Review Letter

8α-SUBSTITUTED FLAVINS OF BIOLOGICAL IMPORTANCE: AN UPDATING

Dale E. EDMONDSON and Thomas P. SINGER

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143 and Molecular Biology Division, Veterans Administration Hospital, San Francisco, California 94121, USA

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1. Introduction

In the two years which have passed since we wrote a review letter on this subject [1] advances in the field of 8α-substituted flavins have been so rapid that a brief summary of major new developments seems desirable. Updating of our review letter is timely, moreover, because the structure of the fourth class of covalently-bound flavins, previously thought to be 8α-*N*-histidyl-8α-hydroxyriboflavin, has just been conclusively identified as 8α-[*N*(1)-histidyl]-riboflavin [2] and this, in turn, necessitates revision of earlier ideas on the influence of acid on 8α-[*N*(3)-histidyl]-riboflavin. Further, a number of interesting applications of accrued knowledge concerning covalently-bound flavins have been reported very recently.

The present article is intended to call attention to these developments. It is only a supplement to our previous review [1], however, to which reference will be made throughout, and to which the reader is referred for a systematic coverage of the field, while the history of the development of the field has been surveyed elsewhere [3].

2. 8α-[*N*(3)]-Histidyl flavins

Pinto and Frisell have now isolated a flavin peptide from the sarcosine dehydrogenase of a strain of *Pseudomonas* [4]. By the use of aminopeptidase M, which had been previously shown [5] to degrade flavin peptides from succinate dehydrogenase to histidyl flavin, the following amino acid sequence has been determined for the peptide:

flavin
|
–Asp–Lys–Ser–Glu–Gly–His–(Asp,Ala, Thr)–

The pK_a value for the fluorescence quenching of the flavin peptide, at the FMN level, is stated to be 4.8 [4]. This is closer to the pK_a of flavin peptides containing imidazole-*N*(3) than imidazole-*N*(1) linkage to the flavin [2], although it coincides with neither. Thus, while the conclusion that sarcosine dehydrogenase contains *N*(3)-linkage to histidine is probably correct, it would be desirable to prove it by methylation of the peptide and isolation of 1-methylhistidine after acid hydrolysis. The report of Pinto and Frisell [4] thus appears to raise the number of enzymes containing 8α-[*N*(3)-histidyl]-flavin to 3: succinate dehydrogenase, D-6-hydroxynicotine oxidase, and bacterial sarcosine dehydrogenase. The question whether dimethylglycine dehydrogenase contains the same structure still awaits isolation and analysis of a pure flavin peptide from this enzyme.

A significant advance in chemical studies on 8α-[*N*(3)-histidyl]-riboflavin has been the elaboration of a method for the cleavage of the histidine–flavin linkage, permitting recovery of the flavin [6,7]. Until recently methods have been available only for the recovery of the histidine residue after cleavage in good yield, but these procedures (drastic acid hydrolysis or reductive cleavage with H_2 and Pd in CF_3COOH) resulted in extensive or complete destruction of the flavin [8]. In order to recover some of the flavin, reductive cleavage of the histidyl flavin by Zn in CF_3COOH–CH_3COOH had been recommended [5], but, in our hands, the recovery of

riboflavin from synthetic 8α-[*N*(3)-histidyl]-riboflavin was only 10 to 20%. It has now been found [6,7], that recovery of the flavin by reduction with Zn in acid media, following methylation of the unbonded imidazole nitrogen with CH_3I, is increased to 80%. Similarly high yields of flavin have been obtained on reductive cleavage of 8α-[*N*(1)-histidyl]-riboflavin and of the acid modification products of *N*(3) and *N*(1) histidylriboflavins.

This methodological advance assumed great importance in the elucidation of the structure of the flavin components of thiamine dehydrogenase and β-cyclopiazonate oxidocyclase and in proving the structure of acid-modified histidylflavins (cf. below).

3. 8α-[*N*(1)]-Histidyl flavins

At the time our review letter on 8α-substituted flavins [1] was written, 8α-[*N*(1)-histidyl]-riboflavin was believed to be the product of acid modification of the *N*(3) isomer. This conclusion was based on the finding that hydrolysis of either the pentapeptide of histidyl flavin isolated from mammalian succinate dehydrogenase or of synthetic 8α-[*N*α-benzoylhistidyl]-tetracetylriboflavin in 6 N HCl resulted in the gradual formation of two histidyl flavins, separable by thin-layer chromatography or by high voltage electrophoresis, one of which was unambiguously identified as 8α-[*N*(3)-histidyl]-riboflavin, the naturally occurring compound, the other assumed to be 8α-[*N*(1)-histidyl]-riboflavin, the formation of which was suggested to be the result of the acid-catalyzed migration of riboflavin from *N*(3) to *N*(1) of the imidazole ring [5]. The weaknesses of this interpretation were recognized by its proponents at the time: thus both the unidirectional nature of the proposed *N*(3)→*N*(1) migration and the conversion of a sterically less hindered to a sterically more hindered structure are contrary to expectations.

The question whether acid modification of 8α-[*N*(3)-histidyl]-riboflavin indeed yields the *N*(1) isomer assumed great importance when it was found that hydrolysis of the flavin peptides from thiamine dehydrogenase [9] and from β-cyclopiazonate oxidocyclase [10] resulted in the formation of a histidyl flavin which differed in pK_a and in other properties and was clearly separable from authentic 8α-[*N*(3)-histidyl]-riboflavin and what was thought to be the *N*(1) isomer, although linkage of the flavin was by way of the 8α-carbon to one of the two ring nitrogens of histidine.

Since the histidyl flavin moiety of thiamine dehydrogenase and of β-cyclopiazonate oxidocyclase is clearly 8α-linked, as judged by the hypsochromic shift of the absorption spectrum, the e.p.r. spectrum, and the formation of 8-formylriboflavin on storage [9,12], and since one of the imidazole nitrogens was the bonding site to the flavin because of the negative Pauly reaction, the difference between the histidyl flavins derived from these enzymes and both the *N*(3) and the supposed *N*(1) isomers of histidylriboflavin left only one alternative, namely, that the 8α-carbon of the flavin is at a higher oxidation state than in previously known histidylriboflavins. There was some evidence indicating, in fact, that these enzymes contain 8α-[*N*(3)-histidyl]-hydroxyriboflavin. Preliminary n.m.r. studies suggested that only one proton is present in the 8α-position [1]. On storage of the unknown histidylriboflavin in the cold, authentic 8-formylriboflavin was formed, as judged by the e.p.r. spectrum of its cationic semiquinone, its mobility in thin-layer chromatography [6], and by its characteristic cationic hydroquinone absorption spectrum (fig.1).

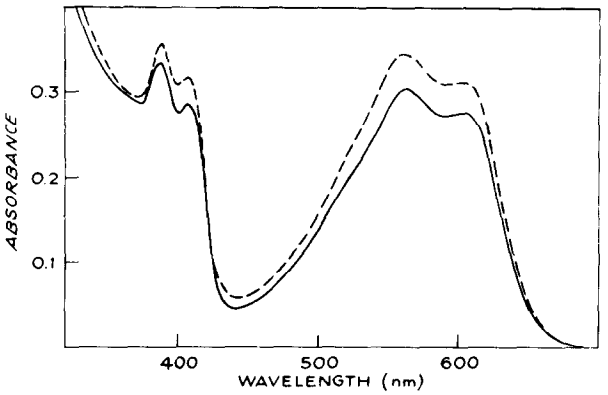
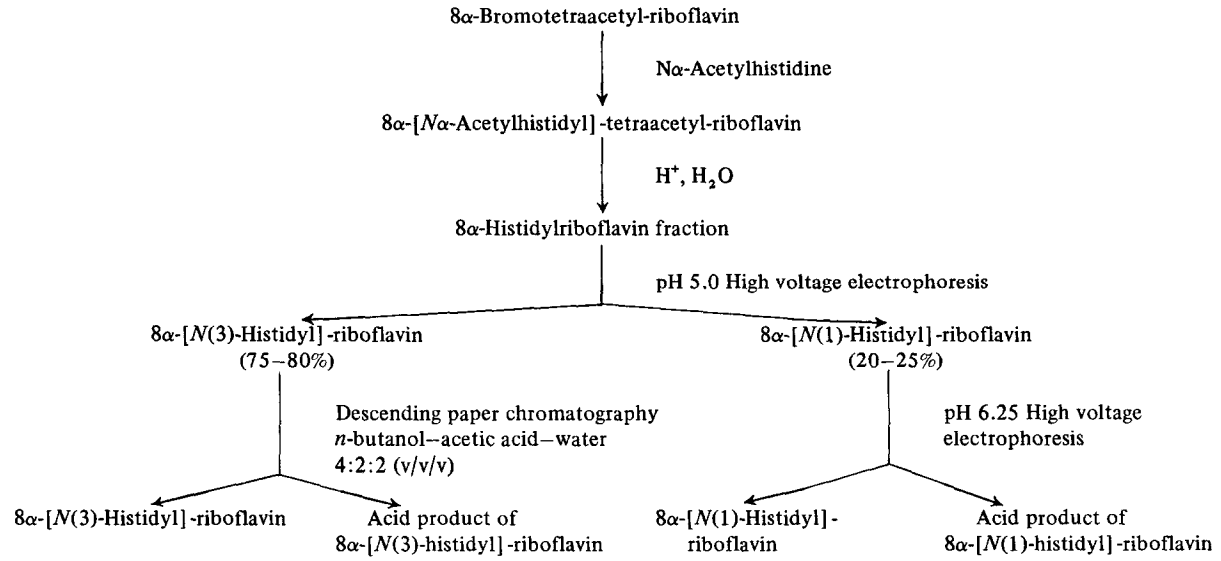


Fig.1. Absorption spectra of the cationic hydroquinone forms of the flavin arising from the breakdown of 8α-[*N*(1)-histidyl]-riboflavin and of 8-formylriboflavin. The histidylriboflavin was refluxed for 8 h in water and the products were separated by high voltage electrophoresis. 8-Formylriboflavin (- - -) and the product of breakdown of 8α-[*N*(1)-histidyl]-riboflavin (——) were dissolved in 6 N HCl and reduced with 1 mM $TiCl_3$.



Scheme 1. Isolation of synthetic 8α-[*N*(1)-histidyl]-riboflavin.

Characterization of the 'histidyl-riboflavin X' component of thiamine dehydrogenase and of β-cyclopiazonate oxidocyclase was facilitated by the finding [2,6] that the condensation of 8α-bromotetraacetylriboflavin with *N*α-acetylhistidine yields, besides 8α-[*N*(3)-histidyl]-riboflavin, histidyl-riboflavin X in 20–25% yield of the total histidyl flavin fraction (scheme 1). With the availability of this new histidyl-riboflavin in micromole quantities, it became possible to show that the compound contained an 8α-methylene group, as judged by integration of this resonance in the Fourier-transform n.m.r. spectrum at 300 MHz [2,6], thus ruling out the contemplated histidyl-hydroxyriboflavin structure.

With further, conclusive evidence from ENDOR and from e.p.r. studies [2,6] that the linkage to histidine was by way of the 8α-carbon, the conclusion was inescapable that the structure of one of the model compounds used in earlier studies had not been correctly assigned. Since proof for the structure of 8α-[*N*(3)-histidyl]-riboflavin was unambiguous [5], whereas the structure of the *N*(1) isomer had never been adequately investigated, the latter compound was subjected to methylation and acid hydrolysis. The product found was 1-methylhistidine [2], proving that acid treatment of 8α-[*N*(3)-histidyl]-riboflavin or of its peptides does *not* cause migration of the flavin from *N*(3) to *N*(1) of the histidine. What it does cause is cyclization of the ribityl chain (cf., below), which leaves the absorption and fluorescence spectral properties unaffected but changes the mobility in chromatographic systems, as well as perturbs the pK_a of the imidazole nitrogen, so as to make it separable from the parent compound on high voltage electrophoresis.

The same experimental procedure, methylation and acid cleavage, showed that the synthetic histidyl-riboflavin, whose properties coincide with those of the natural compound isolated from thiamine dehydrogenase or from β-cyclopiazonate oxidocyclase, yields 3-methylhistidine. This proves that the flavin moiety of these two enzymes is 8α-[*N*(1)-histidyl]-riboflavin. In the enzymes mentioned the flavin occurs at the dinucleotide level [11,12]. Comparison of the properties of the *N*(3) and *N*(1) isomers of 8α-histidyl-riboflavin (fig.2) is given in table 1. It is noteworthy

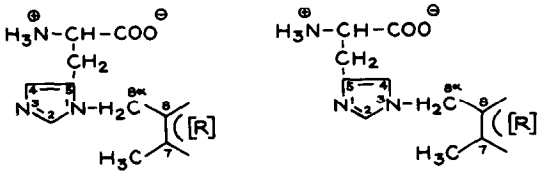


Fig.2. Structures of 8α-[*N*(1)-histidyl]-riboflavin (left) and of 8α-[*N*(3)-histidyl]-riboflavin (right).

Table 1
Comparison of the properties of 8α-[*N*(1)-histidyl]-riboflavin with those of 8α-[*N*(3)-histidyl]-riboflavin

Property	8α-[*N*(1)-Histidyl]-riboflavin	8α-[*N*(3)-Histidyl]-riboflavin
pK_a of imidazole	5.2	4.7[a]
pK_a of imidazole	5.0	4.5[b]
Acid-modified product		
Oxidation–reduction potential ($E_{m,7}$)	−165 mV	−160 mV[b]
K_d for sulfite adduct formation	0.087 M	0.056 M[b]
Second-order rate constant for sulfite adduct formation	1.64 M^{-1} min^{-1}	1.94 M^{-1} min^{-1}[b]
Reduction by BH_4^-	Yes	No
Electrophoretic mobility at pH 5.0 (FMN = + 1)	−1.1	−0.88
Stability to storage in H_2O (30 days at room temperature in the dark)	80% decomposed to 8-formylriboflavin	97% recovery of original material

[a] Data of Walker et al. [5].
[b] Data of Edmondson and Singer [18].

that $NaBH_4$ readily reduces the *N*(1) isomer (although reoxidation by air does not result in recovery of the original compound [2,6]), while the *N*(3) isomer is unaffected.

A recent report from Sato's laboratory [13] describes the purification of a flavin peptide from acid-denatured L-gulono-γ-lactone oxidase, which is obtained from rat liver. The peptide, at the FMN level, exhibited a pK_a of 5.8 for fluorescence quenching, the same value as had been described for the flavin peptide from thiamine dehydrogenase [9]. Moreover, $NaBH_4$ reduces the flavin peptide from either enzyme. It may be tentatively concluded, therefore, that L-gulono-γ-lactone oxidase, along with thiamine dehydrogenase and β-cyclopiazonate oxidocyclase, contains 8α-[*N*(1)-histidyl]-riboflavin at the active site.

4. Structure of acid-modified histidylriboflavins

The flavin product isolated from reductive Zn cleavage of either 8α-[*N*(3)- or 8α-[*N*(1)-histidyl]-riboflavin after methylation is, as expected, riboflavin. Reductive cleavage of either of the corresponding acid-modified methylated histidyl flavin isomers yields a flavin product (flavin X) with identical absorption, fluorescence, and e.s.r. spectral properties to those of riboflavin, but differing from riboflavin in mobility in several thin-layer chromatography systems [2]. These results suggested that acid treatment modified the ribityl side chain rather than the isoalloxazine ring. This was verified by the production of lumiflavin on alkaline photolysis of flavin X. Acid treatment of riboflavin or FMN also yields a flavin compound with identical chromatographic and spectral properties to those of flavin X. Thus, acid modification of the ribityl side chain takes place whether or not the 8α position is substituted.

While riboflavin consumes three moles of periodate with the release of one mole of formaldehyde (originating from the terminal 5′-hydroxyl group), flavin X consumes one mole of periodate with no liberation of formaldehyde [14]. These results suggested the structure of flavin X to be 2′,5′-anhydroriboflavin (fig.3). This structure was unequivocally

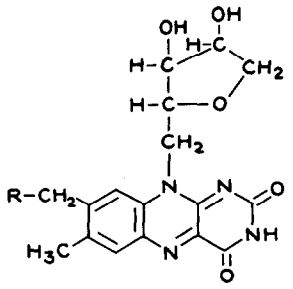


Fig.3. Structure of 2′,5′-anhydroflavin, the acid modification product of histidylriboflavin, where R = histidyl residue.

shown by chemical ionization mass spectrometry of the trimethylsilylated derivative which gave an experimental value for the mol. wt. of 502 (theory = 502 for the cyclic ether with two trimethylsilyl groups present) while the silylated derivative of riboflavin had a mol. wt. of 664 (theory = 664, with four trimethylsilyl groups present).

The effect of acid treatment on either riboflavin or 8α-substituted histidylflavins is to cyclize the ribityl side chain to form a cyclic ether between the 2′ and 5′ carbons of the ribityl side chain. These results are consistent with those of Baddiley and co-workers [15], which showed that acid treatment of ribityl or ribityl phosphate results in cyclization to an anhydro ether. These same authors also postulated that this might also occur in the case of riboflavin or FMN.

5. 8α-*S*-Cysteinyl flavins

We have previously emphasized [1] the instability of cysteinyl flavins, particularly that of 8α-*S*-cysteinyl-riboflavin. In addition to its known tendency to be readily oxidized and hydrolyzed [16,17] and to undergo reductive cleavage by dithionite, following oxidation to the sulfone [18], it has been noted, in preliminary studies performed in collaboration with Dr E. E. Snell, that 8α-cysteinyl-riboflavin rapidly decomposes to yield pyruvate plus a flavin product at room temperature. The mechanism of this cleavage remains to be elucidated. As noted earlier [1], the flavin pentapeptide from monoamine oxidase is considerably more stable, possibly reflecting the influence of the adjacent tyrosyl group.

Recently [19,20], McCormick and co-workers have synthesized various 8α-cysteinyl flavin peptides related to the flavin peptide isolated from monoamine oxidase. The spectroscopic and chemical properties of the synthetic flavin peptides agreed with those of the natural material. An interaction between the tyrosyl residue and the flavin was established from circular dichroism and fluorescence spectra. It was also found [19] that incorporation of an *N*-acetyl blocking group on the amino acid or peptide moiety imparts a stability to the cysteinyl flavin peptides, so that they may be purified and stored under standard conditions, without the decomposition observed with 8α-*S*-cysteinyl-riboflavin itself. The possibility that the tyrosyl residue contributes to stability was not discussed [19].

An extensive study of the fluorescence properties of 8α-*S*-cysteinyl flavin analogs has shown that the fluorescence yield, relative to that of the unsubstituted flavin analog, is markedly dependent on the solvent [20]. The relative fluorescence yield varies from 3% in aqueous buffer to 60% in dioxane [20]. These results show the fluorescence quenching by the non-bonding electrons of the 8α sulfur residue to be dependent on solvent–flavin interactions.

The elaboration of a procedure for the quantitative estimation of the cysteinyl flavin content of proteins (see section 8) has permitted re-examination of the puzzling report [21] that monoamine oxidase from pig brain mitochondria contains non-covalently linked flavin, although the same enzyme in pig liver contains covalently-bound cysteinyl flavin. This conclusion rested on the observations that: (a) at the end of a long purification procedure, but not at any earlier step, trichloroacetic acid released free FAD from the enzyme; and (b) at the terminal stage the acid–ammonium sulfate procedure produced a virtually inactive, non-fluorescent protein, which was interpreted to be an apoenzyme, since the activity was partially restored on adding FAD to it, as in the conventional reconstitution of a holoenzyme. Tipton [21] recognized that this evidence was inconclusive, since if the linkage were non-covalent, FAD should be released at all stages of purification, and, further, the dissociation constant for FAD, determined from reconstitution of the activity, was far too high to account for the observed stability of activity during isolation.

Since Tipton's procedure for the isolation of monoamine oxidase [22] could not be reproduced in other laboratories, other methods were developed for purification of the enzyme from pig brain, one of which appeared to yield a sample of sufficient purity to give a single band on disc electrophoresis [23,24]. With the help of a method for cysteinyl flavin determination recently elaborated (cf. Section 8), all purified samples of the enzyme from pig brain were found to contain covalently-bound cysteinyl flavin, although quantitative estimation was restricted to highly purified samples, in which histidyl flavin originating from succinate dehydrogenase was low. When the turnover number of monoamine oxidase (expressed per mole of covalently-bound flavin) was compared in the most purified preparations from brain and from kidney [25], nearly the same value was obtained. This turnover number remained relatively constant during the last four steps in the isolation of the enzyme from brain [23,24]. It may be concluded, therefore, that the predominant, if not the only, form of monoamine oxidase in brain mitochondria contains covalently-bound flavin, as does the enzyme from liver and kidney. Although even the purest preparation from brain released a small amount of FAD on denaturation with trichloroacetic acid, this FAD probably does not originate from monoamine oxidase [23], since samples of the enzyme inhibited at an earlier stage of purification with [^{14}C]*N, N*-dimethylpropargylamine, a 'suicide inhibitor' which forms an irreversible adduct with the flavin (cf. Section 9), also released free flavin but no radioactivity at the terminal stage on adding trichloroacetic acid [26].

6. 8α-*S*-Cysteinyl flavin thiohemiacetals

At the time our review letter on 8α-substituted flavins [1] was written, the detailed evidence for the conclusion that *Chromatium* flavocytochrome c_{552} contains a cysteinyl flavin thiohemiacetal had not yet appeared. The full papers on the structure of the flavin peptides from *Chromatium* cytochrome c_{552} have appeared in the meantime [27,28]. A logical follow-up of this work was an investigation of the structure of the flavin of the related enzyme, flavocytochrome c_{553} from *Chlorobium thiosulfatophilum*. Bartsch et al. [29] and Meyer et al. [30] have reported that the flavin moiety of this cytochrome behaves like the flavin of *Chromatium* cytochrome c_{552} in that it is not liberated by acid–ammonium sulfate or by trichloroacetic acid but is separable from the protein after incubation with urea for 2–4 days. As in the case of the *Chromatium* flavin [1] the agent responsible for liberation of the flavin may be the cyanate impurity present in urea.

A highly purified preparation of a peptic flavin peptide from *Chlorobium* cytochrome c_{553} has now been isolated by a brief and simple procedure [31]. The peptide is at the FAD level. Its fluorescence is very low, even at the FMN level (7% of the fluorescence of riboflavin or less), but is increased to about 70% of the fluorescence yield of riboflavin on oxidation with performic acid. This is accompanied by a hypsochromic shift of the second absorption band from 367 nm to about 354 nm. These data leave little doubt that the flavin in *Chlorobium* flavocytochrome c_{553} is 8α-linked to cysteine [31]. Since the fluorescence yield of the performic acid-oxidized flavin peptide is higher than that of the sulfur-oxidized flavin thiohemiacetal isolated from *Chromatium* [27,28] but lower than that of the sulfur-oxidized flavin thioether from monoamine oxidase [16], more work is needed to decide which of the two structures is present in the *Chlorobium* flavocytochrome.

7. 8-Substituted flavins

In addition to the naturally occurring 8α-substituted flavins, a new class of flavin coenzymes in which the 8-CH_3 group is replaced by either an oxygen or a nitrogenous group has recently been investigated and their structures elucidated.

8-Hydroxy-FAD has been found in an NADH dehydrogenase from *Peptostreptococcus elsdenii*. The purified flavin analog has absorption maxima at 470 and at 380 nm and is fluorescent with an emission maximum at 530 nm [32]. Proof of the structure was obtained by synthesis of a 7-methyl-8-hydroxy-isoalloxazine and showing the identity of its physical and chemical properties with the isoalloxazine obtained from the photochemical degradation of 8-hydroxy-FMN [33]. The pK_a of the phenolic 8-hydroxyl group was found to be 4.8 [33] and, as

expected, the state of ionization profoundly affected the absorption spectrum of the chromophore. This property has been utilized to probe the environment of the flavin binding site in a number of flavoenzymes by binding the appropriate 8-hydroxyflavin analog to the apoenzyme and determining the pK_a of the 8-OH group of the bound flavin [34].

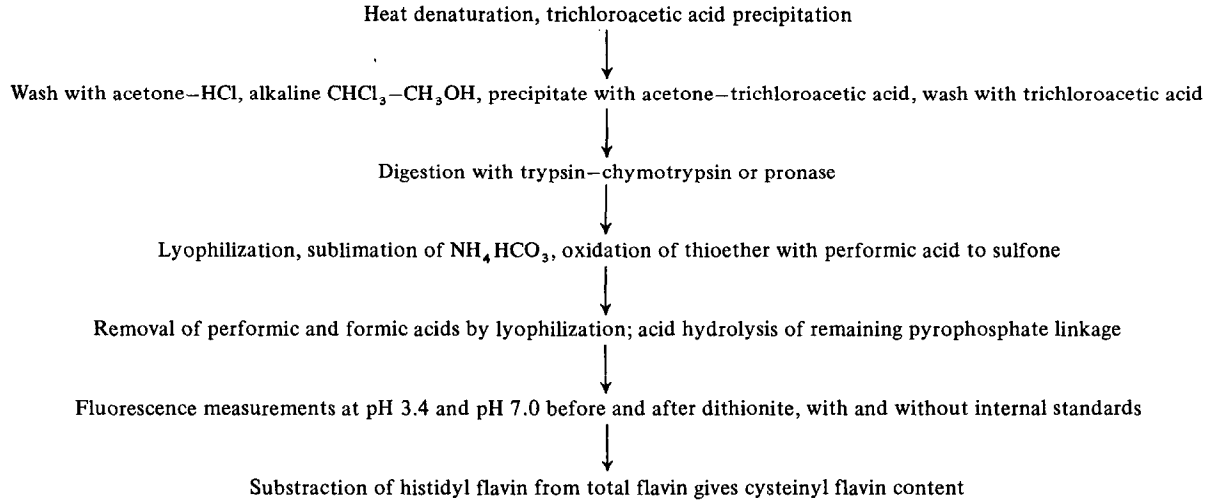
A second 8-substituted flavin (named roseoflavin because of its red color) has been isolated from the culture broth of *Streptomyces* strain No. 768 and the structure determined to be 7-methyl-8-dimethylamino-10-D-ribityl-isoalloxazine [35]. Photolysis of the natural material yields two photoproducts: 7-methyl-8-dimethylamino alloxazine under acidic conditions and 7-methyl-8-methylamino-D-ribityl-isoalloxazine under basic conditions [36]. Their structures were proven by showing that their chemical and physical properties agree with those of synthetic compounds.

Although its oxidation–reduction potential (−222 mV) is only 30 mV more negative than that of riboflavin, roseoflavin has been shown to exhibit an antagonistic effect on riboflavin activity in nutritional studies on weanling rats and also inhibits the growth of *Lactobacillus casei*, a strain which is absolutely heterotrophic with respect to riboflavin [37].

8. Determination of various types of covalently-bound flavins

We have emphasized the numerous difficulties involved in the quantitative determination of covalently-bound flavin in tissues or enzyme preparations in which more than one type of covalently-bound flavin is present [1]. The problem has been accentuated by the discovery that 8α-[*N*(1)-histidyl]-riboflavin is widely distributed and is, in fact, probably present in mammalian liver as a constituent of L-gulono-γ-lactone oxidase. Despite the different pK_a values of the *N*(3) and *N*(1) isomers of histidyl-riboflavin, differential fluorometry at pH 3.2 and at 7.0 would co-determine both types of histidyl flavin.

Progress in the determination of covalently-bound 8α-substituted flavins during the past two years has been reported in connection with the analysis of cysteinyl flavin in monoamine oxidase from brain [23,24] and the determination of covalently-linked 5-deazariboflavin in the presence of histidyl-riboflavin [38]. The analytical scheme evolved for cysteinyl flavin is shown in scheme 2. The procedure appears to yield reliable values only if the histidyl-riboflavin content of the sample does not exceed the cysteinyl-riboflavin content [23,24].



Scheme 2. Determination of cysteinyl flavin in monoamine oxidase.

A method for the quantitative determination of covalently-bound 5-deazaflavin became necessary when it was found [38] that riboflavin-requiring mutants of yeast could utilize 5-deazaflavin for growth, provided that a small concentration of riboflavin, insufficient for optimal growth, was included. Cells grown on mixtures of riboflavin and 5-deazariboflavin appeared to incorporate both in covalent linkage to protein in the mitochondria. Two methods were developed for the simultaneous determination of the two types of covalently-bound flavin in yeast. The first one relied on the fact that the fluorescence emission maximum of covalently-bound riboflavin is 540 nm, while that of covalently-bound 5-deazariboflavin is 470 nm. The second method takes advantage of the much greater affinity of deazaflavin than of riboflavin for sulfite adduct formation. In the flavin peptide fraction from cells grown in the presence of both 5-deazariboflavin and riboflavin, the fluorescence due to deazaflavin is almost completely quenched by sulfite, whereas that due to bound-riboflavin is unaffected. The two methods gave identical values for the content of bound-riboflavin and bound-deazariboflavin in the mitochondria, despite the preponderance of the former [38].

9. Applications

9.1. *Mechanism of inactivation of monoamine oxidase by suicide inhibitors*

Among the inhibitors of mitochondrial monoamine oxidase, acetylenic amines, as exemplified by pargyline, have attracted wide interest, in part because several compounds of this type have been used clinically as antidepressants or antihypertensive agents. Inhibitors of this type have been called 'suicide inhibitors' because an irreversible inhibitor is produced by the action of the target enzyme from a relatively innocuous compound, which acts as a substrate. Determination of the structure of the adduct isolated from the inactivated enzyme may yield a clue to the normal catalytic mechanism.

Pargyline, an acetylenic amine, was shown by Hellerman and Erwin [39] to inhibit kidney monoamine oxidase irreversibly. The substrate protects from the inhibition. The latter results from the formation of a 1:1 covalent adduct between the enzyme and the inhibitor. Later, Chuang et al. [25] suggested that the inhibition may involve the flavin because the 455 nm band of the flavin disappears and is replaced by one at 410 nm and because [7-^{14}C] pargyline is recovered in the flavin peptide fraction after proteolytic digestion of the inactivated enzyme. Isolation of the pure adduct was not attempted, however. Structures for the flavin–inhibitor adduct have been proposed [40,41] on the basis of the similarity between the spectra of the inactivated enzyme and of compounds produced by the photochemical reaction between acetylenic amines and 3-methyllumiflavin. Final proof had to await, however, the isolation and structural characterization of the flavin–inhibitor adduct from inhibited monoamine oxidase.

It has now been shown that the development of the irreversible inhibition of liver monoamine oxidase by 3-dimethylamino-1-propyne obeys first-order kinetics and follows the characteristics of 'dead end inhibition' [42,43]. Development of the inhibition is characterized by the appearance of a new band at 410 nm, concurrently with the loss of the typical spectrum of flavoproteins (fig.4, inset). After proteolytic digestion, the absorption maximum of the cysteinyl flavin–inhibitor adduct shifts to 375 nm (fig.4). The extremely high absorbance (ϵ_{375} = 36 000) is explained by the highly conjugated structure of the adduct (fig.5).

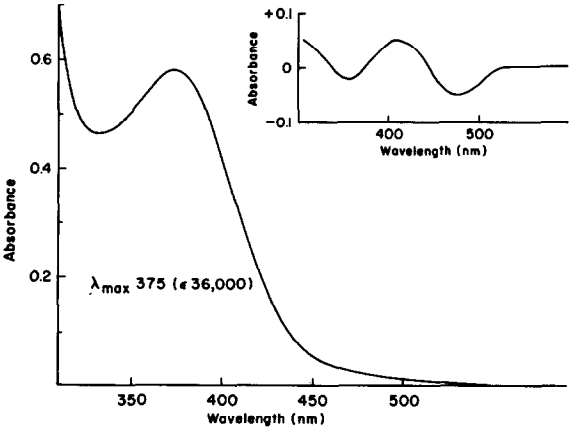


Fig.4. Absorption spectrum of the purified flavin peptide–inhibitor adduct isolated from monoamine oxidase. Inset, difference spectrum of inhibited enzyme versus untreated monoamine oxidase.

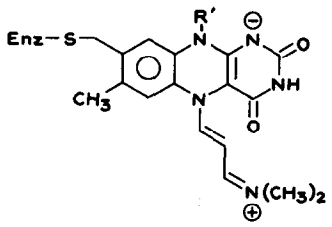


Fig.5. Structure of the covalent adduct formed by the inactivation of monoamine oxidase with 3-dimethylamino-1-propyne.

The structure of the compound has been elucidated by inactivating the purified enzyme with [^{14}C]3-dimethylamino-1-propyne, removing unreacted inhibitor, isolating the flavin peptide–inhibitor adduct, and determining its structure on the basis of its chemical reactivities and physical properties [42–44]. The essential features of the structure agree with those of the model compound derived from the photochemical reaction between this inhibitor and 3-methyllumiflavin [41]. It may be reasonably assumed that other acetylenic 'suicide inhibitors' of monoamine oxidase, such as pargyline, clorgyline, and Deprenyl inhibit the enzyme because of covalent interaction of the 8α-substituted flavin in an analogous manner.

9.2. *The binding of 8α flavins and flavin peptides by apoflavoenzymes*

The binding of 8α-flavin peptides (at the FMN level), isolated from several enzyme sources, to apoflavodoxin from *Azotobacter vinelandii* has been investigated as a potential method for flavin peptide purification, as well as a means to assess the effect of 8α substitution on flavin binding [45]. Shiga et al. [46] have studied the binding and redox properties of several synthetic cysteinyl flavin peptides to the *Azotobacter* flavodoxin. Rather tight binding of natural and synthetic 8α flavins and flavin peptides to the apoprotein has been observed: while the FMN analogs are bound 10–20-fold less tightly then FMN itself, 8α riboflavin analogs are bound to 2–28-fold more tightly than riboflavin itself [45].

The interaction of the 8α flavins and flavin peptides with other apoflavoenzymes has been investigated to a limited extent. Synthetic 8α-[*N*(3)-histidyl]-FMN is tightly bound by apo 'Old Yellow Enzyme' [47] and restores 100% the catalytic activity of the native holoenzyme [7]. In contrast, neither the 8α-[*N*(3)-histidyl]-FAD pentapeptide from succinate dehydrogenase nor the 8α-*S*-cysteinyl-FAD pentapeptide from monoamine oxidase restores any catalytic activity to apo-D-amino acid oxidase or apo-glucose oxidase [7].

9.3. *Flash photolysis studies of 8α-substituted flavins*

Flash photolysis techniques have been used to study the effect of 8α substitution on the flavin triplet, as well as on the reactivity of the neutral and anionic semiquinone forms of the flavin [48,49]. The presence of an 8α histidyl or cysteinyl residue substantially reduces the yield of flavin triplet, since little or no 680 nm absorption is observed at times where riboflavin gives substantial triplet absorption [48]. Of the 8α-substituted flavins investigation only 8-formyl-riboflavin and 8α-cysteinylsulfonetetraacetyl-riboflavin give substantial yields of flavin triplet [48,49].

Edmondson et al. [48] have shown that 8α substitution shifts the pK_a of the neutral flavin semiquinone from 8.4 (the value for riboflavin) to 7.35 for 8α-[*N*(3)-histidyl]-riboflavin and 6.94 for 8-formylriboflavin. These values were obtained by studying the pH dependence of the reaction of 8α flavin semiquinones with oxygen. The chemical rationale for this shift in pK_a has been suggested to be stabilization of the anionic semiquinone form through electron-withdrawing inductive effects by the 8α substituents [48].

9.4. *Studies on the biosynthesis of enzymes containing covalently-bound flavin*

Two laboratories have reported interesting findings on the biosynthesis of enzymes containing covalently-bound flavin. Brühmüller et al. [50] have been studying the induction of D-6-hydroxynicotine oxidase in *Arthrobacter oxidans*, an enzyme which contains covalently-bound 8α-[*N*(3)-histidyl]-riboflavin. Since this is an inducible enzyme, its formation requires special growth conditions. When the bacteria were grown in a medium which fails to induce the formation of D-6-hydroxynicotine oxidase activity, a protein was nevertheless synthesized which lacked flavin but cross-reacted with an antibody to the holoenzyme. This has been interpreted to suggest that a flavin-free apoenzyme is formed under these

conditions and thus the induction of D-6-hydroxynicotine oxidase activity actually involves incorporation of the flavin into a preexisting apoenzyme or related precursor.

Grossman et al. [38] have been investigating the incorporation of riboflavin analogs into the covalently bound flavin of succinate dehydrogenase during the oxygen-induced development of respiration which occurs on exposure of anaerobic yeast cells to O_2. Three analogs were found to be incorporated into succinate dehydrogenase: 7α-methylriboflavin, 8α-methylriboflavin, and 5-deazariboflavin. The first two permit optimal growth on riboflavin-requiring mutants of yeast; 5-deazariboflavin does not promote growth by itself but is incorporated if a very low level of riboflavin is also present. The first two analogs had been previously studied by Lambooy and co-workers [51,52], who reported that weanling rats grown on either of these analogs show reduced succinate dehydrogenase activity in tissues, although incorporation of the analogs in the covalently-bound flavin of succinate dehydrogenase was not demonstrated.

In yeast cells, both of these analogs are incorporated into the covalently-bound flavin fraction and lead to the formation of succinate dehydrogenase with a somewhat reduced turnover number [38]. In the case of 7α-methylriboflavin, the analog-containing enzyme was purified, its flavin peptide separated, isolated in pure form, and the presence of 7α-methylriboflavin linked to histidine was demonstrated by reductive cleavage, following imidazole methylation of the histidyl flavin.

Yeast cells adapted to oxygen in the presence of 5-deazariboflavin and riboflavin contain both types of flavin in covalent linkage to the protein. Interestingly, in such preparations the turnover number of succinate dehydrogenase is several times higher than in cells grown on (or adapted to O_2 with) riboflavin alone. This extraordinary behaviour survives extraction and extensive purification of the enzyme. It is not known, however, whether 5-deazaflavin undergoes oxidation–reduction in the enzyme or whether it modulates the activity of a riboflavin-containing enzyme in some manner. Investigation of this interesting question and elucidation of the site of attachment of 5-deazaflavin to the enzyme awaits further work. It may be mentioned that a histidyl-5-deazaflavin linkage does not seem very likely because flavin peptides containing 5-deazaflavin do not show the expected pH-dependent fluorescence quenching [38].

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