mechanisms which control the synthesis of immunoglobulin heavy chains.

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Syntheses and Properties of Flavine–Histidine Peptides†

Paul G. Johnson and Donald B. McCormick*

ABSTRACT: Flavinylhistidine peptides were synthesized by attaching the amino group of the histidine moiety through amide linkage to aliphatic carboxylic acid chains of varying length at the N-10 position of the isoalloxazine ring. The fluorescence properties of these models were measured in order to understand more fully flavine–histidine interactions, which can occur in certain flavoprotein systems. Measurements of fluorescence quenching indicate interaction between the isoalloxazine and nonprotonated imidazole ring of the histidine moiety. Very little interaction apparently occurs between flavine and protonated histidine. The effects seen when temperature and solvent are varied seem to indicate that there is greater ground-state association for the short-chain peptides, in which the histidine and flavine intramolecularly interact to form a dark, nonfluorescent complex. There is less complexing apparent in the case of the long-chain peptides and more fluorescence quenching due to increased collisions between the light-excited flavine and the histidine. Proton magnetic resonance measurements of these models in D_2O (37°) at various pD values suggest a weak intramolecular interaction between the imidazole ring of the histidine moiety and the benzenoid portion of the flavine. Such conformations are disrupted at higher temperatures.

Interactions of flavine coenzymes and aromatic molecules have been recognized for quite some time (Weber, 1950; Yagi *et al.*, 1959; Strittmatter, 1961; McCormick *et al.*, 1967). Recently, this has led to a study of the degree and type of molecular interactions of flavins with such aromatic amino acids as tryptophan, tyrosine, and phenylalanine. Flavine peptide models were synthesized by attaching the amino acid through amide linkage to the N-10 position of the isoalloxazine ring *via* aliphatic chains of varying length (Föry *et al.*, 1968). A fluorescence spectral investigation of the compounds showed that hydrophobic interactions probably cause complex formation in aqueous solution, while a dipole–dipole type of interaction is responsible for intramolecular association of some of the amino acid residues with the flavine portion in nonaqueous media (MacKenzie *et al.*, 1969). The spatial aspects of the intramolecular interactions of these flavine amino acids, both in aqueous and nonaqueous media, were examined by means of proton magnetic resonance spectroscopy (Föry *et al.*, 1970).

The FMN-dependent enzyme, pyridoxine (pyridoxamine) 5′-phosphate oxidase, is another case where molecular association occurs between the flavine coenzyme and an aromatic compound, in this case, a substrate. Synthesis of spectroscopic flavine-B_6 models (McCormick and Johnson, 1972) and examination of their fluorescence properties (McCormick, 1972) again suggested a significant interaction between such chromophores.

In the last few years, several flavine-containing enzymes have been shown to have a histidine residue at the active site.

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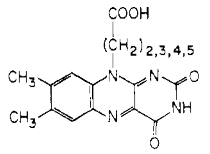
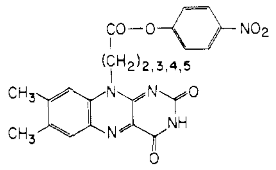
CHART I


$I_{2,3,4,5}$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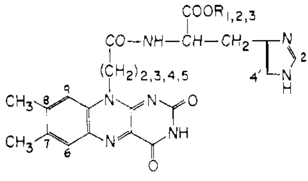
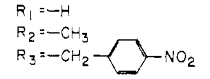

$III_{2,3,4,5}$



For example, succinate dehydrogenase (Walker and Singer, 1970), both D- and L-6-hydroxynicotine oxidases (Möhler *et al.*, 1972), and sarcosine dehydrogenase (Patek and Frisell, 1972) actually have the N-3 imidazole ring of the histidine moiety covalently attached to the 8α position of a FAD. The presence of a histidine at the active site of glucose oxidase was postulated by Müller *et al.* (1970) to explain the pH-dependent blue and red flavine semiquinone stabilization observed for this enzyme.

These developments prompted us to synthesize flavinylhistidines as spectroscopic models for a study on the interactions between the flavine and imidazole ring. The structures of these compounds and the primary synthetic intermediates are shown in Chart I.

Materials and Methods

Materials. L-Histidine methyl ester dihydrochloride was synthesized from L-histidine (Fischer and Cone, 1908) and was converted to the free base as described by Glemzha and Severin (1970).

L-Histidine *p*-nitrobenzyl ester di-*p*-toluenesulfonate was synthesized according to the method of Mazur and Schlatter (1963).

Dry dimethylformamide was prepared by vacuum distillation from calcium hydride, and dimethyl sulfoxide was dried over molecular sieve type 4A and twice distilled *in vacuo* from calcium oxide.

Carboxyalkylflavines and their *p*-nitrophenyl esters were synthesized by known methods (Föry *et al.*, 1968).

Methods. Fluorescence measurements were made with an Aminco-Bowman spectrofluorimeter, using a xenon lamp (150 W), photomultiplier IP21, and slit arrangement 3. Proton magnetic resonance spectra were recorded on a high-resolution Varian Model A-60 at 37 ± 1°. Chemical shifts were measured in cycles per second (cps) from external tetramethylsilane.

The pH of solutions was measured with a Corning Digital 112 Research pH Meter equipped with a Fisher Microprobe No. 13-639-12 electrode. For the fluorescence study, buffers were prepared and used as recommended by Johnson and Metzler (1970). For the nuclear magnetic resonance (nmr) study, the exchangeable protons were removed by lyophilization from D_2O. The samples were then dissolved in fresh D_2O, and the pD was adjusted by additions of acid (0.1 M DCl in D_2O) or base (0.1 M NaOD in D_2O).

Thin-layer chromatography (tlc) was done with MN silica gel N-HR (Brinkman), and the spots were visualized under ultraviolet (uv) light to reveal fluorescent flavine and by spraying with a diazotizing reagent, as in Pauly's reaction (Block *et al.*, 1958), to reveal the imidazole moiety. Elemental microanalyses were performed by Schwarzkopf Microanalytical Laboratories, Woodside, N. Y.

Synthesis of Flavinylhistidine Methyl Esters ($III_{2,3,4}$, R_2). A mixture of 0.25 mmol of $II_{2,3,4}$ and 0.5 mmol of L-histidine methyl ester (free base) in 5 ml of dimethyl sulfoxide was stirred for 30 min (III_2) or 12 hr ($III_{3,4}$). The reaction mixture was then poured into 100 ml of butanol and washed with 75 ml, each, of water and sodium bicarbonate, followed by six 75-ml portions of water. The aqueous solutions were washed twice with 50 ml of butanol. The combined organic layers were stirred into 200 ml of chloroform, dried over sodium sulfate, and concentrated *in vacuo* to 10 ml. This solution was added slowly to anhydrous ether, giving an orange precipitate, which was washed with ether and dried. Recrystallization from dimethylformamide–ether gave $III_{2,3,4}$, R_2 in 48–62% yield. The flavine peptide moved as one spot on tlc in dioxane (R_F 0.10–0.15) and gave a positive Pauly's reaction. *Anal.* Calcd for $C_{22}H_{23}N_7O_5$ (III_2, R_2): C, 56.72; H, 4.94; N, 21.03. Found: C, 56.64, H, 4.90; N, 20.90.

Synthesis of Flavinylhistidine Methyl Ester (III_5, R_2). A mixture of 0.5 mmol of I_5 and 0.5 mmol of *N,N′*-carbonyldiimidazole was stirred in 35 ml of dry dimethylformamide for 24 hr. Histidine methyl ester dihydrochloride (0.5 mmol) was added and stirring continued for another 24 hr. The reaction mixture was concentrated *in vacuo* to 5 ml and poured into 50 ml of butanol. The butanol solution was washed with 50 ml of sodium bicarbonate and three 50-ml portions of water. The aqueous layers were washed twice with 50 ml of butanol and the combined organic layers were added to 150 ml of chloroform, dried over sodium sulfate, and evaporated to 5 ml. This resulted in some flavine peptide precipitate, which was filtered, washed with ether, and dried. Slow addition of the supernatant to anhydrous ether gave more flavine peptide, which was collected on a filter and washed with three 5-ml portions of dioxane and 50 ml of ether. This second crop was dried and added to the original precipitate to give a total yield of 72%. The peptide moved as one spot on tlc and gave a positive Pauly's reaction. *Anal.* Calcd for $C_{25}H_{29}N_7O_5$ (III_5, R_2): C, 59.17; H, 5.72; N, 19.33. Found: C, 58.84; H, 5.84; N, 19.46.

Synthesis of Flavinylhistidine (III_5, R_1). A mixture of 0.51 mmol of L-histidine and 0.5 mmol of triethylamine in 10 ml of dimethyl sulfoxide was kept dry at room temperature for 24 hr. Compound II_5 (0.25 mmol) was then added and the mixture was stirred for 24 hr at room temperature. The mixture was filtered and the filtrate was poured into a mixture of 300 ml of diethyl ether and 100 ml of petroleum ether (bp 30–60°). The solvent was decanted and the residue was washed twice with 100-ml portions of ether. The resulting orange oil was dissolved in 50 ml of 1 mM sodium phosphate buffer (pH 7.8) and washed twice with 50 ml of chloroform. The aqueous solution was concentrated to 15 ml and applied to a 2.5 × 30 cm column of DEAE-cellulose (chloride form). The flavine–histidine was eluted with 500 ml of 1 mM phosphate buffer (pH 7.8), while the unreacted 10-ω-carboxypentylflavine (I_5) remained near the top of the column. The aqueous eluent was evaporated to dryness under reduced pressure at 50°, the residue was suspended in 20 ml of cold methanol, and the mixture was filtered. The filtrate was added dropwise to 200 ml of acetone and the resulting precipitate was collected

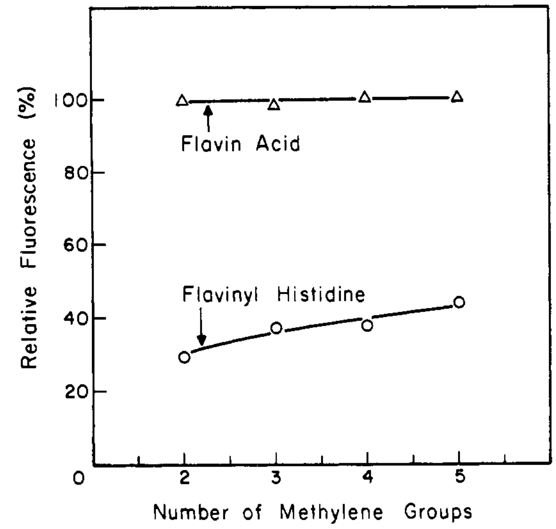


FIGURE 1: Fluorescence of flavinylhistidines (III_{2-5}, R_2) measured relative to the 10-ω-carboxyalkylflavine (I_{2-5}) at 100%. Aqueous solutions contained 10^{-5} M flavines in 0.01 M sodium phosphate buffer (pH 7.6).

on a filter and washed with 10 ml of acetone and 50 ml of ether. Recrystallization from dimethylformamide–acetone gave the sodium salt of the flavine–histidine, III_5, R_1, in 53% yield. The peptide appeared as one spot on tlc and gave a positive Pauly's reaction. *Anal.* Calcd for $C_{24}H_{26}N_7O_5Na$ (III_5, R_1): C, 55.92; H, 5.08; N, 19.02. Found: C, 55.94; H, 5.41; N, 18.95.

Synthesis of Flavinylhistidine p-Nitrobenzyl Ester (*III_2, R_3*). Compound II_2 (1 mmol), anhydrous sodium bicarbonate (400 mg), and L-histidine *p*-nitrobenzyl ester di-*p*-toluenesulfonate (1.25 mmol) in 25 ml of dimethyl sulfoxide were stirred for 6 hr at room temperature. The mixture was added to 150 ml of *n*-butyl alcohol, cooled to 5° for several hours, and filtered. The filtrate was washed successively with 150 ml of water, 100 ml of 5% sodium bicarbonate, and 100 ml of water. Each aqueous extract was washed once with 100 ml of ethyl acetate. The organic layers were combined, dried over sodium sulfate, evaporated to 15 ml, and added slowly to anhydrous ether. The resulting precipitate was collected on a filter, washed with ether, and dried for a 74% yield of III_2, R_3. The peptide gave a positive Pauly's reaction after tlc in dioxane (R_F 0.72). A trace of 10-ω-carboxyethylflavine was present (R_F 0.85); however, the peptide was used in the next synthesis without any further purification.

Synthesis of Flavinylhistidine (*III_2, R_1*). A mixture of 0.5 mmol of III_2, R_3 in 180 ml of *n*-butyl alcohol was hydrogenated over palladium black at 45 psi for 8 hr. The mixture was filtered and the organic filtrate was extracted with 1 mM sodium phosphate buffer (pH 7.8) and purified on DEAE-cellulose as done previously. Two recrystallizations from dimethylformamide–acetone gave the sodium salt of III_2, R_1 in 38% yield. The peptide moved as one spot, with an R_F of 0.18, on tlc in butanol–ethanol–2 N ammonia (3:1:1) and gave a positive Pauly's reaction. *Anal.* Calcd for $C_{21}H_{20}N_7O_5Na$ (III_2, R_1): C, 53.28; H, 4.26; N, 20.71. Found: C, 53.02; H, 4.17; N, 20.55.

Results

Fluorescence Properties. The relative fluorescence of the flavine moiety of a homologous series of 10-ω-carboxyalkylflavines (flavine acids) and flavinylhistidines are shown in Figure 1. As chain length is shortened, there is very little change in the fluorescence of the flavine acids. However, the fluorescence of the flavinylhistidine, $n = 5$, is only 44% of that of its homologous acid. Moreover, as the chain length separating the flavine and histidine is shortened to two methylene groups ($n = 2$), a decrease in fluorescence (30%) occurs.

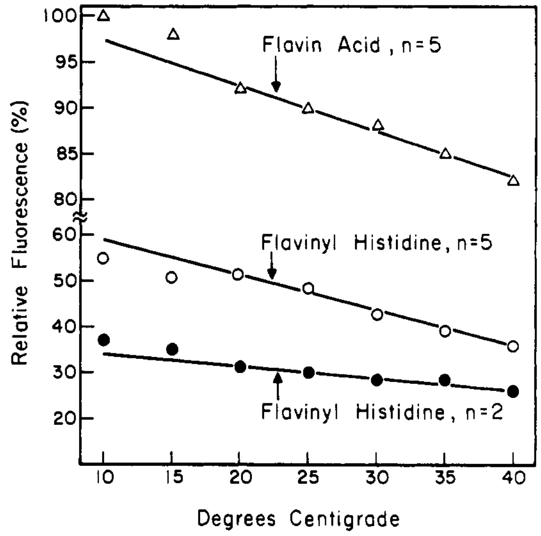


FIGURE 2: The effect of temperature on the flavine fluorescence of 10-ω-carboxypentylflavine and both the longest and shortest chain flavinylhistidine. Aqueous solutions contained 10^{-5} M flavine in 0.01 M sodium phosphate buffer (pH 7.6). Numbers (*n*) refer to the aliphatic methylene groups in the flavine side chain.

The relative fluorescences of flavine acids and flavinylhistidines in 50% aqueous organic solvents are depicted in Table I. The acid behaves similarly to other "free" flavines (Koziol and Knobloch, 1965); *i.e.*, there is an increase in relative fluorescence as the polarity of the solvent decreases. Dimethyl sulfoxide is an exception, as it quenches the fluorescence of flavines (Gascoigne and Radda, 1965). A more noticeable change can be seen in the case of the flavine peptides. For these latter compounds, the increased fluorescence observed as dielectric constant decreases is greater than for "free" flavine. Also, the increase is greater as the chain length is shortened.

The effect of temperature on the fluorescence of flavine acid and flavinylhistidine is shown in Figure 2. There is a decrease of 14% in the fluorescence of the 10-ω-carboxypentylflavine as the temperature increases from 10 to 40°.

TABLE I: Fluorescence of Flavine Acid and Flavinylhistidines in 50% Aqueous Solvents.

	Relative Fluorescence (%)				
Compound	Water	Ethanol	Dioxane	Dimethylformamide	Me_2SO
Flavine acid					
I_5	100	115	124	115	64
Flavinylhistidine (R_2)					
III_5	45	51	60	72	62
III_4	41	47	61	73	62
III_3	39	47	55	68	51
III_2	30	39	48	77	42

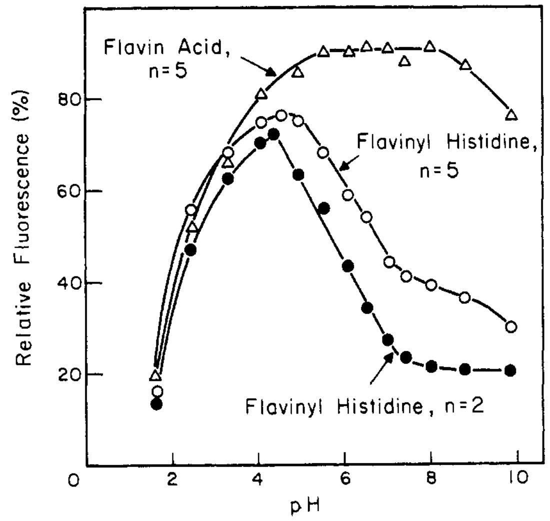


FIGURE 3: The effect of pH on the flavine fluorescence of 10-ω-carboxypentylflavine (I_5) and flavinylhistidines ($III_{2,5}$, R_2). Aqueous solutions contained 10^{-5} M flavine in various buffers (Johnson and Metzler, 1970) at 25°. Numbers (n) refer to the aliphatic methylene groups in the flavine side chain.

Both peptides exhibit a similar quenching; however, the long-chain flavinylhistidine, $n = 5$, shows a much larger curtailment of fluorescence (37%), compared to the short-chain one, $n = 2$ (18%).

A most interesting and important feature, which derives from this fluorescence study, is depicted in Figure 3, which illustrates the effect of pH on the fluorescence of the model peptides. The 10-ω-carboxypentylflavine exhibits a relative fluorescence *vs.* pH curve in aqueous buffer typical of riboflavine or FMN, *i.e.*, a broad maximum at intermediate pH with quenching on both the acid and alkaline sides. However, in the case of the flavine peptides, the fluorescence reaches a maximum at pH 4 and decreases through pH 8. The extent of quenching at each pH is greater for the shorter chain peptide, which agrees, in general, with the data from Figure 1. This fluorescence–pH profile with characteristic optima is reminiscent of those obtained for the natural coenzyme, FAD (Weber, 1950), and such synthetic models as flavinyl-B_6 amides (McCormick, 1972) wherein the non-flavine ring portions are electronically insulated from the flavine as far as the attaching chain is concerned and must, therefore, fold back for interaction leading to fluorescence quenching. Such optima are also found with flavines directly substituted in the isalloxazine rings, where alteration of electronic properties must clearly follow; these latter include such cases as where protonatable electronegative substituents are in the 2 position (Roth and McCormick, 1967) or the natural 8α-(N^3-histidyl)-FAD of certain flavoproteins (Walker and Singer, 1970).

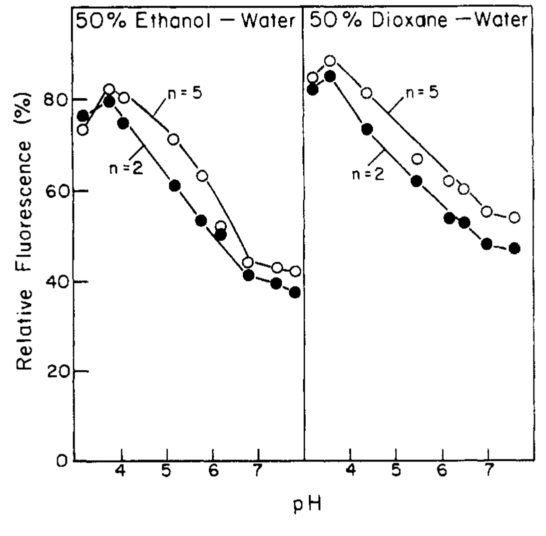


FIGURE 4: The effect of pH on the flavine fluorescence of flavinylhistidines ($III_{2,5}$, R_2). Solutions contained 10^{-5} M flavine in 50% aqueous ethanol or 50% aqueous dioxane buffers at 25°. Numbers (n) refer to the aliphatic methylene groups in the flavine side chain.

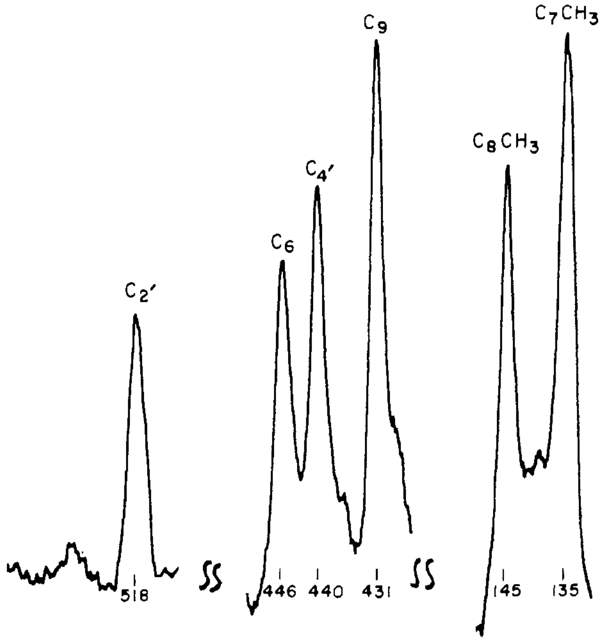


FIGURE 5: The proton magnetic resonance spectrum of 0.1 M flavinylhistidine (III_5, R_1) in D_2O at pD 4.4. Chemical shifts are expressed in cycles per second downfield from tetramethylsilane. The amplitude settings for the $C_{2'}$, C_6, $C_{4'}$, and C_9 protons were three times that of the C_8CH_3 and C_7CH_3.

The effect of pH on the relative fluorescences of the flavinyl peptides in aqueous organic buffer is shown in Figure 4. The shapes of the profiles of both peptides, $n = 2,5$, are similar to those in aqueous buffers, except that the curves are slightly shifted to lower pH values as the solvent polarity decreases.

Proton Magnetic Resonance Properties. The proton magnetic resonance spectrum of the aromatic protons a of flavinylhistidine at pD 4.4 is shown in Figure 5. The experimental assignments of the spectrum of flavine (Bullock and Jardetzky, 1965; McCormick, 1967) and histidine (Sachs *et al.*, 1971) have appeared in the literature. The high-field peaks have been assigned to the C_8CH_3 (145 cps) and C_7CH_3 (135 cps), respectively. The C_8CH_3 peak is assigned downfield from C_7CH_3, since the former has been shown to be slightly acidic by the fact that it exchanges with deuterium at elevated temperatures. The peaks at 518, 446, 440, and 431 cps have been assigned to the $C_{2'}H$, C_6H, $C_{4'}H$, and C_9H, respectively, as warranted by the aforementioned literature survey.

The effect of pD on the chemical shift of these aromatic protons is presented in Table II. Each set of protons of III_5 experience upfield shifts varying from 46 cps ($C_{2'}H$) to 3 cps (C_7CH_3) with increasing pD. Flavinylhistidine III_2 shows upfield shifts similar to III_5, except to a greater degree. At pD 8.0, the peaks in III_5 appear 3.5–8 cps more downfield than those of the shorter chain peptides (III_2).

The effect of temperature on the chemical shift of the C_6H, C_9H, $C_{2'}H$ and $C_{4'}H$ at pD 8.0 is depicted in Table III. It can be

TABLE II: Effect of pD on the Chemical Shift of Flavinylhistidine Protons.[a]

Compound	pD[b]	Flavine Protons C_6H	C_9H	C_8CH_3	C_7CH_3	Histidine Protons $C_{2'}H$	$C_{4'}H$
III_2, R_1	4.4	441	430	143	133	516	435
	5.0	436.5	427	142.5	132	507.5	430
	6.0	435	428.5	140	130	500.5	428.5
	6.5	431.5	425	140.5	131	499	424.5
	7.0	431	424.5	139	129	490.5	418
	7.5	429.5	425	138.5	130	479	413
	8.0	427.5	422.5	138	128.5	464	411
III_5, R_1	4.4	446	431	145	135	518	440
	5.0	440.5	431	144	134.5	510	435
	6.0	438	430	143	134.5	509	432.5
	6.5	435	428.5	142.5	134	505.5	432
	7.0	434.5	428	143	133.5	499	428
	7.5	434	427.5	142.5	133	484.5	420
	8.0	434	426	142	132	472	416

[a] Chemical shifts are in cycles per second downfield from external tetramethylsilane. [b] pD = pH + 0.4 (Glasoe and Long, 1960).

TABLE III: Effect of Temperature on the Chemical Shift of the C_6H, C_9H, $C_{2'}H$, and $C_{4'}H$ Protons[a] of Flavinylhistidines.

Compound[b]	Temp (°C)	C_6H	C_9H	$C_{2'}H$	$C_{4'}H$
III_2, R_1	10	421.5	417.5	457	407
	20	425.5	420	459.5	408.5
	30	427	422	465	409
	40	431.5	425.5	470	411.5
	50	433.5	427.5	474	417
III_5, R_1	10	431	425	462.5	408
	20	432	424	467	410.5
	30	434	427.5	470	412
	40	436	429	474	414
	50	441	431	477.5	415.5

[a] Chemical shifts are in cycles per second downfield from external tetramethylsilane. [b] 0.1 M flavine in D_2O at pD 8.0.

seen that the chemical shift positions of the aromatic protons increase throughout the temperature range of 10–50°.

Discussion

The measurement of fluorescence emission has again proved to be a good indicator of flavinyl peptide interactions. In aqueous solution at pH 7.6, fluorescence quenching of the flavine peptides, compared to "free" flavine, seems to indicate some intramolecular complex formation may occur between the flavine and the histidine. The lack of extensive quenching (60–70%), compared to that observed for flavinyltryptophan peptides with comparable lengths in the separating chains (98–99%), may reflect polarity differences between imidazole and indole rings but is also likely due to the greater hydrophobic surface of the latter. Furthermore, the lower fluorescence of the shorter chain flavinylhistidine, as compared to the longer chain one, is probably due to the contribution of two general types of quenching mechanisms, *i.e.*, collisions in the excited state and ground-state complex formation.

The effect of temperature and organic solvents on the relative fluorescence of the peptides certainly helps to distinguish between these two mechanisms. The large decrease in fluorescence of the longest chain peptide upon increasing temperature can be interpreted as a quenching due to greater molecular collisions between the singlet excited state of the flavine and the imidazole ring of the histidine moiety. The less-pronounced quenching in the case of the shortest chain peptide perhaps reflects more intramolecular ground-state quenching, which would, of course, decrease with increasing temperature due to dissociation of this nonfluorescent complex.

The fluorescent properties of flavines are considerably influenced by solvent characteristics. This may be due to solvent polarity effects in which the flavine acid behaves similarly to other "free" flavines, such as FMN, in that the more polar excited states interact more strongly with polar solvents than the less-polar ground states. Such an interaction would cause fluorescence quenching, as well as a decrease in the energy difference between the excited and ground states (Kotaki and Yagi, 1970). Consequently, these flavines would show an increase in fluorescence as the solvent polarity decreases (*cf.* Table I). The greater solvent effect for the flavinyl peptides, compared to the acids, may be due to a ground-state association of flavine and histidine in water, which dissociates in solvents of organic characteristic. The larger increase in fluorescence for the short-chain peptide again may reflect greater intramolecular, nonexcitable complex formation as the proximity between the two chromophores becomes greater. However, specific interactions between solvent and flavine may occur, and apparently does in the case of dimethyl sulfoxide.

The fluorescence–pH profile of the flavinylhistidines not only shows that complex formation is important in quenching, but also that the ionic state of the imidazole ring controls such dissociations. At pH 4, the histidine is protonated and apparently does not associate appreciably with the flavine moiety. The small amount of quenching (*ca.* 10–20% of "free" flavine) that occurs may be collisional in nature. However, as the acidity decreases through pH 8, the fluorescence quenching may be at least partly attributed to intramolecular complexing between the flavine and histidine as the imidazole ring deprotonates. Again, the profile confirms that the proximity of the flavine and histidine is important, even at a pH other than 7.6. Of course, the fluorescence of both 10-ω-carboxypentylflavine and flavinylhistidines is quenched on the strongly acid side of the profile due to collisions between chloride ion (from the buffer) and flavine, as well as partial protonation of the isoalloxazine ring (Weber, 1950). On the strongly alkaline side, quenching is due to deprotonation of the 3-imino hydrogen of the flavine.

The small acid shift of the fluorescence–pH curves in 50% aqueous–organic solvents may weakly suggest that it is the deprotonated imidazole ring that forms the dark, nonfluorescent complexes. Since the basicity of nitrogen-containing ligands is known to decrease as solvent polarity decreases (Sigel and McCormick, 1970), the basicity of the imidazole should behave similarly.

It has been shown in previous work that the aforementioned flavin interactions can be monitored by the upfield shift of the aromatic protons of the model peptides (Föry *et al.*, 1970). However, since several of the resonance peaks of the isoalloxazine ring are concentration dependent, and the peaks of the histidine pH dependent, such a quantitative study in the case of flavine–histidines would be difficult. Therefore, the objective in this study is to show that the proton magnetic resonance data simply substantiates, more fully, the fluorescence work.

The pD-dependent upfield shifts of the flavine aromatic protons are small, indicating a rather weak interaction with the imidazole ring. However, the short-chain peptide (III_2) experiences consistently more pronounced shifts than the long-chain one (III_5). Therefore, the proton magnetic resonance and fluorescence data complement each other by suggesting a specific involvement of the flavine aromatic moieties in complex formation when the proximity of the chromophores increases. The downfield shift of the flavine protons upon increasing temperature again implies ground-state complex formation at lower temperatures and an apparent unfolding of the peptide as the sample is warmed to 50° at pD 8.0.

The larger upfield shifts, with increasing pD, experienced by the $C_{2'}H$ and $C_{4'}H$ of the peptides are not just due to flavine–histidine interactions. Sachs *et al.* (1971) have measured the chemical shift of these two two protons for *N*-acetylhistidine at various pH values. They reported an upfield shift from approximately 518 to 480 cps for $C_{2'}H$ and from 436 to 422 cps for $C_{4'}H$ as the nitrogen of the imidazole ring is deprotonated. At pD 4.4 (pH 4.0), the chemical shift positions of $C_{2'}H$ and $C_{4'}H$ of the flavinylhistidine (III_5) are very near those of *N*-acetylhistidine, 518 *vs.* 518 cps and 440 *vs.* 436 cps, respectively. However, the resonance peaks of the peptide move further upfield upon deprotonation than those of the histidine model, *i.e.*, 8 cps for the $C_{2'}H$ peak and 6 cps for $C_{4'}H$. Again, the data denote a weak interaction; however, the upfield shift is greater for the short-chain flavinylhistidine (III_2). The chemical shift difference between the peptide and *N*-acetylhistidine of 16 cps ($C_{2'}H$) and 11 cps ($C_{4'}H$) in the case of III_2 is in good agreement with the results from the study of its fluorescence properties.

From all of the foregoing, it is clear that the proximity of an imidazole function, whether a substrate or histidyl residue of a flavoprotein, can considerably influence the spectral (and, hence, electronic) properties of a flavine coenzyme, even though the strength of such interaction may not so markedly contribute to the binding of the flavine as was found for the indole and phenolic systems of tryptophan and tyrosine, respectively.

It is also clear that the simple observation of a fluorescence–pH optimum below neutrality for flavoproteins is not sufficient evidence to suppose that FAD, or even the 8α-(N^3-histidyl)-FAD, must be the coenzyme form present, since a nearby histidine noncovalently bound to the flavine can elicit a similar pH-dependent quenching of flavine fluorescence.

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