Review Letter

8α-SUBSTITUTED FLAVINS OF BIOLOGICAL IMPORTANCE

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1. Introduction

The existence of riboflavin in covalent linkage with proteins was first indicated by concurrent studies in two laboratories in 1955. Boukine [1] reported that a substantial fraction of the flavin in a variety of plant and animal tissues became acid-extractable only after proteolytic digestion or autolysis, whereas Green et al. [2] demonstrated the same for a preparation derived from the inner mitochondrial membrane. Both groups correctly predicted, without solid evidence, however, that the tightly bound flavin is associated with succinate dehydrogenase. Evidence for this was obtained only after isolation of the enzyme in highly purified form [3,4] and the demonstration that its flavin component is released by proteolysis but not by conventional methods of denaturation [4,5].

The historical aspects of the chemistry of covalently bound flavins have been reviewed elsewhere [6], so that it may suffice in the present paper to list merely the major steps in unraveling their structure. Flavin peptides from succinate dehydrogenase were purified both in China [7] and in the USA [4,5,8] but the first pure flavin peptide was obtained by Kearney [9]. She was also responsible for the demonstration that the attachment of the peptide is to the isoalloxazine ring itself and not to the rest of the FAD moiety, since the lumiflavin degradation yielded a lumiflavin peptide [5,9]. She further called attention to the fact that both the isolated pentapeptide, after cleavage to the FMN level, and the product of acid hydrolysis (95°C, 6 N HCl) show major fluorescence quenching on transition from pH 3.2 to 7, with a pK_a of 4.6. Much later [10,11] this fact helped to identify the substituent on the flavin as histidine. The hypsochromic shift of the second absorption band in the neutral flavoquinones was noted early [4,5] and has since become a distinguishing feature of 8α-substituted flavins [12–14]. Later, Chi et al. [15] showed that hydrolysis of the flavin peptide with $Ba(OH)_2$ liberates free urea, rather than a ureido peptide, thus eliminating positions 1, 2, and 3 in the flavin ring system as the site of peptide attachment.

The fact that the peptide is attached to the 8α-CH_2 group of the flavin was demonstrated by the differences in the EPR hyperfine spectra of the free radical cation of the flavin from that of riboflavin and by differences in the absorption spectra, while these properties closely resembled the EPR and optical spectra of 8α-substituted synthetic flavins [12–14]. Confirmatory evidence came from ENDOR and NMR studies [11,16].

Similar, though less extensive, evidence shows that the flavin is also attached to the peptide chain in all other enzymes hitherto reported to contain covalently bound flavin, including monoamine oxidase [17,18], *Chromatium* cytochrome c_{552} [19], sarcosine dehydrogenase [20], thiamine dehydrogenase [21], and β-cyclopiazonate oxidocyclase [22].

The attachment of the peptide to the 8α-CH_2 group is by way of N(3) of the imidazole ring of histidine in succinate dehydrogenase [10,11],

D-6-hydroxynicotine oxidase [23], and probably also in sarcosine dehydrogenase [20]. In thiamine dehydrogenase [21] and β-cyclopiazonate oxidocyclase [22], the histidine substituent is also attached via a nitrogen of the imidazole ring of histidine, but the nature of the bond to the 8α-group of the flavin is different than in succinate dehydrogenase. In mitochondrial monoamine oxidase the linkage is a thioether to cysteine, while in *Chromatium* cytochrome c_{552} it appears to be a cysteinyl thiohemiacetal [6,24]. Thus, while the linkage of the peptide to the flavin shows considerable variation, it involves the highly reactive 8α-CH_3 group of the flavin in each known case. This fact may be of considerable importance in considering the mechanism of the biosynthesis of covalently bound flavins.

2. Approaches used in localizing the site of attachment of the flavin

The chemical approaches, listed in the introduction, eliminated all positions on the succinate dehydrogenase flavin molecule except the benzenoid ring as the site of peptide attachment. To provide unequivocal evidence for the site of attachment, magnetic resonance techniques were applied which included EPR, ENDOR, and NMR spectroscopy.

Flavin cation semiquinones can be conveniently prepared by the anaerobic addition of one molar equivalent of $TiCl_3$ under acidic conditions. EPR studies on various flavin analogs show appreciable isotropic hyperfine coupling between the unpaired electron and the nuclei at the following positions: N(5), H(5), H(8α), N(10), and H(10α) [25]. Thus, substitution of an 8α hydrogen by an amino acid would be expected to influence drastically both the linewidth and resolution of the EPR spectrum, because of the loss of a coupled proton as well as restriction of the rotation of the resulting methylene group. As will be discussed below, other factors could also influence the observed EPR spectra.

The EPR spectrum of the 8α-[*N*(3)]-histidyl flavin peptide from succinate dehydrogenase has a total linewidth of 46 gauss, as compared with a value of 52 gauss for riboflavin [12–14]. A similar reduction in linewidth is also observed in comparing the spectrum of the flavin peptide at the lumiflavin level with that of lumiflavin [12–14]. In the case of 8α-[*N*(3)]-histidylflavin peptides it appears that the reduction in linewidth of the EPR spectrum (6 gauss) can be explained by the loss of a proton by substitution (coupling constant of 3.4 ± 0.2 gauss) and by the loss of another 'EPR active' proton by restricted rotation of the 8-methylene group. This explanation is not satisfactory for other 8α-substituted flavins, as the linewidth values for the cationic semiquinones of 8-carboxyriboflavin [19] and 8α-*S*-cysteinylriboflavin [18,26] are reduced by 13–15 gauss from that of riboflavin. As the cysteinyl flavin has methylene protons available for coupling, this would suggest that other factors induced by substitution may be responsible for the reduced linewidth. It has been pointed out [14] that substitution may influence the radical relaxation time, which, in turn, would affect both the EPR linewidth and resolution. In this regard, the EPR spectra of 8α-*S*-cysteinylriboflavin [18,26] and 8-carboxyriboflavin [19] show considerably more resolution than that of 8α-[*N*(3)]-histidylriboflavin [12,14].

To provide more conclusive evidence for 8α substitution in the flavin peptide from succinate dehydrogenase, the ENDOR spectra were determined [13,14,16]. Methyl groups with appreciable hyperfine coupling to an unpaired electron give intense ENDOR signals, while methylene protons usually give weak, broad signals at low temperatures, since hyperfine coupling to them is dependent on their orientation, which is usually not well defined. Thus, the strong ENDOR signal from the 8-methyl group of the riboflavin cationic semiquinone at 18 MHz is missing in the spectrum of the flavin peptide from succinate dehydrogenase [13,14,16], thereby providing conclusive evidence for 8α substitution. This technique was shown to be quite unambiguous in elucidating the structure of the covalently bound flavin from succinate dehydrogenase. It has not been utilized, however, in the elucidation of structures of the other 8α-substituted flavins.

Due to the relatively large amounts of material required, NMR spectroscopy has been used only in the structural analysis of the 8α-[*N*(3)]-histidylflavin from succinate dehydrogenase [11]. In agreement with conclusions from EPR and ENDOR studies, the CH_3 (7), H (6), and H (9) protons appear, thus proving the 8 position as the site of substitution.

Resonance peaks due to the 2 and 4 position protons of the imidazole ring are the same for the N(1) and N(3) histidylflavin isomers [11]. The main differences seen in comparing the NMR spectra of the two isomers are a shift in the H(9) from 8.00 ppm (N(3) isomer) to 8.7 ppm (N(1) isomer) and a broadening of the resonance peak of the ribityl side chain protons [11]. This latter point suggests a difference in orientation of the ribityl side chain with the isoalloxazine ring for the two isomers.

With the availability of the more sensitive Fourier transform NMR spectrometer, it should be possible to obtain spectra of flavin peptides at quantities as low as 1 μmole. This would be invaluable in the structural analysis of new 8α-substituted flavins, as well as in studies of non-covalent amino acid–flavin interactions.

Perhaps the most commonly used evidence for 8α substitution is the hypsochromic shift of the near ultraviolet absorption maximum. The 372 nm absorption band of riboflavin is shifted to 355 nm at neutral pH upon 8α substitution by the N(3) position of histidine and further shifts to 345 nm upon protonation of the imidazole ring, while the 445 nm band remains unchanged [9]. Substitution of the 8α position by a thioether linkage results in an absorption maximum at 367 nm which, on oxidation of the sulfur to a sulfone, shifts to 354 nm [26]. Spectral shifts of similar magnitude to those listed above are observed for the flavin peptides from *Chromatium* cytochrome c_{552} [19,27], thiamine dehydrogenase [2], and β-cyclopiazonate oxidocyclase [22].

3. Properties of 8α-[*N*(3)] and of 8α-[*N*(1)]-histidylflavins

Anaerobic acid hydrolysis (95°C, 6 N HCl, 16 hr) of flavin peptides from mammalian succinate dehydrogenase liberates all amino acids, except the one attached to the 8α group of the flavin [10,11]. The resulting acid hydrolyzed flavin, which is at the riboflavin level, exhibits fluorescence and absorption characteristics very similar to those of the flavin peptides. The absorption coefficients at the maxima (in parentheses) of the neutral, oxidized form are 12×10^3 (445 nm), 9.5×10^3 (355 nm), 37×10^3 (268 nm), and 46×10^3 (219 nm). The corresponding values for the oxidized cation are 10×10^3 (405 nm), 14.4×10^3 (373 nm), and 34.5×10^3 (264 nm) [10]. The pK_a of fluorescence quenching, in agreement with titration and electrophoresis data, is 4.6 ± 0.1 at 25°C [10,11]. The compound gives a positive ninhydrin but negative Pauly test for histidine, showing that the imidazole ring is substituted.

Drastic acid hydrolysis of this compound (6 N HCl, 125°C, 16 hr) yields a mole of free histidine with extensive destruction of the flavin ring [10]. The same occurs on reductive cleavage with H_2 and Pd in CF_3COOH. The use of CF_3COOH, in lieu of stronger acids, is essential to make sure that hydrogenation of the benzenoid ring of the flavin does not occur prior to reductive cleavage of the histidine-flavin bond [11]. In order to recover both the histidine and some of the flavin, reductive cleavage by Zn in CF_3COOH–CH_3COOH has been recommended [11], but the yield of flavin is modest even in this procedure. Neutral photolysis of the compound also liberates histidine, along with a lumichrome-type compound. On the basis of these data the structure shown in fig. 1 has been assigned to the acid hydrolyzed flavin peptide [10,11]. The compound has been synthesized by condensation of 8α-bromotetraacetylriboflavin and N_α-benzoyl histidine, followed by mild acid hydrolysis of the protective groups [11,28]. The resulting synthetic compound is a mixture of two isomers (*N*(3) and *N*(1) histidyl riboflavins), which may be separated by paper chromatography in *n*-butanol–acetic acid [11]. Histidyl riboflavin, obtained by acid hydrolysis of the flavin peptide from succinate dehydrogenase, contains the same two isomers. Digestion of the peptide with aminopeptidase M to the histidyl flavin level, however, yields only a single isomer. This has been identified as the less hindered N(3) isomer by methylation, followed by reductive cleavage, and identification of the product as 1-methylhistidine.

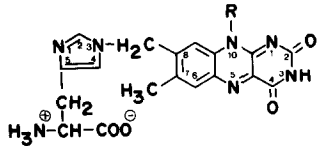

Fig. 1. Structure of histidyl 8α-FAD from succinate dehydrogenase. R is rest of FAD.

This shows that the N(3) position in the parent histidyl riboflavin was substituted by the flavin. The N(3) isomer is also the initial product of the reaction of 8α-bromotetraacetylriboflavin and benzoyl histidine. The N(1) isomer arises by an acid-catalyzed migration of the riboflavin from the N(3) to the N(1) position both during synthesis and on isolation from natural sources. This isomerization may be avoided by careful control of the pH. Curiously, the reverse (migration from N(1) to the less hindered N(3) position) does not seem to occur [11].

Synthetic and natural *N*(3) histidyl riboflavins have identical absorption, NMR, and EPR spectra, pH–fluorescence curves, and mobilities in high voltage electrophoresis, and in paper and thin layer chromatography. The N(1) isomer differs in having a slightly (~ 0.2 pH) lower p*K*, reflected in a shifted pH–fluorescence curve and lower mobility in electrophoresis at pH 5.5 and significantly different R_F values in chromatography in several solvents. The NMR spectra of the N(1) and N(3) isomers differ, however, only as regards the C(9) and ribityl hydrogens [11].

8α-[*N*(3)]-histidyl FAD has also been demonstrated in D-6-hydroxynicotine oxidase from *Arthrobacter oxidans* [29]. The covalently bound flavin was released from the purified enzyme, the flavin peptide purified, and histidyl riboflavin demonstrated in aminopeptidase digests of the peptide by essentially the same methods [29,30] as had been used for succinate dehydrogenase [9,10,12].

8α-[*N*(3)]-histidyl FAD may also be present in sarcosine and dimethylglycine dehydrogenases from rat liver, although evidence for the former enzyme is incomplete and for the latter at best suggestive. The presence of covalently bound flavin (along with acid-extractable flavin) in partially purified preparations of these two enzymes was reported many years ago [31]. Since the preparations were impure and the degree of enrichment during isolation very modest, the possibility that the covalently bound flavin originated from succinate dehydrogenase, present as an inactive impurity, could not be ruled out. The situation with regard to dimethylglycine dehydrogenase has not changed over the years. In the case of sarcosine dehydrogenase, however, recent evidence showing that in a strain of *Pseudomonas* sarcosine dehydrogenase activity and covalently bound flavin rise at similar rates during induction [32] supports the interpretation [31–33] that this enzyme contains covalently bound flavin. The hypsochromic shift of the second absorption band of purified flavin peptides from both the liver and *Pseudomonas* enzyme indicate 8α substitution [20,33] and the pK_a of the fluorescence quenching of the flavin peptide from the rat liver enzyme (4.7) is compatible with N(3) histidine substitution [20]. Definite assignment of the structure must await isolation and identification of 8α-[*N*(3)]-histidylriboflavin from the peptide. Also, it remains to be seen whether or not the relatively high (25%) fluorescence of the flavin peptide at pH 7 is due to an impurity.

4. Properties of 8α-*N*-histidyl–8α-hydroxyflavins

While the compounds described in the previous section are adducts of histidine and 8α-hydroxyriboflavin, evidence has recently been obtained for the existence in certain flavoenzymes of adducts of histidine and 8α-formylriboflavin. These differ, therefore, from the succinate dehydrogenase type of histidylriboflavin in that the 8α group is at the oxidation state of carbonyl.

The discovery of this new class of covalently bound flavins resulted from studies on two flavoenzymes: thiamine dehydrogenase and β-cyclopiazonate oxidocyclase. The former enzyme, isolated from a soil bacterium [34], oxidizes thiamine to thiamine acetic acid and contains FAD in covalent linkage to the protein. β-Cyclopiazonate oxidocyclase from *Penicillium cyclopium*, an enzyme which catalyzes the dehydrogenation and cyclization of β-cyclopiazonic acid to α-cyclopiazonic acid, has been isolated in homogeneous form and reported to contain covalently linked flavin [35]. Tryptic–chymotryptic FAD peptides have been obtained and studied from both enzymes in this laboratory [21,22]. Substitution of the peptide is at the 8α position in both enzymes, as judged by the hypsochromic shifts of the second fluorescence excitation and absorption bands of the flavin peptides, at the FMN level, from 372 nm to 345 nm (in thiamine dehydrogenase) and 348 nm (in the oxidocyclase) [21,22]. The pure FMN peptide from the former shows extensive quenching of the fluorescence between pH 3.5 and 8, similar to the FMN peptide

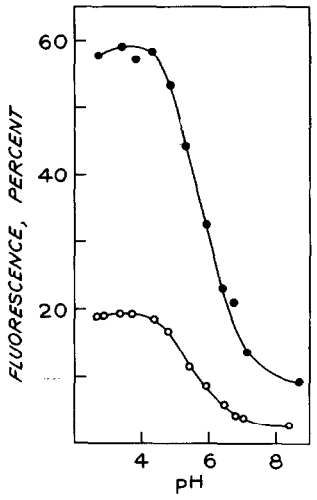

Fig. 2. pH–Fluorescence profile of tryptic–chymotryptic flavin peptide from β-cyclopiazonate oxidocyclase (FMN level). Fluorescence is expressed relative to riboflavin. Open circles, before performic acid oxidation; solid circles, after performic acid oxidation at 0°C.

from succinate dehydrogenase [9], but the pK_a of this fluorescence quenching is over one pH unit higher ($pK_a = 5.8 \pm 0.1$) than in 8α-[*N*(3)]-histidyl-riboflavin.

On hydrolysis with 6 N HCl (95°C) an aminoacyl riboflavin was obtained, with a negative Pauly reaction, and a pK_a (from fluorescence quenching and from electrophoretic mobility) of 5.1 ± 0.1. Drastic acid hydrolysis liberated 1 mole of histidine, showing that histidine is the 8α substituent.

Similar observations were made with the flavin peptide (FMN level) from the oxidocyclase, except that in that case, superimposed on the fluorescence quenching by the imidazole nitrogen, extensive additional quenching due to noncovalent interaction with another amino acid was also observed [22]. As shown in fig. 2, the maximal fluorescence was only 19% of that of FMN and the pK_a was 5.4 before performic acid oxidation, but increased to 59% ($pK_a = 5.8$) after oxidation. That this additional quenching is due to interaction with an amino acid (probably a tryptophan) other than the 8α substituent is shown by the fact that the histidyl flavin obtained by acid hydrolysis (95°C, 6 N HCl) showed the same fluorescence as FMN before and after performic acid oxidation and the pK_a was shifted to 5.0 [22]. The 'histidyl riboflavins' isolated from the oxidocyclase and from thiamine dehydrogenase appear to be indistinguishable from each other and from a synthetic compound recently obtained [36]. It differs clearly from 8α-[*N*(1)]- and 8α-[*N*(3)]-histidylriboflavin (section 3) in pK_a value and may be readily separated from these by high voltage electrophoresis at pH 5 and by TLC.

While final proof of its structure awaits unambiguous synthesis, the following evidence [36] suggests that it is the 8α-hydroxy analog of histidyl riboflavin. The compound gives a negative Pauly test; thus, linkage of the flavin is to the imidazole nucleus. The extensive fluorescence quenching suggests a direct substitution on a ring nitrogen. Further, NMR data show that C(2)H and C(4)H of the imidazole ring are present. NMR data also show that a single proton is present at 8α, while histidyl flavin from succinate dehydrogenase shows two protons. On storage this hystidyl flavin adduct hydrolyzes to 8-formylriboflavin [36]. Decision as to whether N(1) or N(3) of the imidazole is involved in the linkage is under investigation by the methods previously used to decide this question in the case of succinate dehydrogenase [11], but by analogy with the latter, N(3) is the probable site of attachment.

In view of these data and those in Sections 5 and 6, it seems that adducts of both 8-hydroxy FAD and 8-formyl FAD with both histidine and cysteine occur in nature.

5. Properties of 8α-*S*-cysteinylflavin thioethers

Studies in the laboratories of Yasunobu [37,38] and of Hellerman [39] indicated that liver and kidney mitochondrial monoamine oxidase contains FAD covalently linked to the protein. The presence of covalently bound flavin has been detected to date only in mitochondrial monoamine oxidase preparations. The structure of the covalently bound flavin could not be elucidated until enzyme preparations free of succinate dehydrogenase were obtained, since existing methods of purification were likely to inactivate but not necessarily remove succinate dehydrogenase. This problem was resolved by the isolation of liver mitochondrial outer membranes containing monoamine oxidase but free of succinate

dehydrogenase (an inner membrane enzyme) [17,18]. The flavin peptide was then isolated from tryptic–chymotryptic digests of the enzyme and the flavin moiety shown to be linked to a cysteinyl group on the peptide via a thioether bond to the 8α position [17,18,26]. As expected from the known reactivity of thioethers, the purified flavin peptide gave a positive chloroplatinic acid but a negative iodine–azide test [18]. The properties of the flavin peptide were quite similar to those of synthetic 8α-*S*-cysteinyl-riboflavin [40].

Substitution of the 8α position by cysteine in a thioether linkage results in almost complete quenching of flavin fluorescence (10% of that of riboflavin) [18] because of the π-electron donating properties of the sulfur atom. Oxidation of the thioether by either performic acid or peracetic acid at 0°C yields the sulfone, rather than the sulfoxide, as shown by infrared spectral analysis [40]. Since the oxidized sulfur no longer has non-bonding electron pairs, the fluorescence of the isoalloxazine ring increases to 85–90% of that of riboflavin [26,40].

8α-*S*-cysteinylflavin thioethers are quite unstable as compared with 8α-[*N*(3)]-histidylflavin [18,26]. Under conditions where histidyl flavin is reasonably stable (6N HCl, 95°C), the cysteinylflavin thioether readily hydrolyzes, presumably to 8-hydroxyriboflavin [26]. Because of the electron donating properties of bivalent sulfur, cysteinyl flavins are readily oxidized by air [18,26] and by an internal redox reaction in which the flavin ring is reduced [26]. In the experience of the authors 8-formylflavins are the major degradation product of 8α-*S*-cysteinylflavins stored either in solution or as a lyophilized powder.

Reductive cleavage of the cysteinyl flavin thioether readily occurs by treatment with Zn in acidic media [26,40] to form cysteine and riboflavin. An alternative reductive cleavage of the flavin sulfone has recently been published [41], which takes advantage of the electron-deficiency of the 8α-sulfone. Upon reduction of 8α-*S*-cysteinylsulfoneriboflavin with a stoichiometric amount of dithionite, the resulting flavin hydroquinone spontaneously reoxidizes under anaerobic conditions to form riboflavin and cysteinesulfinate. The reaction occurs apparently in first-order manner with a maximal rate at pH 6.0. The electron-deficient sulfone is required for the cleavage reaction, as the thioether is stable towards reduction by dithionite. Analysis of the pH–rate profile suggests that two ionization processes can influence the rate of electron migration to the 8α-sulfone [41]. One is the ionization of the N(1) nitrogen of the flavin hydroquinone ($pK_a \simeq 6.5$), which influences the degree of folding across the N(10)–N(5) axis [42]. For maximal electron migration the molecule should be in an all-coplanar form to maximize *p*-orbital overlap. A more folded molecule (the anionic hydroquinone [42]) would thus be expected to have a smaller population in the all-coplanar form and, therefore, undergo cleavage of the 8α-sulfone more slowly. The effect of the second ionization on the rate is not well understood. It is probable, however, to be due to the effect of pH on the rate of proton abstraction from the N(5) position.

6. 8α-*S*-cysteinylflavin thiohemiacetals

On chemical grounds thiohemiacetals are generally regarded as being unstable. Yet, all available evidence suggests that the peptide chain of *Chromatium* cytochrome c_{552} is linked by a thiohemiacetal bridge to the 8α position of FAD [24,27], although the flavin is very tightly bound to the enzyme, and that even flavin peptides are stable enough for isolation in the pure state. This raises the question as to the additional bonds or interactions responsible for the stable binding of the FAD to the apoenzyme in this protein.

It was reported many years ago [43] that the flavin component of this flavocytochrome is not released by acid ammonium sulfate or trichloroacetic acid treatment but is cleaved from the protein by tryptic digestion, prolonged incubation with saturated urea solution, exposure to pH > 9, and treatment with organic mercurials [44]. A related enzyme, flavocytochrome c_{553} from *Chlorobium thiosulfatophilum* has also been reported to contain a covalently bound flavin, the release of which required prolonged incubation with saturated urea solution [44,45]. While the nature of the flavin component of the *Chlorobium* enzyme has not been further investigated, the *Chromatium* flavin has been extensively studied in two laboratories.

Release of the flavin from the protein on prolonged incubation with 8 M urea has been confirmed [46], but it appears likely [47] that the agent

responsible is the cyanate impurity in urea preparations. On the other hand, release of the flavin on exposure to alkaline pH or organic mercurials could not be confirmed by the present authors.

The product released by digestion with urea is an FAD derivative, substituted at the 8α position, as shown by the hypsochromic shift of the absorption spectrum [19,46] and the hyperfine EPR spectrum of the radical cation [19]. Its properties strongly suggest that is 8-carboxy FAD. Oxidation of the denatured cytochrome with performic acid also releases 8-carboxyriboflavin in excellent yield [19], showing that the peptide is indeed substituted on the 8α-carbon.

Digestion with trypsin–chymotrypsin or with pepsin releases FAD peptides, which have been isolated in homogeneous form [24]. These exhibit a hypsochromic shift of the second fluorescence excitation maximum from 372 to 365 nm and extensive quenching of fluorescence, independent of pH between 3.2 and 7.0 (5% of the quantum yield, compared with riboflavin, in the case of tryptic–chymotryptic peptide and 1% in the peptic peptide, both at the FMN level). On performic acid oxidation the fluorescence increases, with further hypsochromic shift of the second excitation band [27]. The properties are strongly reminiscent of the flavin thioether isolated from monoamine oxidase [17] and suggest that the substituent is cysteine. The presence of cysteine was confirmed by the positive chloroplatinic reaction and by analysis for cysteine after acid hydrolysis of the two flavin peptides [24,27].

The properties of the flavin in the flavocytochrome, however, differ in important respects from those in monoamine oxidase, so that a thioether linkage is highly unlikely. (1) Performic acid oxidation of the cytochrome c_{552}, but not of monoamine oxidase, releases 8 carboxyriboflavin. (2) Reduction by Zn releases cysteine in the case of flavin peptides from monoamine oxidase (or cysteinyl riboflavin thioether) but not from the *Chromatium* flavin peptides. (3) The flavin peptide from monoamine oxidase, after performic acid oxidation, shows 80–85% of the fluorescence of riboflavin, while those from *Chromatium* only 50%. (As discussed below, with the peptic peptide this degree of fluorescence is obtained only on oxidation at 40°C) [6,24]. Alternate structures compatible with the negative I_2–azide test of the *Chromatium* peptides are: disulfide, thioester, and thiohemiacetal. The first two are ruled out since neither dithionite nor hydroxylamine liberate the flavin from the protein. This leaves a flavin thiohemiacetal as the only possibility.

Direct evidence for this structure was obtained by acid hydrolysis of the peptic peptide (6 N HCl, 95° C, N_2) and demonstration of the release of 8-formylriboflavin [6]. Supporting evidence came from the observation [24] that digestion of the peptic peptide with aminopeptidase M yields two flavin components (electrophoresis, pH 6.5). One has 0.95 of the mobility of FAD, as expected for an aminoacyl FAD derivative, the other a much greater anionic mobility than FAD, suggesting that the amino group of the cysteine is blocked. This is compatible with cyclization to a thiazolidine derivative during aminopeptidase digestion (fig. 3).

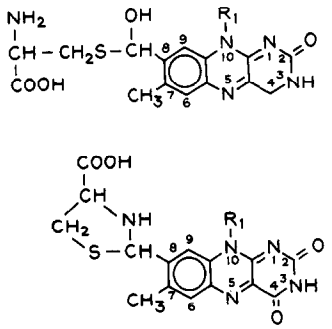

Fig. 3. Proposed structure of cysteinyl flavin from *Chromatium* cytochrome c_{552} in linear and thiazolidine forms.

Synthetic flavin thiohemiacetals have not been described in the literature. Studies in this laboratory [48] show that at pH ~ 5 and room temperature cysteine and *N*-acetylcysteine readily react with 8-formyllumiflavin and 8-formyltetraacetylriboflavin to form a thiohemiacetal adduct at 8α, but they hydrolyze with time to yield the reactants (K_D = 1 mM). These observations are in line with the expected lability of thiohemiacetals. 8-Formylriboflavin exists in a hemiacetal form by reaction with the ribityl 5′ hydroxyl group [49], and does not react until after hydrolysis in 6 N HCl. On the other hand, at high (~ 50 mM) concentrations of 8-formyl

riboflavin and cysteine, in 6 N HCl (30°C to 38°C) an adduct is formed which is stable enough for isolation (10% to 20% yield) [48]. Conceivably, the high concentrations of the reactants pushes the equilibrium in favor of the thiohemiacetal which might then be stabilized by mixed acetal formation with the ribityl 5′ hydroxyl group. Digestion of the dephosphorylated peptic peptide from *Chromatium* with aminopeptidase M, however, does not yield the same compound. Instead, 8-formylriboflavin is the product of hydrolysis [48]. This breakdown may be either the result of the low (micromolar) concentration of flavin peptide prevailing during digestion (a limitation imposed by available starting material) or of reaction with –SH groups in the aminopeptidase. It should be noted that dithiothreitol or serum albumin hydrolyze the peptic peptide, as well as the synthetic flavin thiohemiacetal, to 8-formylriboflavin [48].

The fact that the peptic flavin peptide is relatively stable does not contradict the proposed thiohemiacetal structure, since it is stabilized by interaction of the N-terminal tyrosine with the flavin and, perhaps, to a lesser extent by interaction with the adenine moiety [24]. In accord with this, the tryptic–chymotryptic peptide, lacking this tyrosine group, is far less stable, particularly after dephosphorylation to the riboflavin level. Since studies of models [49] indicate that C(5) OH of the ribityl is the only one capable of acetal formation with the 8-formyl group and since it is substituted in the FAD peptide, stability cannot be ascribed to mixed acetal formation. Since the $-NH_2$ group is peptide-linked, thiazolidine formation is also ruled out.

7. Properties of other 8α-substituted flavins

With the availability of a chemical procedure by which 8α-substituted flavins may be chemically synthesized through the nucleophilic displacement of the bromide from 8α-bromotetraacetylriboflavin [28], several 8α-substituted flavin analogs which could possibly exist in nature have been synthesized and their properties compared with those previously characterized [50]. The flavins thus synthesized include 8α-*O*-tyrosylriboflavin and 8α-(N_ϵ)-lysylriboflavin as well as 8α-sulfonylriboflavin. The latter flavin analog could arise by performic acid oxidation of an 8α disulfide bond between a flavin moiety and a cysteine residue.

The tyrosylflavin analog shows a quenched fluorescence (20%), as compared with riboflavin, which is an expected property of an 8α substituent with π-electrons available for donation. The tyrosyl residue is readily cleaved from the flavin by acid hydrolysis (6 N HCl, 110°C, 15 hr) [50]. The EPR spectrum of the cationic semiquinone shows a well resolved hyperfine pattern with a similar linewidth to that given by the cationic semiquinone of 8α-*S*-cysteinylriboflavin.

8α-(N_ϵ)-Lysylriboflavin shows a characteristic pH-dependent fluorescence yield, as seen with other flavins with a nitrogeneous group at the 8α position. A pK_a of 7.2 for the ionization of the secondary amino group was found [50]. At pH 4.0 the fluorescence was equal to that of riboflavin, while at pH 9.0 the molar fluorescence was reduced 10-fold. The protonated 8α-(N_ϵ)-lysylflavin showed absorption maxima at 345 and 448 nm, which shifted to 355 and 445 nm upon deprotonation. The influence of protonation of the 8α nitrogen on these spectral properties is similar to that seen with 8α[*N*(3)]-histidylriboflavin and 8α-morpholinolumiflavin [14] and differ only in the apparent pK_a of the respective nitrogeneous substituent.

8-Formylriboflavin has been used as an intermediate in the synthesis of other 8α-substituted flavin analogs [14,51], but has only recently been characterized in terms of chemical reactivity. A comparison of the properties of 8-formylriboflavin with those of 8-formyl-3-methyllumiflavin and 8-formyltetraacetylriboflavin shows the 5′-hydroxyl group of 8-formylriboflavin to form an intramolecular hemiacetal bond with the 8-formyl group [49]. The bond is quite stable in neutral or acidic conditions at ambient temperature but is hydrolyzed upon refluxing in 6 N HCl or by reducing the flavin to its hydroquinone form under acidic conditions.

Aside from differences in reactivity with 2,4-dinitrophenylhydrazine, the hemiacetal form may be distinguished from its 'open' form by differences in spectral properties. The hydroquinone form of 8-formyltetraacetylriboflavin has absorption maxima at 520 and at 392 nm, while that of 8-formylriboflavin has a maximum in the 350 nm spectral region

[49]. Circular dichroism data show 8-formyltetraacetylriboflavin to be similar to that of tetraacetylriboflavin, while the CD spectrum of 8-formylriboflavin is much more intense and of different shape than that of riboflavin [49]. This increased optical activity results from the rigidity of the optically active ribityl side chain, induced by two points of attachment to the optically inactive isoalloxazine ring. In the case of riboflavin, the ribityl chain is free to rotate in a number of orientations with respect to the isoalloxazine ring, which is the reason for its weak optical activity

8. Amino acid sequences and flavin–amino acid interactions in flavin peptides

The covalent linkage of the flavin to the peptide chain in this group of enzymes provides a simple means for isolating and sequencing peptides of different chain lengths (depending on the proteolytic enzyme used in the digestion) derived from the active center. Thus, digestion of mammalian succinate dehydrogenase with trypsin–chymotrypsin yields predominantly the pentapeptide [52]

Ser–His (FAD)–Thr–Val–Ala,

while tryptic digestion releases a flavin peptide containing 23 amino acids, the structure of which has also been determined [53]. Knowledge of the peptide sequence permits verification or interpretation of data on the action of active site directed inhibitors of the given enzyme and may be used to trace the evolutionary development of the enzyme, at least in regard to the flavin site. Moreover, as illustrated below for the *Chromatium* flavocytochrome, knowledge of the amino acid sequence of flavin peptides may be essential for explaining their chemical and physical properties.

With the exception of current studies in the authors' laboratory, comparing the structures of the flavin sites of yeast and mammalian succinate dehydrogenases, little has been done to exploit the advantage of flavin peptides for tracing the evolution of enzymes, probably because of the scarcity of purified enzymes of this class from various sources. It seems clear, however, that enzymes of different catalytic function containing 8α-[*N*(3)]-histidyl FAD may have widely varying amino acid sequences at the flavin site. Thus the tryptic–chymotryptic peptide from D-6-hydroxynicotine oxidase [54] has the sequence: Ser–Gly–Gly–Asn–Asn–Pro–Asp–His(FAD)–Tyr–(Gln,Pro)Ala, which appears to have nothing in common with the corresponding pentapeptide from beef heart succinate dehydrogenase.

An example of how sequencing of flavin peptides complements information derived from inhibition studies is as follows. It has been known since 1938 [55,56] that substrates and malonate protect succinate dehydrogenase from inhibition by –SH reagents. Although this made it likely that –SH groups are present in the active center, Kearney et al. [8] in 1956 showed that the tryptic–chymotryptic flavin peptide contains no cysteine. Although Wang et al. [7] debated this finding, the cysteine they found in the peptide was obviously a contamination, since neither the pure pentapeptide [9] nor the longer tryptic peptide [53] contains any thiol. It seems likely, therefore, that the substrate site is on a different segment of the polypeptide chain (or on another subunit) and is juxtaposed to the flavin site in the quaternary structure to form the active center [53].

Knowledge of the amino acid sequence is also necessary at times to rationalize the physical–chemical properties of flavin peptides. Thus, although the peptic and tryptic–chymotryptic flavin peptides from *Chromatium* cytochrome c_{552} differ only in an N-terminal tyrosine (fig. 4), the former is far more stable, has a lesser fluorescence, and requires oxidation with performic acid at 40°C to yield the same molar

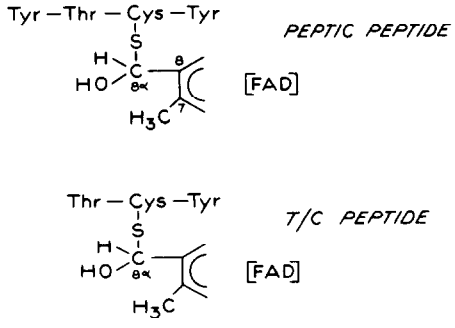

Fig. 4. Amino acid sequences of flavin peptides isolated from *Chromatium* cytochrome c_{552}.

fluorescence as is obtained on oxidation of the tryptic–chymotryptic peptide at 0°C [24]. The seeming resistance to cold performic acid is due to quenching of the fluorescence by the tyrosyl residue, since removal of this residue with aminopeptidase following exposure to cold performic acid yields the same fluorescence as the performic acid treated tryptic–chymotryptic flavin peptide. The fact that the N-terminal tyrosine interacts with the flavin is clearly shown in the CD spectrum of the peptic flavin peptide [24] which monitors perturbation of the electronic transition of the flavin. Its spectrum is composed of two negative bands at 375 and at 305 nm, but on removal of the N-terminal tyrosine (tryptic–chymotryptic peptide) a positive band at 340 nm and a weak negative band at 380 nm are seen. The spectrum of the peptic peptide is quite similar in shape to those of the flavodoxins [57,58]. Recent X-ray crystallographic studies of two flavodoxins [59,60] reveal the presence of a tyrosyl residue in a planar 'stacking' arrangement with the flavin. Thus, it may be concluded that in the case of the peptic peptide the N-terminal tyrosyl residue interacts with the flavin ring in a parallel 'stacking' manner.

A C-terminal tyrosine is also present in both flavin peptides from *Chromatium* (fig. 4) and in the flavin pentapeptide from monoamine oxidase [18], which has the sequence: Ser–Gly–Gly–Cys(FAD)–Tyr. Whether this tyrosine also interacts with the flavin and contributes to the much greater stability of the flavin pentapeptide from monoamine oxidase than of cysteinyl riboflavin, remains to be investigated.

Interaction of the flavin with an amino acid (other than the 8α substituent) may also explain the dramatic fluorescence enhancement of flavin peptides from β-cyclopiazonate oxidocyclase on performic acid oxidation and the lack of effect of performic acid on the histidyl flavin derived from this peptide [22] (see above). Unpublished data suggest that this may be due to a tryptophan residue, which has been found in equimolar ratio to the flavin in the tryptic–chymotryptic peptide.

9. Oxidation–reduction properties of 8α-substituted flavins

As a first step to understanding the influence of covalent binding on the chemistry of the isoalloxazine ring, the oxidation–reduction potentials and sulfite affinities of a number of 8α-substituted flavin analogs were determined [41]. Previous results had shown that sulfite formed an adduct with the isoalloxazine ring at the N(5) position with an affinity paralleling the redox potential of the particular flavin analog [61]. Measurements of this type would provide information on the inductive effect of a particular 8α substituent on the electron affinity of the isoalloxazine ring.

The data in table 1 show that 8α substitution raises the redox potential by 0.02–0.03 V. The nature of the substituent had little effect on the electron affinity with the exception of the 8-formylflavins (to be discussed below). In agreement with the redox potentials, the substituted flavin analogs showed an increased affinity for sulfite (10–20 times that of riboflavin). Inasmuch as the rate of sulfite complex formation for the various 8α flavin analogs varied no more than 2–3-fold compared with riboflavin (table 1), the lower K_D values (increased sulfite affinity) reflect a *decrease* in the magnitude of the rate of dissociation.

The oxidation–reduction potential of the *Chromatium* cytochrome c_{552} peptic peptide (FAD level) also shows a higher potential than FAD (table 1) although the influence of the N-terminal tyrosyl residue (see section 8) may have an influence. Attempts to determine the potential of the tryptic–chymotryptic tripeptide have been unsuccessful because of breakdown of the flavin peptide under reducing conditions.

Substitution of the 8-CH_3 by a formyl group has a greater effect on the redox potential of the isoalloxazine ring. 8-Formyltetraacetylriboflavin has a redox potential 0.190 V more positive than tetraacetylriboflavin (table 1). A similar potential difference is observed in the case of 8-formyl-3-methyllumiflavin [49]. 8-Formylriboflavin, however, because of intramolecular hemiacetal formation (section 7) has a potential only 0.035 V more positive than riboflavin ([49], table 1). Hydrolysis of the hemiacetal bond raises the potential further to a value 0.100 V more positive than riboflavin ([49], table 1). The results demonstrate the electron-withdrawing effect of the 8-formyl group, which gives the isoalloxazine ring a greater electron affinity. Modification of the formyl group by hemiacetal formation decreases the

Table 1
Oxidation–reduction potentials (E_0'), dissociation constants of sulfite complexes (K_d), and sulfite complex formation rate constants (k) for various 8α-substituted flavins

8α Substituent	Flavin	E_0' (mV)	K_d (M)	k ($M^{-1}min^{-1}$)
H	RF	−190	1.16	1.36
Hydroxy	RF	−170	0.22	0.98
Carboxy	RF	−165	0.081	1.91
N(3)-histidyl	RF	−160	0.056	1.94
S-cysteinyl	RF	−169	0.104	1.85
Formyl (hemiacetal)	RF	−159		
Formyl (open)	RF	−90		
H	TARF	−195	1.21	1.12
S-glutathionyl	TARF	−168	0.11	1.74
Sulfonyl	TARF	−159	0.10	2.49
S-cysteinylsulfone	TARF		0.027	3.67
Formyl	TARF	−6		
Formyl	3-MeLF	−48		
H	FAD	−209		
Chromatium cytochrome c_{552} peptic flavin peptide	FAD	−187		

RF, riboflavin; TARF, tetraacetylriboflavin; 3-MeLF, 3-methyllumiflavin.

electron deficiency on the 8α-carbon and thus decreases the electron affinity of the isoalloxazine ring. The difference in potential between 8-formyl-tetraacetylriboflavin and 8-formylriboflavin (open form) (table 1) suggests the electron affinity of 8-formylflavins to be very sensitive to the nature of the ribityl side chain. Because of the affinity of the 8-formyl group for sulfite, an unambiguous determination of the sulfite affinity at the N(5) position was not possible.

10. Determination of various types of covalently bound flavins

The practical importance of determining total covalently bound flavin content is clear from the early work of Boukine [1], showing that a major part of the vitamin B_2 content of many plant and animal tissues is covalently bound, all the more, since it is not established whether this fraction is utilized by higher organisms as a source of riboflavin. For the biochemist methods for the chemical determination of each form of covalently bound flavin would be of great value, since this would permit the determination of the concentration of enzymes containing this form of flavin, regardless of the state of activation or the degree of inactivation of the enzyme. Thus, the availability of a chemical method for determining the succinate dehydrogenase content of enzyme preparations [62] permitted the determination of its turnover number and the unambiguous demonstration [63,64] that 'reconstituted' succinoxidase preparations contain one mole of inactive succinate dehydrogenase for each mole of active one and that, therefore, 'reconstitution' of this system is not merely the combination of succinate dehydrogenase with a dehydrogenase-free respiratory chain, as speculated by others [65].

Despite this need, a method for determining the *total* covalently bound flavin content of tissues is not yet available and a satisfactory method exists only for the determination of the type of histidyl flavin present in succinate dehydrogenase. The latter was developed many years ago [62] on the basis of Kearney's studies [9] and the details have been recently reviewed [66,67], including modifications required in work with microorganisms and higher plants. The method involves removal of non-covalent flavin with trichloracetic acid, tryptic–chymotryptic

release of flavin peptides, mild acid hydrolysis to the FMN level, and determination of the fluorescence difference between pH 3.2 to 3.4 and 7. This procedure will determine total histidyl flavin, including > 90% of that at the histidyl–8α-hydroxyflavin level, provided that non-covalent interaction between an amino acid and the flavin does not quench the fluorescence, as in the FMN peptide from β-cyclopiazonate oxidocyclase [22] (fig. 2). In samples in which only one enzyme containing histidyl flavin is known to be present, this method yields a satisfactory value for the content of the particular enzyme. Thus, in heart and probably also in yeast mitochondria succinate dehydrogenase seems to be the only source of covalently bound flavin, but liver mitochondria have been reported to contain at least three other enzymes with covalently bound flavin prosthetic groups [6,66].

There are two problems to be overcome in evolving a method for the quantitation of total covalently bound flavin in biological samples. One concerns the fact that in flavin thioethers and thiohemiacetals the fluorescence is profoundly quenched and even after performic acid oxidation only 50% to 80% of the fluorescence of an equivalent amount of riboflavin is seen [24,26]. Thus, if a particular sample contains a large amount of 8α-*S*-linked peptide, the fluorescence of proteolytic digests at pH 3.4 and after performic acid oxidation would underestimate the covalently linked flavin. The second problem is the extensive quenching of the fluorescence by non-covalent interaction between the flavin and certain amino acids, as in flavocytochrome c_{552} [24] and in β-cyclopiazonate oxidocyclase [22]. This source of error may be theoretically overcome by further digestion of flavin peptides to the aminoacyl flavin with aminopeptidase or hydrolysis with 6 N HCl at 95°C, but the former procedure is too lengthy for routine use and the latter leads to variable loss of flavin by destruction.

In purified enzyme preparations containing flavin covalently linked to cysteine the flavin may be determined fluorometrically, provided that the histidyl flavin content of the sample is less than the cysteinyl flavin content. In this laboratory satisfactory estimates of the cysteinyl flavin thioether concentration have been obtained in outer membrane and in purified monoamine oxidase preparations from liver (which contain relatively little histidyl flavin), as follows. The fluorescence of tryptic–chymotryptic peptides, after hydrolysis to the FMN level and performic acid oxidation, is measured at pH 3.2–3.4 and at pH 7. At the former pH the fluorescence equals the histidyl FMN plus 80% of the cysteinyl FMN content. At the latter pH the fluorescence equals 80% of the cysteinyl FMN plus 10% of the histidyl FMN content. If the histidyl FMN concentration exceeds that of the cysteinyl FMN, the error due to correction for the histidyl FMN content becomes too great for accurate calculation of the cysteinyl flavin concentration. Cleavage of flavin from enzymes or flavin peptides containing a thioether by performic acid oxidation, followed by reduction with dithionite (section 5), appears to be unfeasible in analytical work.

In purified preparations of *Chromatium* cytochrome c_{552} the thiohemiacetal linkage is readily cleaved by performic acid oxidation, with quantitative liberation of the flavin as 8-carboxyriboflavin [19], but in flavin peptides this procedure results in formation of the sulfone, without release of the flavin. It remains to be seen whether these observations will hold for all enzymes containing covalently bound flavin in thiohemiacetal linkage and thus provide a means for estimating 8α-thiohemiacetals.

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