text{Nicotinamide ring: } & 1.8853x - 0.6254y + 13.3514z = 3.346 \text{ Å} \\ \text{Nitrate ion: } & 3.7718x - 4.1153y + 10.1831z = 3.3109 \text{ Å} \\ \text{Amide group: } & 2.8725x + 5.0389y + 12.8468z = 8.9796 \text{ Å} \end{aligned}$$

Atom	Pyrimidinoid ring	Benzenoid ring	Atom	Nicotinamide ring
N(1)	-0.012	-0.529*	N(1N)	-0.009
C(2)	0.008	-0.740*	C(2N)	0.007
O(2)	0.000	-0.983*	C(3N)	0.003
N(3)	-0.002	-0.709*	C(4N)	-0.012
C(4)	0.001	-0.448*	C(5N)	0.011
O(4)	-0.003	-0.439*	C(6N)	-0.001
C(4a)	0.001	-0.219*	C(7N)	0.016*
N(5)	0.134*	0.181*	N(7N)	-0.599*
C(5a)	-0.262*	0.014	O(7N)	0.566*
C(6)	-0.548*	-0.008	C(3')	-0.077*
C(7)	-0.748*	-0.009	R.m.s.	
C(7a)	-1.038*	0.022*	deviation	0.008
C(8)	-0.722*	0.019		
C(8a)	-0.899*	0.090*		
C(9)	-0.485*	-0.013		Nitrate ion
C(9a)	-0.247*	-0.004	NX	-0.004
N(10)	0.022*	-0.011*	O(1X)	0.001
C(10a)	0.006	-0.248*	O(2X)	0.001
C(1')	0.125*	0.060*	O(3X)	0.001
R.m.s.			R.m.s.	
deviation	0.006	0.012	deviation	0.002

* Atoms not included in the least-squares fit.

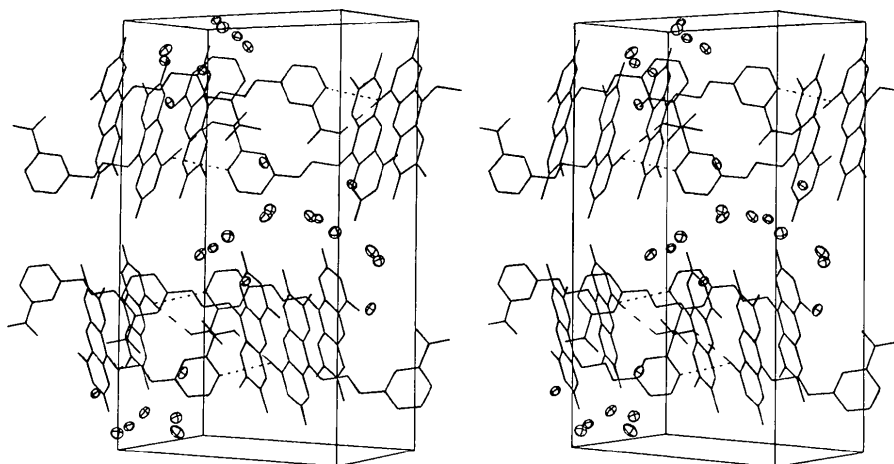


Fig. 5. A stereodiagram of the $\text{Fl}_{\text{ox}}^{-}\text{-C}_3\text{-Nic}^{+} \cdot 7\text{H}_2\text{O}$ structure illustrating the packing of the molecules in the unit cell. The view is roughly along the a axis with the b and c axes extending vertically and horizontally respectively. Water oxygen atoms are shown as thermal ellipsoids at the 50% probability level. Hydrogen bonds and $\text{C-H}\cdots\text{O}$ hydrogen-bond-like interactions between the flavin and nicotinamide residues are shown as dashed and dotted lines, respectively. Hydrogen atoms have been deleted for the sake of clarity.

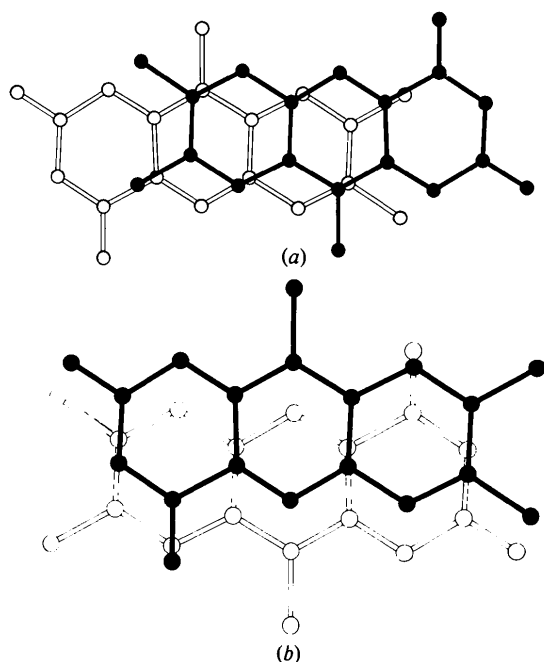


Fig. 6. Projections on the least-squares plane of the $\text{Fl}_{\text{ox}}^{-}\text{-C}_3\text{-Nic}^{+}$ flavin residue showing the stacking relations between neighboring flavin residues. The view is approximately along the a axis with the b and c axes extending roughly horizontally and vertically respectively. Illustrated are the flavin moiety listed in Table 2 and those related to it by the centrosymmetric transformations (a) $(1-x, 1-y, -z)$ and (b) $(-x, 1-y, -z)$. Atoms are shown as spheres of arbitrary radii. H atoms have been omitted for the sake of clarity.

can be seen in Fig. 5, the nicotinamide residues are too far from one another to associate.

The hydrogen-bonding interactions in the $\text{Fl}_{\text{ox}}^{-}\text{-C}_3\text{-Nic}^{+} \cdot 7\text{H}_2\text{O}$ structure are tabulated in Table 6. The

Table 6. *Hydrogen-bonding associations in*
 $\text{Fl}_{\text{ox}}^{-}\text{-C}_3\text{-Nic}^{+}$

The symmetry code, relative to atoms in Table 2, is (i) $x, y, z + 1$; (ii) $1-x, 1-y, 1-z$; (iii) $1-x, -\frac{1}{2}+y, \frac{1}{2}-z$; (iv) $1+x, \frac{1}{2}-y, \frac{1}{2}+z$; (v) $1-x, -y, -z$; (vi) $-1+x, y, z$; (vii) $x, \frac{1}{2}-y, \frac{1}{2}+z$.

Hydrogen bond $D\text{-H}\cdots A$	Distance $D\cdots A(\text{\AA})$	Distance $H\cdots A(\text{\AA})$	Angle $D\text{-H}\cdots A(^{\circ})$
(1) Flavin-nicotinamide			
$\text{N}(7N)\text{-H}(20)\cdots\text{O}(4^{\text{I}})$	2.929	2.12	170
$\text{C}(4N)\text{-H}(16)\cdots\text{O}(4^{\text{I}})^*$	3.222	2.30	167
(2) Nicotinamide-solvent			
$\text{N}(7N)\text{-H}(19)\cdots\text{O}(2W^{\text{II}})$	2.959	2.06	168
$\text{O}(3W)\text{-H}(3W)\cdots\text{O}(7N^{\text{III}})$	2.795	1.97	163
(3) Flavin-solvent			
$\text{O}(1W)\text{-H}(1W)\cdots\text{O}(2^{\text{IV}})$	2.774	1.89	170
$\text{O}(3W)\text{-H}\cdots\text{O}(4^{\text{IV}})$	2.739	—	—
$\text{O}(4W)\text{-H}\cdots\text{N}(3^{\text{IV}})$	2.755	—	—
$\text{O}(6W)\text{-H}\cdots\text{N}(1)$	2.914	—	—
(4) Solvent-solvent			
$\text{O}(1W)\text{-H}(1'W)\cdots\text{O}(7W)$	2.824	1.92	161
$\text{O}(2W)\text{-H}(2W)\cdots\text{O}(6W^{\text{V}})$	2.958	2.08	147
$\text{O}(2W)\text{-H}(2'W)\cdots\text{O}(1W)$	2.806	1.88	167
$\text{O}(5W)\text{-H}(5W)\cdots\text{O}(3W)$	2.797	2.04	123
$\text{O}(6W)\text{-H}(6W)\cdots\text{O}(4W^{\text{VI}})$	2.624	1.67	165
$\text{O}(1W)\cdots\text{O}(5W^{\text{VII}})$	2.890	—	—
$\text{O}(4W)\cdots\text{O}(5W)$	2.852	—	—
$\text{O}(5W)\cdots\text{O}(7W)$	2.962	—	—
$\text{O}(6W)\cdots\text{O}(7W)$	2.863	—	—

* $\text{C-H}\cdots\text{O}$ hydrogen-bond-like interaction.

only H atoms of the $\text{Fl}_{\text{ox}}^{-}\text{-C}_3\text{-Nic}^{+}$ zwitterion that are capable of forming normal hydrogen bonds are those of the amide group. Of these, only one participates in a hydrogen bond between the nicotinamide and flavin moieties of the structure. This is the

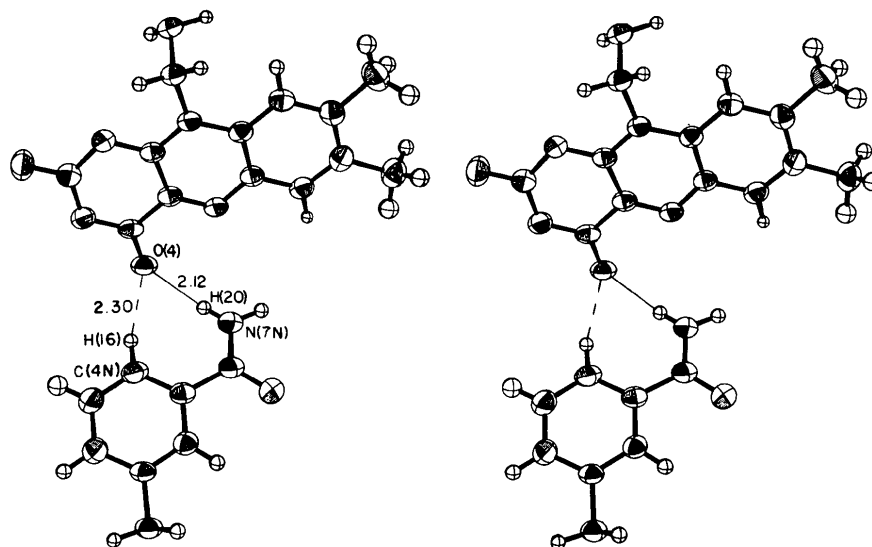


Fig. 7. A stereodrawing illustrating the intermolecular hydrogen-bonding associations between the nicotinamide and flavin moieties of $\text{Fl}_{\text{ox}}^{-}\text{-C}_3\text{-Nic}^{+}$. Hydrogen bonds and $\text{C-H}\cdots\text{O}$ hydrogen-bond-like interactions are shown as thin and dashed lines, respectively, accompanied by their $\text{H}\cdots\text{O}$ distances in Å.

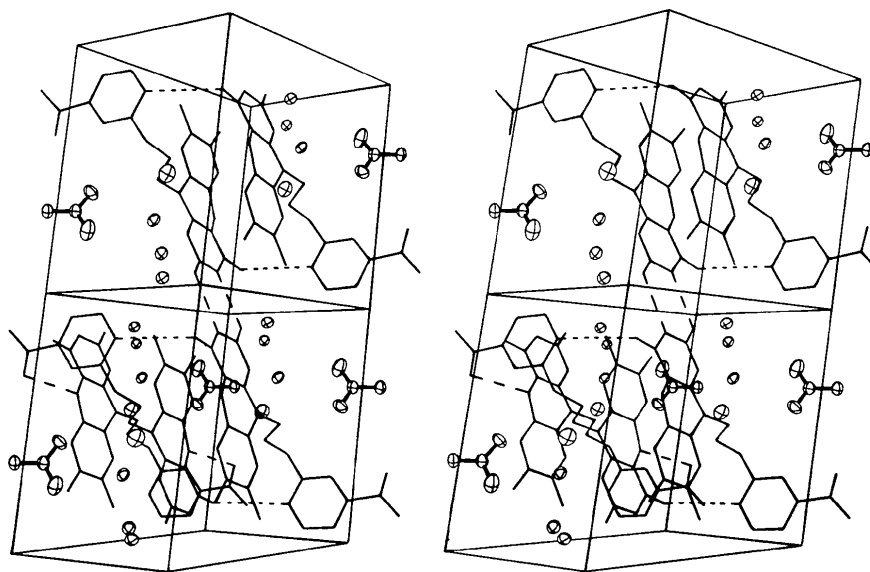


Fig. 8. A stereodiagram of the $(\text{H}_2\text{Fl}_{\text{red}}\text{-C}_3\text{-Nic}^{+})\text{NO}_3^{-}\cdot 4\text{H}_2\text{O}$ structure illustrating the packing of the molecules in the unit cell. The view is roughly along the c axis with the a and b axes extending horizontally and vertically, respectively. Nitrate and water oxygen atoms are shown as thermal ellipsoids at the 50% probability level. Hydrogen bonds and $\text{C-H}\cdots\text{O}$ hydrogen-bond-like interactions between the flavin and nicotinamide residues are shown as dashed and dotted lines respectively. Hydrogen atoms have been deleted for the sake of clarity.

$\text{N}(7\text{N})\text{-H}(20)\cdots\text{O}(4)$ ($x, y, z + 1$) hydrogen bond. This interaction is flanked by a relatively short $\text{C}(4\text{N})\text{-H}(16)\cdots\text{O}(4)$ ($x, y, z + 1$) hydrogen-bond-like interaction. Both of these interactions, which can be seen in Fig. 5 and in greater detail in Fig. 7, are unusual in that the nicotinamide and flavin residues are more nearly perpendicular than parallel to each other. Flavin atom $\text{N}(5)$ ($x, y, z + 1$), a potential hydrogen-bond

acceptor, is 2.86 Å distant from amide $\text{H}(20)$. However, this is too large a distance to be considered a significant interaction.

The bands containing the $\text{Fl}_{\text{ox}}^{-}\text{-C}_3\text{-Nic}^{+}$ zwitterions are interspersed by parallel bands containing the solvent molecules. These are arranged in a complex hydrogen-bonded network that also involves the hydrophilic groups of the $\text{Fl}_{\text{ox}}^{-}\text{-C}_3\text{-Nic}^{+}$ zwitterions (Table 6).

(ii) $(\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+)\text{NO}_3^- \cdot 4\text{H}_2\text{O}$

The packing of $(\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+)\text{NO}_3^- \cdot 4\text{H}_2\text{O}$ in its unit cell is shown diagrammatically in Fig. 8. It can be seen there that the flavin residues associate, *via* stacking and hydrogen-bonding interactions, into bands of ring molecules endlessly extending parallel to the (001) plane. Neighboring heterocycles within the columns of stacked flavins are related by centers of symmetry. Their butterfly conformation causes them to alternately fold towards and away from each other, as can be seen in Fig. 9(a). Despite the folded conformation of the 1,5-dihydroflavin residues there is considerable ring overlap between neighboring molecules in the stack, as is illustrated in Fig. 9(b) and (c) and also in Fig. 8. There are no interatomic contacts closer than the minimum van der Waals distances within these stacks.

Table 7 lists the parameters describing the hydrogen bonds found in the structure. Neighboring flavins base-pair across a center of symmetry in the manner in which flavins have been frequently observed to associate (Bear, Waters & Waters, 1970; Wang & Fritchie, 1973; Kuo, Dunn & Fritchie, 1974; Fritchie & Johnston, 1975). The $\text{N}(3) \cdots \text{O}(2)$ ($1-x, -y, 1-z$) hydrogen-bond length of 2.796 Å in this cyclic dimer is within the normal range for such interactions (Voet & Rich, 1970).

The nicotinamide residues alternately extend to opposite sides of the flavin stacks and are oriented such that they are nearly parallel to the flavin stacking axis. The nicotinamide groups are too far removed from one another to associate in any manner.

Nicotinamide atom $\text{O}(7N)$ accepts a tenuous hydrogen bond from the flavin $\text{N}(5)-\text{H}(3)$ group of a centrosymmetrically related $\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+$ ion, thereby forming a dimer in which the stacked flavin molecules are folded away from one another. This association, which is illustrated in Figs. 8 and 10, appears to be quite strained. The $\text{N}(5) \cdots \text{O}(7N)$ ($2-x, 1-y, 1-z$) and the $\text{H}(3) \cdots \text{O}(7N)$ ($2-x, 1-y, 1-z$) distances of 3.184 and 2.53 Å, respectively, are very long for $\text{N}-\text{H} \cdots \text{O}$ hydrogen bonds and the $\text{N}(5)-\text{H}(3) \cdots \text{O}(7N)$ ($2-x, 1-y, 1-z$) angle of 152° deviates significantly from the expected near linearity for such interactions [note, however, that the relatively unreliable $\text{N}(5)-\text{H}(3)$ distance of 0.72 Å is much shorter than is to be expected]. Furthermore, although an amide group is usually observed to be nearly coplanar with the $\text{N}-\text{H}$ group to which it is hydrogen bonded, flavin atoms $\text{N}(5)$ and $\text{H}(3)$ deviate 2.399 and 1.98 Å, respectively, from the plane of the amide group with which they are associated. In fact, without the large deviation of the amide group from the plane of the pyridinium ring, the foregoing hydrogen bond could not be formed.

The nicotinamide group associates with the flavin

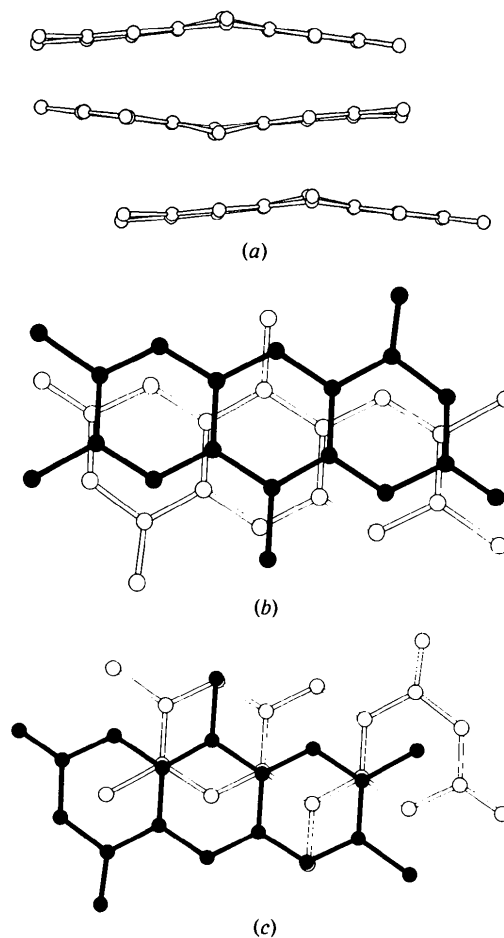


Fig. 9. (a) A projection, along the least-squares plane of the $\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+$ flavin residue, of three consecutive stacked 1,5-dihydroflavin groups, illustrating their folded 'butterfly' conformation. The view is approximately along the c axis. The upper and lower residues are related by the a -axis translation and the b axis extends roughly horizontally. (b) A projection on the least-squares plane of the 1,5-dihydroflavin residue illustrating the stacking relation between the flavin moiety listed in Table 3 and that related to it by the centrosymmetric transformation ($1-x, 1-y, 1-z$) [the upper pair in (a)]. The view is approximately along the a axis with the b and c axes extending roughly horizontally and vertically respectively. (c) A projection on the least-squares plane of the 1,5-dihydroflavin residue illustrating the stacking relation between the flavin moiety listed in Table 3 and that related to it by the centrosymmetric transformation ($2-x, 1-y, 1-z$) [the lower pair in (a)]. The direction of view is the same as that in (b). Atoms are shown as spheres of arbitrary radii. H atoms have been omitted for the sake of clarity.

group that is stacked on and folded towards its covalently attached flavin residue. This is done *via* the $\text{C}(6N)-\text{H}(17) \cdots \text{O}(4)$ ($1-x, 1-y, 1-z$) hydrogen-bond-like interaction that can be seen in Fig. 8 so as to form a third type of centrosymmetric dimer.

The regions in the crystal structure between the associating $\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+$ ions are occupied by the

Table 7. *Hydrogen-bonding associations in*
 $H_2Fl_{red}-C_3-Nic^+$

The symmetry code, relative to atoms in Table 3, is: (i) $1-x, -y, 1-z$; (ii) $2-x, 1-y, 1-z$; (iii) $1-x, 1-y, 1-z$; (iv) $1+x, 1+y, z$; (v) $2-x, 1-y, -z$; (vi) $-1+x, y, z$; (vii) $1-x, 1-y, -z$.

Hydrogen bond $D-H \cdots A$	Distance $D \cdots A$ (Å)	Distance $H \cdots A$ (Å)	Angle $D-H \cdots A$ (°)
(1) Flavin-flavin $N(3)-H(2) \cdots O(2)$	2.796	1.93	164
(2) Flavin-nicotinamide $N(5)-H(3) \cdots O(7N^{II})$	3.184	2.53	152
$C(6N)-H(17) \cdots O(4^{III})^*$	3.352	2.42	155
(3) Flavin-solvent $N(1)-H(1) \cdots O(2W)$	2.796	1.91	158
$O(1W)-H(1W) \cdots O(2)$	2.959	2.57	123
$O(1W) \cdots O(4^I)$	2.787	—	—
(4) Nicotinamide-nitrate $C(2N)-H(14) \cdots O(1X)^*$	3.358	2.42	158
(5) Nicotinamide-solvent $N(7N)-H(18) \cdots O(4W^{IV})$	2.976	2.02	174
$N(7N)-H(19) \cdots O(4W^V)$	2.916	2.04	153
$O(3W) \cdots O(7N^I)$	2.902	—	—
(6) Nitrate-solvent $O(2W)-H(2'W) \cdots O(3X)$	2.904	2.00	167
$O(3W)-H(3W) \cdots O(2X^VI)$	2.806	2.22	151
$O(4W)-H(4W) \cdots O(2X^VI)$	2.767	1.93	174
(7) Solvent-solvent $O(1W)-H(1W) \cdots O(2W)$	2.990	2.47	142
$O(2W)-H(2W) \cdots O(3W)$	2.756	1.94	164
$O(4W)-H(4'W) \cdots O(1W)$	2.684	1.68	172
$O(3W) \cdots O(3W^{VII})$	2.952	—	—

* C-H \cdots O hydrogen-bond-like interaction.

NO_3^- ions and the solvent molecules. These entities associate *via* hydrogen-bonded networks that also involve the hydrophilic groups of the heterocycles (Table 7). All of the potential hydrogen bonds of the structure appear to have been formed.

Discussion

The biological implications of the two flavin-nicotinamide biscoenzyme structures have been discussed elsewhere (Porter, Bright & Voet, 1977).

The observed 12.7° bending angle of the 1,5-dihydroflavin moiety of $H_2Fl_{red}-C_3-Nic^+$ is less than half of that expected on the basis of the four other published structural studies of 1,5-dihydroflavin derivatives. These latter compounds, 5-acetyl-3,7,8,10-tetramethyl-1,5-dihydroalloxazine (Norrestam, Kierkegaard, Stensland & Torbjörnsson, 1969), 5-acetyl-9-bromo-1,3,7,8,10-pentamethyl-1,5-dihydroisalloxazine (Werner & Rönquist, 1970), 5-acetyl-9-bromo-1,3,7,8-tetramethyl-1,5-dihydroisalloxazine (Leijonmarck & Werner, 1971) and 9-bromo-1,3,7,8,10-pentamethyl-1,5-dihydroisalloxazine (Norrestam & von Glehn, 1972), all have more and bulkier substituents than does the more biologically realistic flavin residue of $H_2Fl_{red}-C_3-Nic^+$. Hence, it might seem that the large bending angles in these more highly substituted reduced flavin compounds are due to steric interference among their substituents. However, molecular models of these compounds reveal that such overcrowding is relieved at

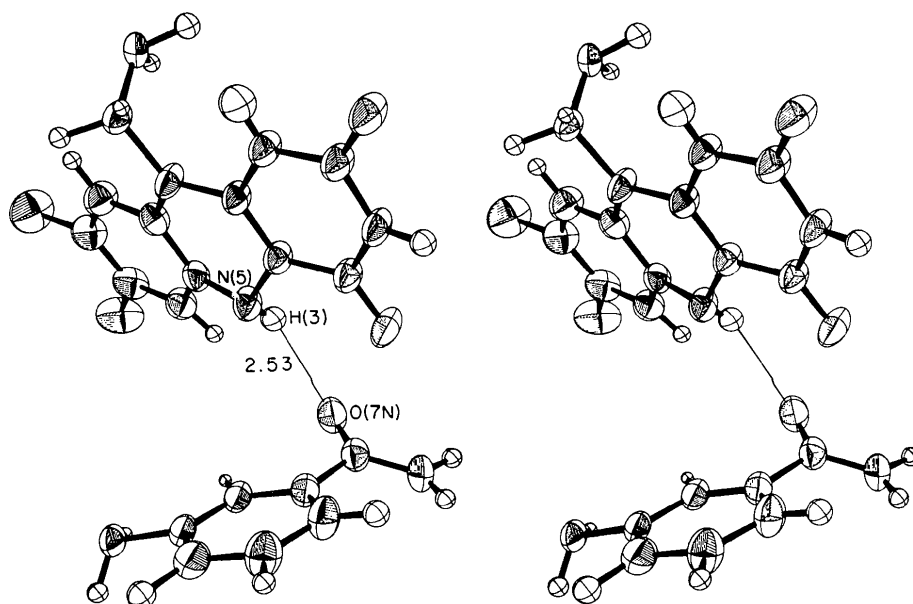


Fig. 10. A stereodrawing illustrating the intermolecular hydrogen-bonding association between the nicotinamide and 1,5-dihydroflavin moieties of $H_2Fl_{red}-C_3-Nic^+$. The hydrogen bond is shown as a thin line accompanied by its H \cdots O distance in Å.

bending angles in the neighborhood of 10° . This is confirmed by the fact that a similarly substituted oxidized flavin, 9-bromo-3,7,8,10-isoalloxazine (von Glehn & Norrestam, 1972), is observed to have a bending angle of 7.0° .

Self-consistent-field molecular orbital calculations using the partial retention of diatomic differential overlap approximation (PRDDO) indicate that the barrier for ring inversion in 1,5-dihydrolumiflavin has a surprisingly low value of almost 4 kcal mol^{-1} (Lindner, Branchaud, Dixon & Lipscomb, 1978). NMR measurements at low temperatures on a number of reduced flavin derivatives yield a larger but still relatively low value of 10 kcal mol^{-1} for this barrier (Tauscher, Ghisla & Hemmerich, 1973).

It can be seen from Fig. 9 that there is considerable ring overlap in the flavin stacks of the $(\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+)\text{NO}_3^-$ structure. However, there is little such overlap in the four previously mentioned structures of reduced flavins (probably due to the steric requirements of their bulky substituents). This suggests that the small flavin bending angle in $\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+$ is due to the influence of stacking interactions. This, in turn, is possible because of the apparent flexibility of the 1,5-dihydroflavin bending angle.

X-ray studies of the enzyme flavodoxin from *Clostridium MP* have shown, as expected, that the flavin moiety of the bound flavin mononucleotide (FMN) cofactor is planar in both the oxidized (Burnett, Darling, Kendall, LeQuesne, Mayhew & Ludwig, 1974; Ludwig, Burnett, Darling, Jordan, Kendall & Smith, 1976) and the semiquinone (Anderson, Apgar, Burnett, Darling, LeQuesne, Mayhew & Ludwig, 1972) forms of the flavoprotein. The flavin residue is also nearly planar in reduced *Clostridial* flavodoxin (Ludwig, Burnett, Darling, Jordan, Kendall & Smith, 1976); its bending angle is estimated to be 8.6° . This is corroborated by the essential identity of the NMR spectra of oxidized and reduced *Clostridial* flavodoxins (James, Ludwig & Cohn, 1973).

The red color of the $(\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+)\text{NO}_3^- \cdot 4\text{H}_2\text{O}$ crystals, in contrast to the reported yellow color of the crystals of the four previously mentioned reduced flavin derivatives (the color of neutral reduced flavins in solution), might reasonably be attributed to either a charge-transfer complex or a spectral shift of the reduced flavin molecule caused by its flattening (Dudley, Ehrenberg & Hemmerich & Müller, 1964). The lack of any close flavin/nicotinamide association rules out the existence of a crystalline reduced flavin/nicotinamide charge-transfer complex such as that which has been observed in solutions of $\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+$ (Proffitt, Ingraham & Blankenhorn, 1974; Blankenhorn, 1975a,b, 1976a,b). Charge-transfer interactions have been observed between stacked oxidized flavin molecules in the crystalline complex lumiflavin-2,6-diamino-9-ethylpurine (Scarborough, Shieh & Voet,

1976, 1977) but have not been previously reported to occur between reduced flavin molecules. This, together with the fact that there are no contacts closer than the van der Waals radii in the stacks of reduced flavin residues illustrated in Fig. 9, indicates that any such charge-transfer interactions would be weak, at best. Likewise, the relatively small changes in the visible absorption spectrum of the nearly planar flavin molecule of reduced flavodoxin from *Clostridium MP*, in comparison with that of free reduced flavin mononucleotide (FMNH_2) (Ghisla, Massey, Lhoste & Mayhew, 1974), do not account for the red color of $(\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+)\text{NO}_3^-$ crystals. Hence, the origin of this color remains obscure.

The use of simple resonance theory indicates that the C(2), C(4) and C(6) positions of N(1)-quaternized nicotinamide are positively charged. Hence, the H atoms at these positions are relatively acidic. This is corroborated by the formation of a C-H...O hydrogen-bond-like interaction involving atom C(4N) in the structure of $\text{Fl}_{\text{ox}}^--\text{C}_3-\text{Nic}^+$ and two similar interactions involving atoms C(2N) and C(6N) in the structure of $(\text{H}_2\text{Fl}_{\text{red}}-\text{C}_3-\text{Nic}^+)\text{NO}_3^-$.

In both biscoenzyme structures the nicotinamide residues are too far from one another to interact. There is a similar lack of intermolecular associations between nicotinamide residues in almost all other nicotinamide-containing structures. This is probably due to Coulombic repulsions between the positively charged atoms on the nicotinamide residue. This results in an intrinsic lack of affinity of nicotinamide for self-stacking.

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