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Identification and Structure of a Novel Flavin Prosthetic Group Associated with Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase from *Peptostreptococcus elsdenii**

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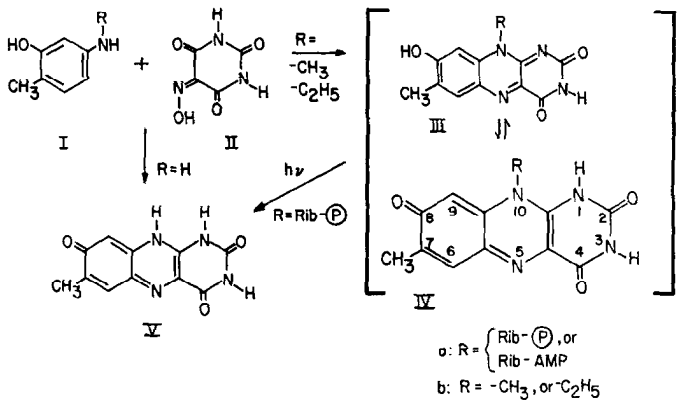
SUMMARY

A novel prosthetic group, which is present in NADH dehydrogenase (EC 1.6.99.3) from *Peptostreptococcus elsdenii*, has been purified and its physicochemical properties investigated. The chemical structure of this species has been established as the FAD analogue of 7-methyl-8-hydroxy-isoalloxazine. The proposed structure is confirmed by: (*a*) chemical synthesis of 7-methyl-8-hydroxy-isoalloxazine models and comparison of their spectral and physical properties with those of the natural chromophore, and (*b*) photochemical degradation of the N(10)-polyhydroxy side chain of the FMN portion of the isolated coenzyme to yield 7-methyl-8-hydroxy-alloxazine. The synthesis of these models is outlined.

In a previous communication Mayhew and Massey (1) presented evidence for the occurrence of a novel orange prosthetic group which is associated with NADH-dehydrogenase from the strict anaerobe *Peptostreptococcus elsdenii*. They concluded from their preliminary investigation of its properties that it is a oxidation-reduction active molecule probably related to FAD. The chromophore was easily released from the protein and therefore seems not to be related to the class of (8α)-substituted flavocoenzymes which are covalently linked to the protein, *e.g.* succinate dehydrogenase (2) or monoamine oxidase (3). The spectral properties reported by Mayhew and Massey suggest that the new coenzyme contains a pronouncedly modified flavin group. This has now been confirmed, and in the present communication we wish to report the elucidation of the structure and some properties of this novel coenzyme.

In order to obtain the amounts of material required for physicochemical investigations, large quantities of *P. elsdenii* were grown in low iron medium as described previously (4). The NADH dehydrogenase was then partially purified according to the method of Mayhew and Massey (1). The prosthetic groups were extracted from the enzyme by precipitation of the apoprotein with trichloroacetic acid, and further purified by chromatography on DEAE-cellulose. This chromatography step yields three fractions: (*a*) a yellow fraction (FAD), (*b*) a fraction containing a green chromophore, the structure of which will be reported elsewhere,[1] and (*c*) a fraction containing the orange chromophore. This orange compound is homogeneous and is probably structurally related to FAD as concluded from its chromatographic behavior and from its tight binding to apo D-amino acid oxidase. On incubation with phosphodiesterase it yields AMP and a molecule related to FMN (1). This orange mononucleotide is bound to apoflavodoxin (1) and the resulting complex shows a catalytic activity similar to that of native flavodoxins. In turn the phosphate group of this FMN analogue can be hydrolyzed to yield a molecule which has properties similar to those of riboflavin. Although the orange flavin has certain features common to normal flavins, it can be readily differentiated by its atypical absorption spectrum (Fig. 1) and by the effects of pH on its absorption and fluorescent properties. The pK values (11.5, 4.8, and 0.7) that can be estimated from these pH-dependent changes are in sharp contrast to the ionization characteristics of normal isoalloxazines (pK values at ~0 and ~10) (5) and indicate the presence of an additional functional group. The dramatic effects that the modification or substitution has on the spectral properties indicate that it must be in direct conjugation with the assumed isoalloxazine chromophoric system.

More information about the nature of this modification was obtained from an evaluation of the chemical properties, oxidation-reduction behavior, thin layer chromatography, electrophoretic mobility at different pH values, pH dependence of absorption and fluorescence spectra of the orange chromophore at its FAD, FMN, and riboflavin levels, its NMR spectrum, and from the ESR spectrum of its radical cation. From the results of these experiments and by comparison with the data obtained from available isoalloxazine model compounds, it was concluded that the orange flavin is probably similar to a paraquinoid system (exocyclic double bond at position C(8), *cf. IV* in Scheme 1,



SCHEME 1

in which the 8-methyl group is either substantially modified *and* substituted with an acidic group or replaced by an acidic substituent. The spectral properties of "paraquinoid flavins,"

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[1] S. G. Mayhew, C. Whitfield, S. Ghisla, and M. Schuman-Jorns, manuscript in preparation.

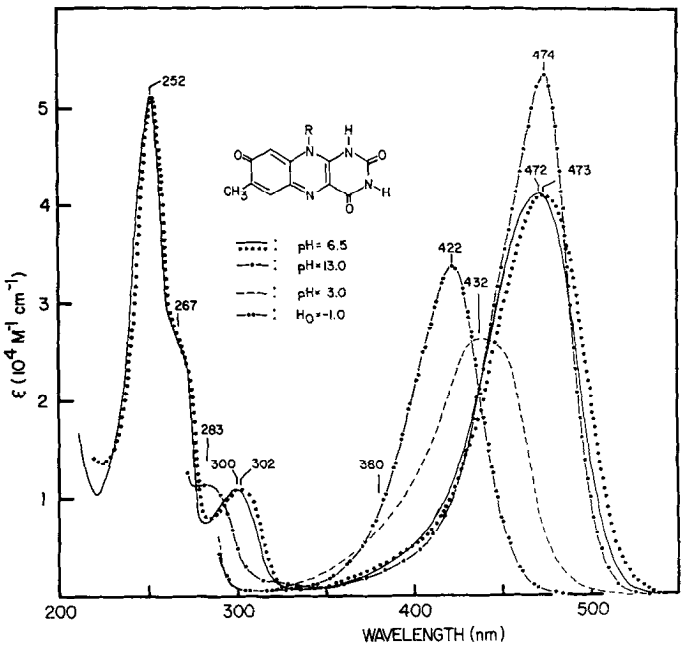


FIG. 1. Absorption spectra of 7-methyl-8-hydroxy-isoalloxazine derivatives (*III* ⇄ *IV*) at different pH values. *Curves* (——), (-·-), (- - -), and (-···-) show the absorption of the synthetic model compounds *III* ⇄ *IVb*, whereas *Curve* (····) represents the absorption of the natural chromophore *III* ⇄ *IVa* in its mononucleotide form (FMN) at pH 6.5 in 0.05 M phosphate buffer. The spectra of the natural orange flavin at pH 13, pH 3, and $H_0 = -1$, which are not shown in this figure, are practically identical with those of the models. Further absorption maxima (omitted in this figure) are: 262 (31,000) and 220 nm (37,000) at pH 3.0 in 0.05 M citrate buffer; 258 (25,000) and 223 nm (32,000) at $H_0 = -1$ in dilute sulfuric acid; and 248 nm (58,000) at pH 13.0 in 0.1 N NaOH solution (ε values are given in brackets as M^{-1} cm^{-1}).

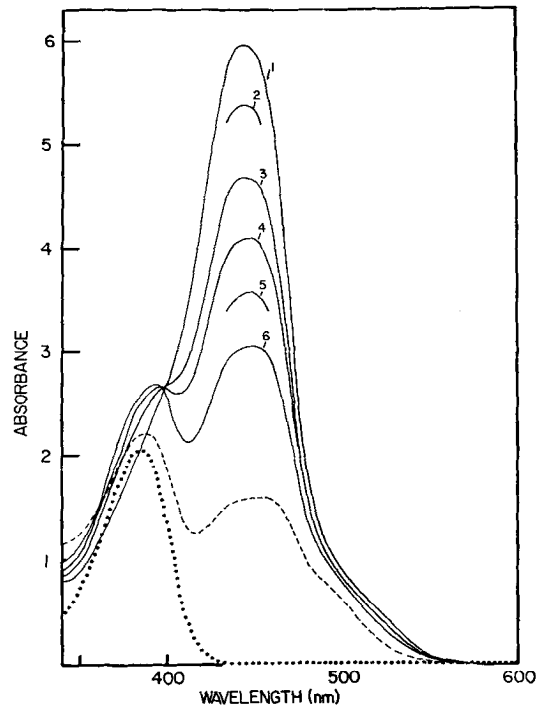
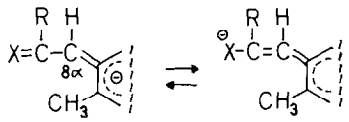


FIG. 2. Spectral course of the photodecomposition of the natural orange flavin *III* ⇄ *IVa* at pH 3.0. *Curves 1, 2, 3, 4, 5,* and *6* after 0, 6, 15, 25, 35, and 50 min of illumination, respectively, with a tungsten-iodine lamp at 20°. The light intensity at the surface of the cuvette was ~10^8 erg cm^{-2} s^{-1}. *Curve* (- - -) after a total of 120 min of illumination; at this point no starting material can be detected by thin layer chromatography in the solution. *Curve* (····) shows for comparison the spectrum of synthetic 7-methyl-8-hydroxy-alloxazine (*V*) at pH 3.

which were synthesized according to a published procedure (6), allowed the exclusion of a C(8α) substitution with a function such as



On the other hand, chemical considerations suggested that a phenolic hydroxyl group, but not a thiol or an amide function, would be the best candidate for a direct substitution at position C(8) of the isoalloxazine. To test this hypothesis 7-methyl-8-hydroxy-10-alkyl-isoalloxazines (*cf.* Scheme 1, *III* ⇄ *IVb*) were synthesized in an unequivocal way: 5-amino-*o*-cresol (*I*, R = H) was converted into its di-trifluoroacetyl derivative and this alkylated *in situ* with methyl iodide and sodium hydride in tetrahydrofuran. The alkylated derivative was hydrolyzed with alkali to yield 5-methyl-amino-*o*-cresol (*I*, R = methyl) and this condensed with violuric acid (*II*) (7) in the presence of sodium borate to yield the product (*III* ⇄ *IVb*, R = methyl) in 10% over-all yield. Alternatively 5-acetylamino-*o*-cresol (8) was reduced with lithium aluminum hydride to the 5-ethyl-amino-*o*-cresol (*I*, R = ethyl), which was reacted with violuric acid (*II*) in the same way to yield *III* ⇄ *IVb*, R = ethyl. The structure of these compounds is confirmed by elemental analysis and NMR spectroscopy. The effects of pH on the absorption and fluorescence emission spectra of the synthetic compounds are almost identical with those of the natural orange chromophore *III* ⇄ *IVa* (*cf.* Fig. 1). A further independent structural proof was obtained by photodegradation of the N(10)-side chain of the "orange FMN" at pH 3 (Fig. 2). This reaction, which is typical of riboflavins and their mononucleotides (9), yields as a main product a compound which is identical with 7-methyl-8-hydroxy-alloxazine (*V*) on thin layer chromatography in four different systems. *V* was synthesized by condensation of 5-amino-*o*-cresol (*I*, R = H) with violuric acid (*II*) in the way outlined above (*cf.* Scheme 1) and shows the expected analytical results. As in the case of the photodegradation of riboflavin and of FMN (9) the reaction leads only to partial degradation of the polyhydroxy N(10)-substituent. Although at present there is no substantial proof for the stereochemical configuration of this chain, it is reasonable to assume that it constitutes a C_5 ribityl unit as in the case of the naturally occurring riboflavin derivatives.

The structure of the orange flavin can be represented in several different tautomeric forms, *e.g.* as an 8-hydroxy-isoalloxazine (*III*) or as an 8-oxo-(iso)alloxazine (*IV*) or in similar forms in which the proton is located at the positions C(2)=O or C(4)=O. *Structure III* would constitute a chromophore with an absorption probably similar to those of isoalloxazines. This is supported by the fact that *III* ⇄ *IVb* yields on acetylation a derivative with spectral characteristics typical of isoalloxazines, namely absorption maxima at 437 (ε ~ 12,000), 350, 263, and 220 nm. From this it is tentatively concluded that acetylation occurs at position C(8)—O (to yield a derivative of *III*) and not in the pyrimidine ring. In contrast to this, methylation yields as a main product a derivative which exhibits a pH-independent spectrum (range pH 3 to 13) very similar to the spectrum of *III* ⇄ *IV* at pH 3. The NMR spectrum of this product shows the presence of two methyl groups in addition to the original signals with chemical shifts suggestive of a nitrogen substitution. From these results it is tentatively concluded that in the o ıge flavin the pK at 4.8 reflects deprotonation at the N(1)H—

C(2)=O amide function (or at its tautomeric C(8) phenolic group), the pK at 11.5 deprotonation at the N(3)H—C(4)=O amide function to form a dianionic species, and that the pK at 0.7 is similar to the protonation of isoalloxazines at low pH (pK $\sim$ 0). Work is in progress to elucidate the exact positional sequence of protonation, and the role and origin of the orange flavin in NADH dehydrogenase.

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