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# Structural Analysis of a Stereochemical Modification of Flavin Adenine Dinucleotide in Alcohol Oxidase from Methylotrophic Yeasts

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**Abstract** Alcohol oxidase (MOX), a major peroxisomal protein of methanol-utilizing yeasts, contains two different forms of flavin adenine dinucleotide, one of which is identical with natural FAD whereas the other (mFAD) is a stereochemical modification of the natural coenzyme. This modification occurs spontaneously with FAD (but not FADH) bound to alcohol oxidase. mFAD was degraded with diphosphatase to provide authentic AMP and mFMN. The latter was degraded further with phosphatase to m-riboflavin. Analysis by $^{1}H$ and $^{13}C$ NMR spectroscopy of mFAD revealed that the isoalloxazine and adenine rings were intact and not modified structurally. However, significant differences were observed in the proton spectra in the sugar chains attached to the isoalloxazine ring (ribitol in the case of FAD). Similar observations were made for mFMN and m-riboflavin. Most striking in COSY spectra is the virtual absence of coupling between protons 2′ and 3′ in the sugar chain attached to the isoalloxazine ring, whereas this coupling is strong in the natural materials. Moreover, the nature of the coupling of proton 2′ to protons 1a′ and 1b′ of the sugar chain is different in modified material. All these observations are consistent with the hypothesis that in modified cofactor the absolute configuration of carbon 2′ of the sugar chain attached to the isoalloxazine ring has changed from R to S. This indicates the presence of an arabityl sugar chain rather than the ribitol present in natural FAD. A possible mechanism for this conversion is suggested.

## Introduction

Alcohol oxidase is the first enzyme involved in the oxidation of methanol in methylotrophic yeasts. A homogeneous preparation of this enzyme, once obtained, was assumed to contain flavin adenine dinucleotide (FAD) as a prosthetic group.[1] This assumption was based on the results of paper chromatography[2a-d] or thin layer chromatography.[3a-c] Therefore the

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discovery that in alcohol oxidases of various methanol utilizing yeasts the cofactor occurred in a modified form (mFAD, the prefix "m" will be used further to identify this structural modification of FAD and degradative products thereof) with FAD as the minor component was unexpected [4,5] An influence of the growth rate of various yeasts on the rate of appearance of mFAD as well as the catalytic properties of alcohol oxidase have been demonstrated.[4,6] These findings suggested that the modification has physiological significance and most probably reflects a special case of adaptation of the yeast cells to a low concentration of substrate.

We present here results of a structural analysis of mFAD and argue that it is an arabinoflavin adenine dinucleotide.

**Experimental**

Source of mFAD. A commercial preparation of alcohol oxidase (Provesta, USA) was used. The cleavage products, mFMN and m-riboflavin, were also derived from this material.

Preparative purification of modified flavins. Commercial alcohol oxidase (50-100 mg) in a sucrose solution as provided by the manufacturer was diluted twice with bidistilled water mainly to reduce its viscosity. The enzyme was precipitated with a 75% solution of cold aqueous acetone, and resuspended in 100 ml of 0.1M potassium phosphate buffer containing ice. Trichloroacetic acid was gradually added to a final concentration of 5%. During this addition the suspension was gently stirred with a glass rod to which a large quantity of white denaturated protein attached itself and which could be removed in this manner. The remaining protein was sedimented by centrifugation at 25,000 g, 10 min, 4°C, and the pellet was repeatedly washed with 5% TCA to extract all flavins. This was sedimented again in the same way and discarded. The supernatant was combined and diluted 3 times with methanol, and then passed through $NH_2$-Adsorbex minicolumns (Merck, Germany) to retain all phosphorylated flavins. Elution was done with 0.5 M ammonium acetate. This crude preparation of mFAD was further purified by preparative HPLC as described above and freeze dried.

Extraction of FAD. This was done as described earlier[3] by treatment of the sample with aqueous 5% trichloroacetic acid (TCA) followed by centrifugation in a minifuge for 5 min (Hettich) or a Sorvall 5b centrifuge, 18,000 g, for 10 min at 4°C (TCA extract). The supernatant was used either for the direct measurement of coenzyme content (analytical use) or for further preparative purification.

Analytical HPLC of flavins. A μ-Bondapack C-18 (39x300mm, Waters) column was employed using 0.1M ammonium formate/formic acid, pH 2.7, mixed with

acetonitrile (100 ml/l) and methanol (20 ml/l) (buffer A). The flow rate was 0.5 ml/min, and detection was at 280 nm using a setting of 0.05 A full scale on the detector.

Preparative HPLC of flavins. A Supelco semi-prep column was used with an elution solvent containing 0.1M ammonium acetate, pH 3.7, mixed with 80 ml/l of acetonitrile and 20 ml/l of methanol (buffer B). The flow rate was 1 ml/min, room temperature, detection the same as for analytical HPLC

HPLC of AMP. The same column and elution and detection conditions were used as for the analytical HPLC of flavins, except that buffer C was used. This contained 0.1M ammonium formate, pH 6.1, and 2 ml/l of methanol.

Preparation of mFMN. mFAD was used as a source. This was cleaved enzymatically in an appropriate buffer by phosphodiesterase following the procedure of Sherry & Abeles.[4] Cleavage was carried out on analytical as well as preparative scales For analysis of cleavage products the reaction mixture (1 ml) containing 0.1M Tris-HCl buffer, pH 8.0, FAD or mFAD, 0.025 mM, was treated with phosphodiesterase from ***Crotalus durissus*** (Boehringer, Germany), 0.03 U (10 ul). The mixture was incubated for 30 min at 30°C in the dark, and was then subjected to HPLC analysis. Final preparations were stored dry in the dark at -80°C.

Preparative cleavage of mFAD into AMP and mFMN was done in 50 ml of 0.1M Tris-HCl buffer , pH 8.0, containing 2 mg of mFAD, and 0.3 U (100 ul) of phosphodiesterase. Incubation was carried out for 1 h under the same conditions as above.

Preparation of m-riboflavin. Either mFAD or mFMN was used as a source. The same assay mixture as for preparation of mFMN was employed whereby 15-75 U (10-50 ul) of alkaline phosphatase (from calf intestine, Cat No 108138, Boehringer) was added for analytical or preparative scale reactions, respectively. When mFMN was used as a source, phosphodiesterase was omitted from the reaction mixture. Other conditions were the same as above.

**NMR Apparatus.**

300 MHz $^{1}$H-NMR data and 75 MHz $^{13}$C-NMR data were recorded on a Varian VXR 300s. The chemical shifts are given in δ units (ppm) relative to TMS. The coupling constants are in Hz. Solutions were in $D_2O$ and the temperature was as indicated in the figures. In many cases an optimal temperature had to be sought whereby the water peak was shifted away from the sugar absorptions.

## Results

Presence of mFAD in alcohol oxidase.

HPLC analysis (Fig 1) revealed that the commercial preparation of alcohol oxidase used here (Provesta, USA) contained almost exclusively

modified FAD (Fig 1A). The HPLC retention time was identical to that of mFAD found earlier in alcohol oxidases of various methylotrophic yeasts, namely *Hansenula polymorpha* CBS 4732, *H polymorpha* DL-1,[5] and *Candida boidinii* ATCC 32195.[6] This modification is clearly one in which neither the modified nor the natural cofactor is covalently bound to the enzyme

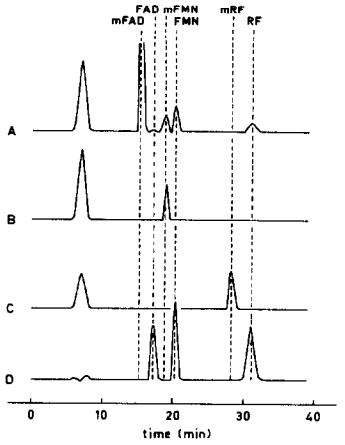



Fig 1. A comparison of the HPLC retention times of the various cofactor segments is given. **A** is the HPLC of the TCA extract of the alcohol oxidase used (Provesta, USA). **B** is the HPLC of mFAD after cleavage by phosphodiesterase. **C** is the HPLC of mFMN after cleavage by phosphatase. D gives the retention times of the standards used. See text for solvent systems.

HPLC analysis of modification site.

An enzymic degradation was carried out to aid in ascertaining in which part of the cofactor molecule the structural differences reside. As shown in Figs 1b,c, both mFMN and m-riboflavin (Fig 1, inset d) have retention times not identical (Fig 1d) to natural FMN and riboflavin. Although not illustrated in Fig 1 AMP derived from cleavage of mFAD was shown to have a retention time on HPLC identical to that of authentic material.

**Structural Determination.**

In view of the fact that mFAD could be obtained on a milligram scale (individual batches were about 0.5 to 1 mg) a total structural elucidation of this material as well as the products obtained by enzymatic degradation (see previous section) was undertaken. NMR spectroscopy was chosen as the most promising method to carry out total structural elucidation in solution with the quantities of material available. Attempts to obtain suitable crystals of mFAD for crystallographic structure determination have been thus far unavailing.

The $^{1}H$ NMR spectrum of natural FAD in $D_2O$ is shown in Fig 2a and that

of mFAD (about 0.5 mg) in Fig 2b.

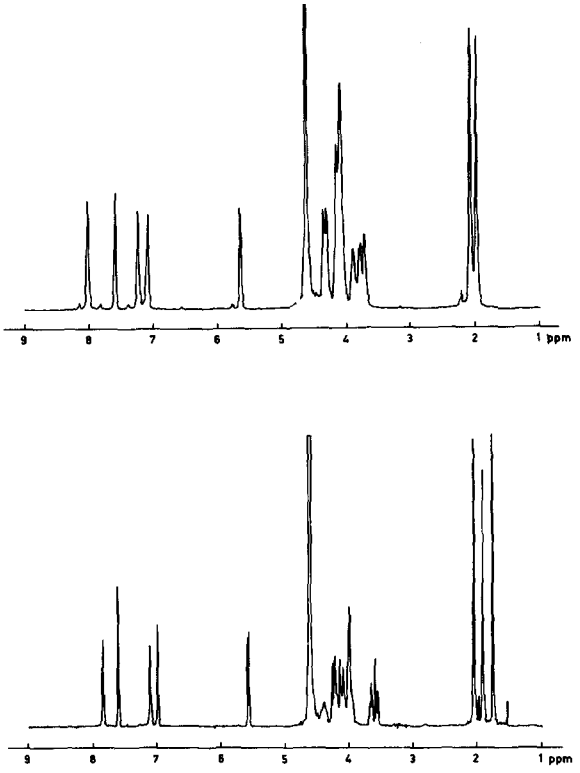



Fig 2 The $^{1}H$ NMR spectra of FAD (Fig a) and mFAD (Fig b) in $D_2O$ at 25°C. The water peak has been suppressed by irradiation. The temperature was optimized to shift this peak away from the sugar absorptions. See Table 1 for assignments.

The decoupled $^{13}C$ NMR spectra of FAD and mFAD are shown in Figs 3a, and 3b The chemical shifts for the $^{1}H$ and $^{13}C$ spectra are tabulated in Tables 1 and 2, respectively. The numbering scheme given in Fig 4 is used for the identification of protons and carbons. The assignments of proton spectra of the sugar chains have been made with the aid of COSY techniques (see further discussion). COSY spectra were recorded with spectral widths of $F_1$ and $F_2$ covering all peaks, which gave 1024 x 512 blocks of data, which were processed using sinusoidal multiplication in each dimension followed by symmetrization of the final data matrices. $^{13}C$ spectra have been assigned with the aid of proton coupling and APT (Attached Proton Test) techniques. The assignments for FAD agree in essence with literature precedent.[7,8,9]

Large quantities of formaldehyde are present during reaction making condensation of FAD with formaldehyde a viable possibility. However, general comparison of the $^{1}H$ and $^{13}C$ spectra of mFAD with those of FAD

clearly show that the total number of carbon atoms (23) and protons (23 carbon bound) is equal to that in natural FAD.

From the $^{1}H$ spectra it may be concluded that the isoalloxazine ring of mFAD is intact (2 methyls and 2 aromatic protons) and that adenine is also

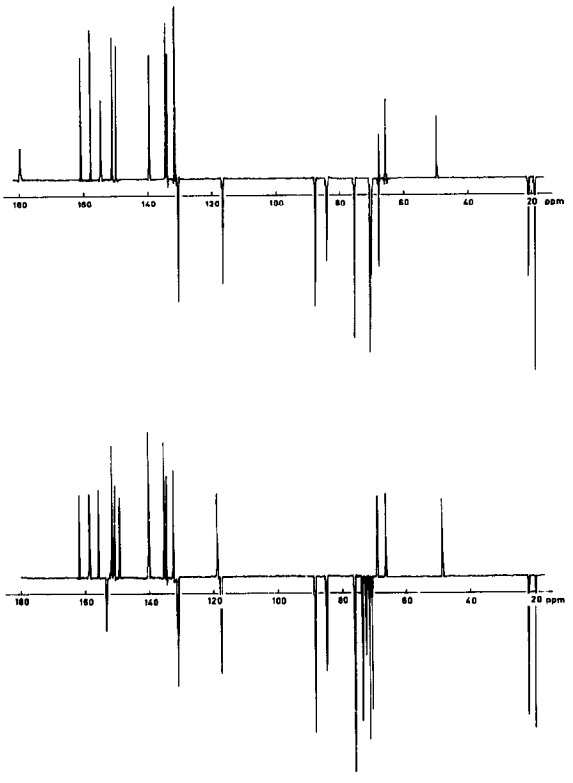
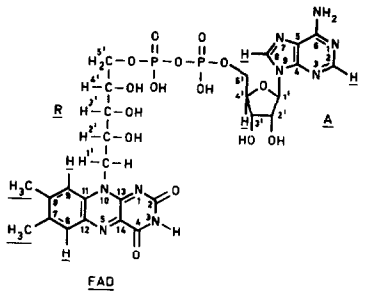



Fig 3 The APT $^{13}C$ NMR spectra of FAD (Fig a) and mFAD (Fig b) in $D_2O$ at 25°C. See Table 2 for peak assigments.

present as revealed from the presence of two aromatic protons. The anomeric proton of ribose is also clearly present in mFAD.

Inspection of the proton spectra of FAD and mFAD in the sugar region makes clear that detailed investigation of this area will be difficult. Seven ribitol and 5 ribose protons lie in an ca 1 ppm range (the anomeric proton of ribose is farther downfield). The water peak also intrudes in this area. By repeated freeze drying virtually all the water can be removed, and by careful adjustment of temperature to shift the water peak as well as by irradiation techniques it is possible with difficulty to obtain full spectra of the sugar area. Because limited amounts of material were available further analysis was done using $^{1}H$ spectra both because of increased sensitivity relative to $^{13}C$. The degree of overlap of



FAD

Fig 4 The numbering scheme used for identification of protons and carbons in FAD and the modified derivative.

various proton absorptions precluded extraction of the magnitude of coupling constants either by first order analysis and/or simulation. COSY spectra, however, were expected to provide insight in the couplings along the chain.

The full assignment of the protons in the sugar chains of FAD with the aid of COSY spectra as given in Table 1 requires some comment. Despite the narrow absorption range the assignment of the ribose and ribitol chains is quite straightforward. That for the ribose chain starts from the anomeric proton and via cross peaks proceeds unambiguously from that point. Assignment of the ribitol chain begins at diastereotopic protons $F1'_{a,b}$, which are recognized by their downfield position and geminal coupling.[9] We were unable with our apparatus to resolve coupling of $F5'_{a,b}$ or of $A5'_{a,b}$ (end methylenes of ribitol and ribose, respectively) with $^{31}P$ in phosphate. Proton-phosphorus coupling was also not observed in the FMN derivatives although examples of such coupling have been reported.[9]

NOESY spectra (not illustrated) suggest that mFAD in solution adopts a folded conformation. No efforts were made, however, to define these conformational aspects further.

In analogy to FAD the proton-proton COSY spectrum of mFAD was recorded (not shown). The ribose chain was readily followed but analysis of the ribitol segment (see also Table 1) was incomplete because of severe overlap at F2' with the ribose absorptions, and because the cross peak between between F2' and F3' could not be identified.

The decision was taken to concentrate further on mFMN and m-riboflavin owing the anticipation that the spectra would be much simpler without the AMP unit. As detailed in the previous section cleavage with phosphodiesterase provided AMP, identical with authentic material as established by HPLC analysis and reaction with myokinase, pyruvate kinase and lactate dehydrogenase (see also Experimental Section).

Sufficient material was obtained to record both $^{1}H$ and $^{13}C$ NMR spectra of mFMN. Considerable care is necessary because it is known that the

**Table 1 $^1$H NMR Shifts for FAD and mFAD[a]**

| FAD | | mFAD | |
|---|---|---|---|
| proton | chem shift (δ) | proton | chem shift (δ) |
| A8H | 8.05 | A8H | 8.15 |
| A2H | 7.60 | A2H | 8.10 |
| A1'H | 5.65 | A1'H | 5.75 |
| A2'H | 4.31 | A2'H | 4.22 |
| A3'H | 4.27 | A3'H | 4.18 |
| A4'H | 4.17 | A4'H | 4.10 |
| A5'$H_a$ | 4.10 | A5'$H_a$[b] | 4.00 |
| A5'$H_b$ | 4.10 | A5'$H_b$[b] | 4.00 |
| F6H | 7.10 | F6H | 7.30 |
| F9H | 7.22 | F9H | 7.42 |
| F1'$H_a$ | 4.73[b] | F1'$H_a$ | 4.50 |
| F1'$H_b$ | 4.20[b] | F1'$H_b$ | 4.35 |
| F2'H | 4.16 | F2'H | 4.25 |
| F3'H | 3.70 | F3'H | 4.08 |
| F4'H | 3.85 | F4'H | 3.70 |
| F5'$H_a$ | 3.87 | F5'$H_a$[b] | 3.60 |
| F5'$H_b$ | 2.10 | F5'$H_b$[b] | 4.00 |
| F8$CH_3$ | 1.95 | F8$CH_3$ | 2.25 |
| F7$CH_3$ | 2.10 | F7$CH_3$ | 2.12 |

a) $D_2O$ solution, neutral pH, 25°C, nitrogen bonded proton fully exchanged.
b) assignment with other diastereotopic proton may be reversed.

phosphate group in FMN migrates spontaneously along the ribitol chain on extended standing.[10] The purity was carefully established by HPLC and samples were stored at -20°C prior to spectral analysis. Again general examination of the spectra of mFMN and natural material (chemical shifts compiled in Table 3) reveals that the unmistakable structural differences between the two are not reflected in profound differences in spectral behaviour. The $^{13}$C NMR spectra (Fig 5) show small but unmistakable differences in the sugar regions.

COSY spectra (Fig 6) revealed the same phenomenon seen in mFAD, namely that the cross peak between the protons 2' and 3' in the sugar chain was virtually absent. Only on extended pulsing and data analysis could a weak cross peak be resolved.

Two structural interpretations seemed plausible at this point. Either

**Table 2 $^{13}C$ NMR Chemical Shifts for FAD and mFAD[a]**

| FAD carbon | chem shift (δ) | mFAD carbon | chem shift (δ) |
|---|---|---|---|
| A2 | 153.14 | A2 | 152.7 |
| A4 | 148.90 | A4 | 149.0 |
| A5 | 118.50 | A5 | 118.7 |
| A6 | 155.43 | A6 | 155.0 |
| A8 | [b] | A8 | [b] |
| A1′ | 88.10 | A1′ | 88.2 |
| A2′ | 71.00 | A2′ | 70.8[c] |
| A3′ | 75.80 | A3′ | 75.9 |
| A4′ | 84.44 | A4′ | 84.6 |
| A5′ | 66.10 | A5′ | 66.1 |
| F2 | 158.20 | F2 | 158.2 |
| F4 | 161.44 | F4 | 161.4 |
| F6 | 131.00 | F6 | 131.9 |
| F7 | 134.37 | F7 | 134.4 |
| F8 | 135.00 | F8 | 134.8 |
| F9 | 117.43 | F9 | 117.2 |
| F11 | 132.10 | F11 | 132.0 |
| F12 | 139.90 | F12 | 139.9 |
| F13 | 151.31 | F13 | 151.6 |
| F14 | 150.54 | F14 | 150.2 |
| F7$\underline{C}H_3$ | 19.49 | F7$\underline{C}H_3$ | 19.5 |
| F8$\underline{C}H_3$ | 21.60 | F8$\underline{C}H_3$ | 21.6 |
| F1′ | 48.33 | F1′ | 50.0 |
| F2′ | 70.15 | F2′ | ? |
| F3′ | 73.39 | F3′ | 70.8[c] |
| F4′ | 72.14 | F4′ | 70.8[c] |
| F5′ | 68.53 | F5′ | 68.2 |

a) $D_2O$ solution, neutral pH, 25°C
b) not seen in spectrum
c) severe overlap

the phosphate (diphosphate in FAD) had migrated along the ribitol chain or the configuration of one or more carbons in the ribitol chain was different from that in FMN and FAD. The order of chemical shifts is the same in both FMN and mFMN: (proceeding upfield) F1a′, F1b′ < F2′ < F4′,$F5_{a'}$,$F5_{b'}$ (diastereotopic protons) < F3′. The pertinent chemical shifts are compiled in Table 4. This suggests, but does not prove, that the

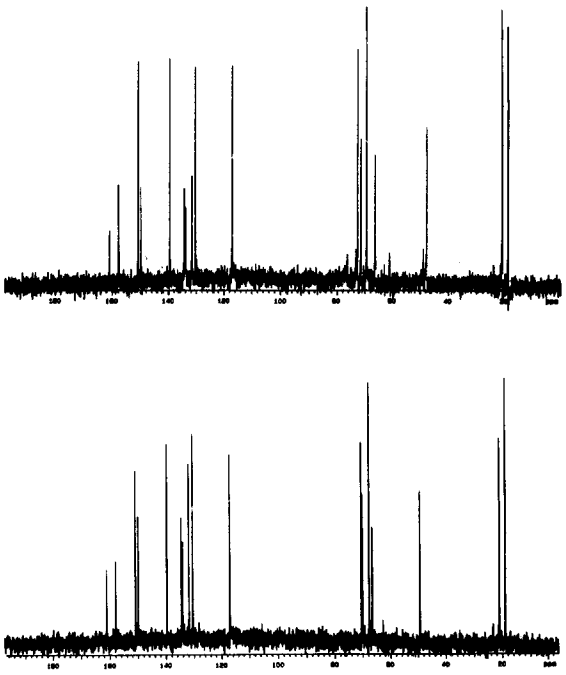


Fig. 5. The decoupled $^{13}C$ NMR spectra of FMN (Fig a) and mFMN (Fig b) in $D_2O$. Assignments are as indicated in Table 3.

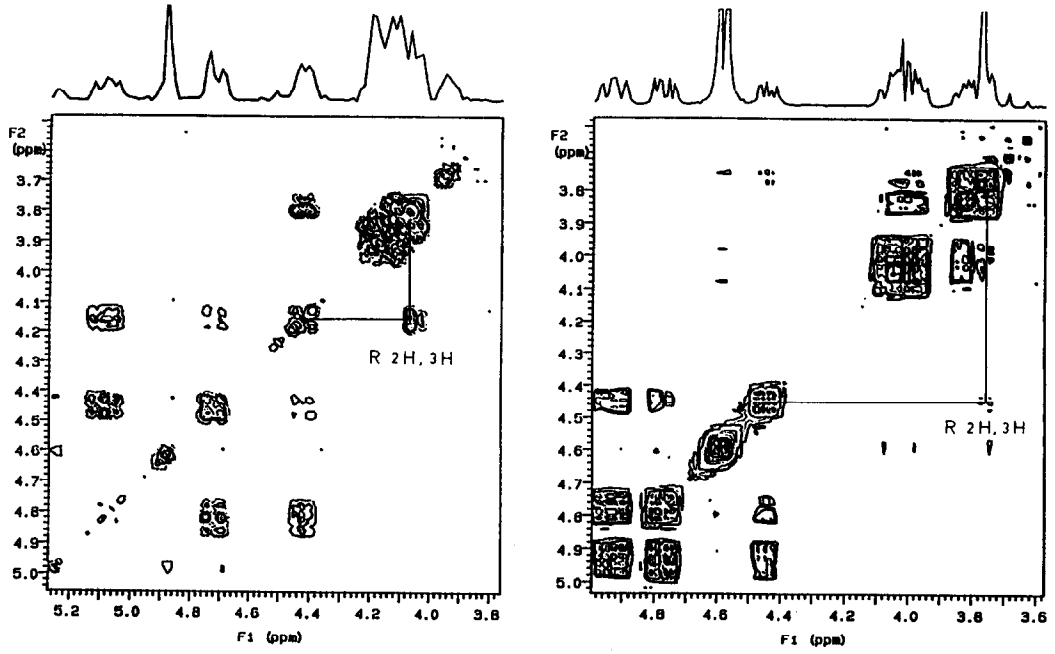



Fig. 6. COSY spectra of the ribitol sugar chain of FMN (Fig a) and the sugar chain of mFMN (Fig b) in $D_2O$. The connectivities between F2' and F3' are indicated. The sequence of chemical shifts is given in the text and the absolute values are given in Table 4 (the shifts in the two dimensional spectra are not absolute).

phosphate substituent has not shifted along the sugar chain. On the other hand, the nature of the proton-proton coupling changes dramatically between H2'-H3'in the modified materials, which is consistent with expectation for a modification of configuration about a stereogenic carbon.

Table 3 $^{13}C$ NMR Chemical Shifts for FMN and mFMN

| FMN | | mFMN | |
|---|---|---|---|
| carbon | chem.shift (δ) | carbon | chem.shift (δ) |
| F2 | 158.2 | F2 | 158.3 |
| F4 | 163.4 | F4 | 163.6 |
| F6 | 133.3 | F6 | 130.9 |
| F7 | 134.8 | F7 | 134.9 |
| F8 | 135.3 | F8 | 135.0 |
| F9 | 137.6 | F9 | 117.4 |
| F11 | 132.5 | F11 | 132.3 |
| F12 | 140.0 | F12 | 140.0 |
| F13 | 151.3 | F13 | 151.3 |
| F14 | 150.7 | F14 | 150.3 |
| $F7CH_3$ | 19.4 | $F7CH_3$ | 19.3 |
| $F8CH_3$ | 21.5 | $F8CH_3$ | 21.4 |
| F2' | 48.1 | F1' | 49.6 |
| F2' | 70.0 | F2' | 68.0 |
| F3' | 73.1 | F3' | 70.9 |
| F4' | 72.0 | F4' | 70.3 |
| F5' | 66.7 | F5' | 66.9 |

The COSY spectra of riboflavin and m-riboflavin (Fig 7) reveal a picture of the sugar chain very similar to that seen with FAD and FMN compared to the modified materials (previous discussion and Fig 6). The fact that phosphatase removed the phosphate from mFMN to provide material not identical with natural riboflavin establishes that a shift of the phosphate group along the ribitol chain in FAD is not the cause of the structural isomerism. It is also a moot point whether diphosphatase would have reacted with material in which the diphosphate was linked to a secondary hydroxyl of the ribitol chain.

The change of magnitude of coupling constant (absolute magnitude could not be determined) between H2' and H3' indicates that a stereochemical modification has occurred at one of these positions. In view of the obviously identical chemical composition of FAD and mFAD this change must be stereochemical. A change of absolute configuration at one of these

carbons would entail a change of angle between vicinal protons. This clearly has happened with F2′-F3′ causing the angle to approach 90° with concomitant virtual loss of vicinal coupling (the coupling between F2′-F3′ in natural FMN is estimated to be 4.5 Hz).[9] A change of configuration also will be communicated via other vicinal couplings to neighbouring protons. There is no significant change in coupling between F3′-F4′. There is, however, a noteworthy change in the coupling between F2′ and diastereotopic protons $F1'_{a,b}$. The conclusion is clear: the configuration of F2′ has changed to S (R in riboflavin).

**Discussion**

On the basis of degradative studies Sherry and Abeles[4] drew the conclusion that mFAD was an "optical isomer" of FAD in the sense that some type of change had occurred in the ribitol segment of FAD. These experiments were carried out on a very small scale. After purification by HPLC a small amount of pure mFAD was obtained, which was treated with phosphodiesterase to provide, as established from HPLC retention times, AMP and material (mFMN) with a retention time different from that of FMN. Removal of the phosphate group from the mFMN obtained by this route afforded material nonidentical with "D- and L-lyxoflavin" as established by HPLC. Cleavage of a very small amount of this modified riboflavin derivative with $NaIO_4$ provided, as established by HPLC retention times, a substituted isoalloxazine derivative identical to that obtained on analogous cleavage of natural FMN. From these observations Sherry and Abeles[4] concluded that some undefined type of optical isomerization had occurred in the ribitol chain attached to $N_{10}$. By use of a similar enzymatic route with larger amounts of material and supplemented with NMR data we are now able to define the nature of the stereochemical modification.

The stereochemical conclusions drawn from NMR data are supported also by other spectral observations. One of us has shown that there are pronounced differences in the CD spectra of natural and mFAD.[5] In particular the sign of the 450nm absorption changes from negative (natural material) to positive (unnatural material) and that of the 335nm absorption from positive to negative. There is, however, no significant shift in the frequency of the various bands. This is consistent with modification in the sugar chain rather than in the electronic structure of the chromophores, the isoalloxazine and adenine heterocyclic rings. The CD spectra of riboflavin and m-riboflavin are uncomplicated by the contributions from the adenine chromophore. We observe that the strongly negative long wavelength band in natural riboflavin is strongly positive in the stereochemical modification. This is fully consistent with the

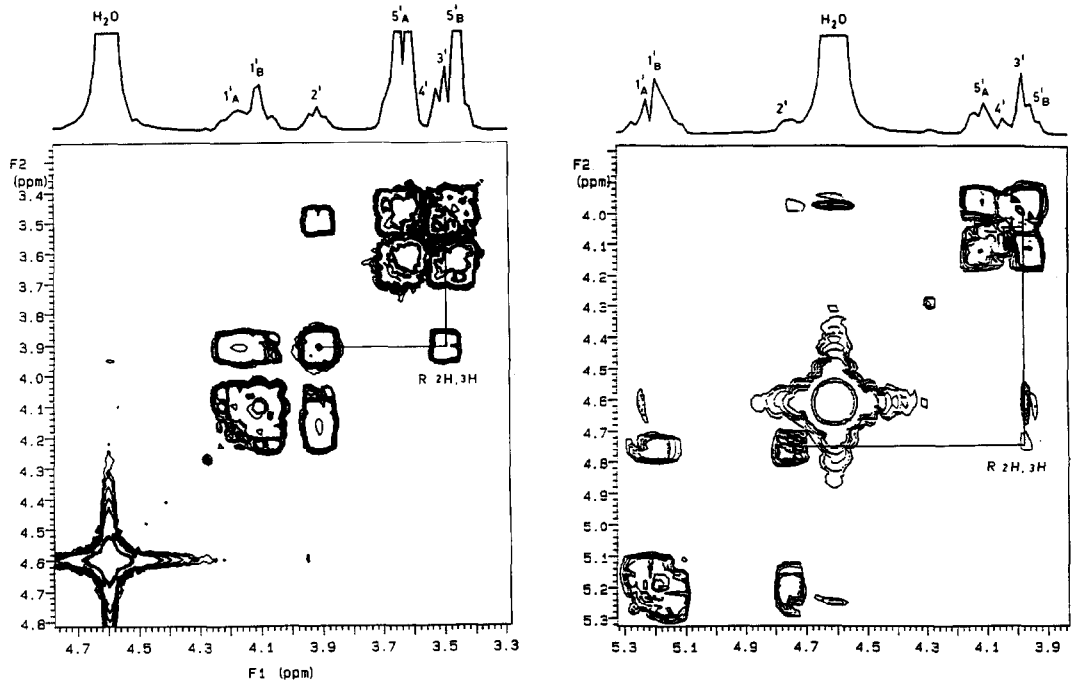



Fig 7 COSY spectra of the sugar portion of natural riboflavin (Fig a) and m-riboflavin (Fig b) in $D_2O$. The connectivities between F2' and F3' are indicated.

modification being closely located to the chromophore.[11] The closest position is at F3' as assigned from NMR arguments.

We note that there is in principle another way to achieve "optical isomerism". Atropoisomerism, i.e. optical isomerism of the biphenyl type, could arise in principle if the diastereotopic methylene protons F1,1' of the ribitol (or other sugar chain in a modified version) interact severely enough with neighbouring proton H9 of the benzene ring of the alloxazine heterocyclic system. Molecular models reveal that the two diastereotopic protons can either straddle H9 or both be turned away from H9. The latter situation seems for steric reasons far more probable. There is to our knowledge no precedent for this type of atropoisomerism in flavins and existing crystal structures give no hint that this might occur.[12,13]

We find that all available evidence points to stereochemical modification in the form of a change of absolute configuration at F2 of the sugar chain. Unfortunately all attempts to obtain crystals of a derivative of modified material in order to carry out a crystal structure determination have been so far unsuccessful.

There are a number of cases of FAD-containing oxidases where modification of FAD has been observed. The types of modification that have been reported are by no means identical.[14] All lead to formation of a

**Table 4 $^1$H NMR Chemical Shifts for Sugar Chains in FMN, mFMN, Riboflavin and m-Riboflavin**

| FMN[a] | | mFMN[a] | |
|---|---|---|---|
| proton | chem.shift (δ) | proton | chem.shift(δ) |
| F1'$H_a$ | 4.95 | F1'$H_a$ | 4.80 |
| F1'$H_6$ | 4.55 | F1'$H_b$ | 4.70 |
| F2'H | 4.30 | F2'H | 4.35 |
| F3'H | 3.85 | F3'H | 3.65 |
| F4'H | 3.90 | F4'H | 3.70 |
| F5'$H_a$ | 3.95 | F5'$H_a$ | 3.95 |
| F5'$H_b$ | 4.00 | F5'$H_b$ | 3.90 |
| **riboflavin** | | **m-riboflavin** | |
| F1'$H_a$ | 4.18 | F1'$H_a$ | 4.70[b](5.2)[c] |
| F1'$H_b$ | 4.10 | F1'$H_b$ | 4.70 (5.2) |
| F2'H | 3.90 | F2'H | 4.28(4.75) |
| F3'H | 3.48 | F3'H | 3.50(3.98) |
| F4'H | 3.60 | F4'H | 3.58(4.04) |
| F5'$H_a$ | 3.-- | F5'$H_a$ | 3.68(4.10) |
| F5'$H_b$ | 3.45 | F5'$H_b$ | 3.45(3.95) |

a) $D_2O$, 30°C; b) 23°C; c) 70°C

covalently bound FAD via a bond of the 8(α)-methyl group with the $N_1$ atom of a histidyl residue of polypeptide chain (L-gluconolactone oxidase,[15] cholesterol oxidase,[16] L-galactolactone oxidase,[17]) or the $N_3$ atom of histidine (6-hydroxy-D-nicotine oxidase,[18] and choline oxidase[19]). In the case of monoamine oxidase a covalent bond is formed with a thiol group of a cysteinyl residue of the polypeptide chain.[20] Very little is known about mechanisms of FAD modification among oxidases. In the case of 6-hydroxy-D-nicotine oxidase the reaction was shown to be catalyzed by the enzyme itself and requires phosphoenolpyruvate to form a covalent bond.[21] The example described here with alcohol oxidase is clearly different; the modified FAD is clearly not covalently bound.

Recently, it has shown that modification of FAD in the alcohol oxidase occurs spontaneously when the enzyme stays in the oxidized form; addition of any reducing agent (including any alcohol substrate) inhibits the reaction.[6] One possible explanation why modification takes place with the oxidized form of the enzyme (FAD) is that oxidized flavin itself catalyzes this modification. The following scheme (Fig 8) illustrates how this

might possibly occur.

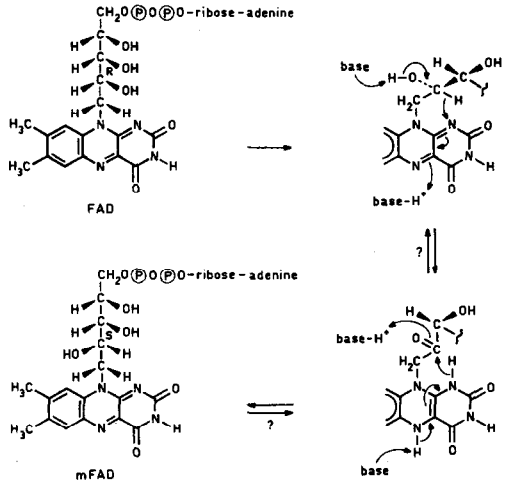



Fig. 8. A suggested mechanism for the stereochemical modification of FAD.

The coenzyme FAD oxidizes itself, probably with the aid of some external base. The carbon-bound hydrogen at F2′ of the ribityl chain is positioned exactly six atoms away from $N_1$ of the isoalloxazine ring. A reduced flavin is formed wherein F2′ is oxidized to the keto oxidation level. Re-reduction at F2′ from the other (prochiral) side of the carbonyl group generates mFMN. This mechanism is consistent with the inhibitory effect of oxygen (dihydro form of the coenzyme is trapped) and with the fact that FAD itself is the source of mFMN. It is, however, not clear why the self reduction of the keto group at F2′ seemingly yields only the modified coenzyme. This may perhaps be a consequence of at this time undefined conformational factors in the peptide to which the coenzyme is bound.

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