Synthetic Flavinyl Peptides Related to the Active Site of Mitochondrial Monoamine Oxidase. II. Fluorescence Properties†

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ABSTRACT: The fluorescence properties of various 8α-sulfur-linked flavinyl peptides and related flavin analogues were investigated as the pH, solvent, temperature, and flavin concentration were varied. Substitution in the 8α position by a thioether-linked peptide brings about a marked quenching of fluorescence (up to 98% in water), a slight bathochromic shift and broadening of the fluorescence emission spectra, and a slight decrease in the fluorescence lifetimes. Oxidation of the thioether function to a sulfone partially releases this fluorescence quenching without further changes in the fluorescence emission spectra. The primary effect on the fluorescence intensity is due to an interaction between the nonbonding electrons of the thioether, the hydrogen-bonding, polar solvent, and the isoalloxazine ring. Dissolving these flavinyl peptides in nonaqueous solvents increases the fluorescence intensity as much as 20-fold. A secondary effect on flavinyl fluorescence can be attributed to a collisional quenching by the vicinal tyrosyl residue within tyrosine-containing flavinyl peptides. The fluorescence properties provide further confirmation of the identity of the synthetic and naturally obtained flavinyl peptides and of the interaction between the free-hydroxyl functions of the ribityl side chain and the thioether.

The fluorescence properties of flavins provide a very sensitive and useful probe of both the microenvironment that surrounds the flavin molecule and the resulting effects on the electronic properties of the flavin. Complex formation between various compounds and flavins in solution, both in the ground and excited states, and binding to most flavoproteins have been shown to quench flavin fluorescence (Penzer and Radda, 1967).

Flavin fluorescence has been shown to be quenched most markedly by two classes of amino acids, viz., aromatic and sulfur-containing ones (Penzer and Radda, 1967). The nature of the quenching mechanisms involved has not been delineated for either of these classes of amino acids when covalently attached to flavin in a natural manner, although some information has been derived from investigations on model compounds of covalently attached aromatic amino acids and flavins (Föry et al., 1968; MacKenzie et al., 1969; Wu and McCormick, 1971; Johnson and McCormick, 1973; Johnson et al., 1975). These model compounds have a lower quantum yield of fluorescence (Φ_F), which has shown to be due predominantly to ground-state complexes and, to a lesser extent, to collisional quenching of the excited state. The stabilities of such complexes in aqueous media decrease in the order tryptophan > tyrosine > phenylalanine > histidine. Less detail has been reported for the fluorescence quenching of flavins by sulfur-containing amino acids, i.e., cysteine, cystine, methionine, and related compounds.

The 8α position of the flavin nucleus of FAD is the site of attachment of amino acid moieties in such covalently bound flavoproteins as are now known to occur in nature (Singer and Edmondson, 1974; Singer and Kenney, 1974). Substitution of the isoalloxazine ring in the 8α position by substituents with nonbonding electrons capable of interacting with the π system of the isoalloxazine ring leads to a dramatic decrease in Φ_F of the flavin (Salach et al., 1972). Negation of nonbonding electron interactions with the π system by protonation of the imidazole ring in histidyl-substituted flavins, oxidation of the thioether in cysteinyl-substituted flavins, or substitution by compounds without available nonbonding electrons all result in flavin derivatives that retain most or all of the fluorescence of the parent 7,8-dimethylisoalloxazine.

The synthetic 8α-*S*-linked flavinyl peptides described in the preceding paper (Falk et al., 1976) and N^{10}-flavinyl-L-methionine are utilized in the present investigation to study the flavin–amino acid interactions outlined above. The effects of such sulfur-containing 8α substituents and the additional effects of such nearby amino acids as tyrosine on the fluorescence properties of flavinyl peptides of the monoamine oxidase type are delineated.

Materials and Methods

The syntheses of the 8α-substituted flavinyl peptides were described in the preceding paper (Falk et al., 1976). N^{10}-Flavinyl-L-methionine ($n = 5$) was synthesized according to Föry et al. (1968). All solvents were spectral or analytical grade.

Fluorescence measurements were made on an Aminco-Bowman spectrophotofluorometer with 3-mm slits and an RCA 1P28 photomultiplier tube. The temperature of the cell compartment was controlled with a Haake Model F constant-temperature circulating bath. Depending on the

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particular flavin and conditions used, flavins were excited at their λ_{max}^{excit}, around 450 nm, and the relative fluorescence intensity determined at their λ_{max}^{emis}, around 530 nm, and corrected for concentration and solvent blank. Flavin stock solutions were made daily, 10^{-3} M in methanol, and diluted just before use so that the final methanol concentration was less than 1% (v/v). Flavin concentrations were determined by absorbance at 448 nm (ϵ 1.2 × 10^4 l. mol^{-1} cm^{-1}).

Fluorescence–pH profiles were determined for 4 × 10^{-5} M flavin solutions in the following 0.05 M buffers: glycine-HCl, pH 2, 3; sodium acetate, pH 4, 5; sodium phosphate, pH 6, 7; Tris, pH 8, 9; and sodium bicarbonate, pH 10.

Fluorescence–temperature studies were conducted in an anaerobic fluorescence cell designed by Hodgson et al. (1973) and constructed by Precision Cells, Inc.; anaerobic conditions were maintained with deoxygenated N_2 during temperature equilibration.

Fluorescence polarization was measured with a Glan prism assembly and glycerol–water mixtures of 4 × 10^{-5} M flavin at 20 ± 0.5 °C. Desired viscosities were achieved according to Sheeley (1932), and the glycerol concentration was checked by refractive index measurements on a Bausch & Lomb Type 33-45-58 refractometer. The values of fluorescence polarization were obtained using the formula of Azumi and McGlynn (1962), after first correcting for solvent blanks.

Fluorescence lifetimes were determined on an apparatus which was described previously (Andrews et al., 1974). An Avco-Everett N_2 laser transversely pumped a dye laser, which was tuned to the wavelength required and focused into selected crystals to generate the second harmonic. The resulting beam was used to excite the flavin solutions. The resulting fluorescence was detected with an RCA 1P28 photomultiplier tube, the output from which went to a sampling oscilloscope, then to a Mnemotron CAT 400B, or to a real-time oscilloscope, and recorded on film. The data were then analyzed by deconvolution techniques formulated by Formoso and Forster (1975) to extract the rapid-decay parameters.

Results

Relative Fluorescence Intensities. As reported earlier (Walker et al., 1971), flavin derivatives with an 8α-thioether function fluoresce with only a small fraction of the intensity exhibited by 8-methylflavins. As shown by the data for phosphate buffer in Table I, the fluorescence quenching effect attributable to thioethers is partially released upon oxidation of such derivatives to their respective sulfones. Flavinyl peptides containing a tyrosyl residue are also somewhat more quenched than those without. The values reported in Table I (phosphate buffer) are essentially in agreement but somewhat lower than those reported by Walker et al. (1971). This probably reflects a higher degree of purity for the synthetic compounds. Acetylation of the ribityl side chain of riboflavin leads to a modest increase in fluorescence intensity by decreasing the interaction between the quenching hydroxyl functions and the isoalloxazine ring. (RF or FMN, which are not listed in Table I, have relative fluorescence intensities of 88% in phosphate buffer.) This trend is reversed for the flavinyl peptides studied, wherein ribityl chain acetylation brings about a decrease in fluorescence intensity, which may indicate an additional interaction between the ribityl side chain hydroxyl function and perhaps the thioether bond.

Fluorescence Emission Spectra. The fluorescence emis-

Table I: Effects of Solvents on Flavin Fluorescence.

Flavin (10^{-5} M)	Phosphate Buffer	Ethanol (mol %)		Acetone (mol %)		Dioxane (mol %)		Dimethylformamide (mol %)			Dimethyl Sulfoxide (mol %)			$CHCl_3$
		50	100	50	100	50	100	50	100	Formamide	50	100		
	(λ_{max}^{emis})[a]		(λ_{max}^{emis})[a]		(λ_{max}^{emis})[a]		(λ_{max}^{emis})[a]		(λ_{max}^{emis})[a]	(λ_{max}^{emis})[a]		(λ_{max}^{emis})[a]	(λ_{max}^{emis})[a]	(λ_{max}^{emis})[a]
Ac_4RF	100 (525)	148	169 (515)	163	209 (510)	172	232 (509)	152	158 (512)	137	51	48 (515)		194 (515)
$AcCysAc_4RF$	3 (528)	9	20 (519)	13	73 (510)	44	138 (511)	14	23 (515)	44	15	19 (517)		73 (517)
$AcCysTyrAc_4RF$	2 (528)	8	21 (519)	12	55 (510)	11	128 (512)	14	29 (515)	28	12	17 (517)		53 (517)
$AcGlyCysTyrAc_4RF$	2 (528)	7	18 (519)	12	62 (510)	27	121 (512)	14	55 (515)		8	17 (517)		61 (517)
$AcCys(O_2)Ac_4RF$	72 (528)	86	94 (520)	68	147 (512)	121	190 (511)	12	120 (515)		81	22 (518)		155 (519)
$AcCys(O_2)TyrAc_4RF$	63 (528)	84	90 (520)	95	134 (512)	126	157 (511)	106	150 (515)		67	18 (518)		79 (519)
$AcGlyCys(O_2)TyrAc_4RF$	60 (528)	82	95 (520)	97	110 (512)	120	157 (511)	112	152 (515)		63	19 (518)		64 (519)
AcCysRF	8 (528)	21	33 (521)	21	63 (517)	40	92 (512)	26	55 (520)		15	39 (520)		20 (517)
AcCysTyrRF	6 (528)	15	31 (521)	19	60 (517)	42	96 (512)	26	51 (520)		12	51 (520)		11 (517)

[a] λ_{max}^{emis} are uncorrected.

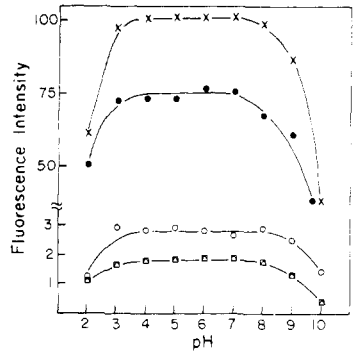

FIGURE 1: Fluorescence–pH profile of flavins, 4×10^{-5} M at 25 °C, in the buffers described under Materials and Methods. Compounds are: Ac_4RF (×); $AcCys(O_2)Ac_4RF$ (●); $AcCysAc_4RF$ (○); $AcCysTyrAc_4RF$ (△); and $AcGlyCysTyrAc_4RF$ (□).

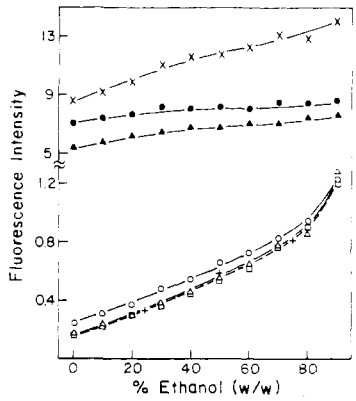

FIGURE 2: Fluorescence of tetraacetylated flavinyl peptides, 10^{-5} M in ethanol–water solutions (w/w), at 25 °C. Compounds are: Ac_4RF (×); $AcCys(O_2)Ac_4RF$ (●); $AcCys(O_2)TyrAc_4RF$ (▲); $AcCysAc_4RF$ (○); $AcCysTyrAc_4RF$ (△); $AcCysTyr(O\text{-}Me)Ac_4RF$ (+ – +); and $AcGlyCysTyrAc_4RF$ (□- -□).

sion spectra of the synthetic flavinyl peptides also compare favorably to the spectra of the naturally obtained flavinyl pentapeptide of Kearney et al. (1971). The 8α-substituted flavins exhibit a bathochromic shift of 5–7 nm and a slight broadening of the bandwidth relative to 8-methylflavins with $\lambda_{max}^{emis} \approx 525$ nm. As the widths and positions of the fluorescence emission bands of the various flavinyl peptides are essentially identical, relative measurements in aqueous solutions correlate well with Φ_F.

Fluorescence–pH Profiles. Fluorescence intensities as a function of pH exhibit relatively flat optima between pH 3 and 8 for Ac_4RF and the flavinyl peptides, as seen in Figure 1. These results are identical with those reported by Kearney et al. (1971) for the naturally obtained flavinyl pentapeptide from monoamine oxidase and the synthetic 8α-cysteinylriboflavin. The corresponding 8α-sulfone derivatives, previously unreported, exhibit the same fluorescence–pH effect as the 8α-thioether derivatives. The flavin fluorescence decreases on the acidic side due to quenching by chloride and protonation of the N^1-nitrogen of the isoalloxazine ring, and on the basic side due to deprotonation of the N^3-nitrogen and decomposition of the isoalloxazine ring.

The excellent correspondence exhibited between the synthetic *N*-acetyl flavinyl peptides (Falk et al., 1976) and the naturally obtained flavin (Kearney et al., 1971; Walker et al., 1971) indicates that the synthetic compounds are good representatives of the properties of the naturally obtained

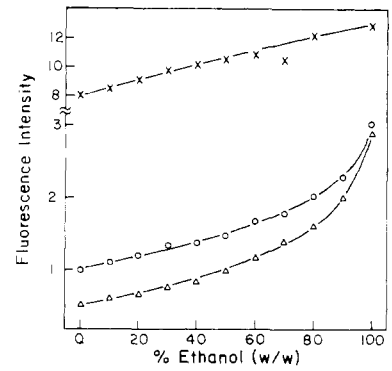

FIGURE 3: Fluorescence of deacetylated flavinyl peptides, 10^{-5} M in ethanol–water solutions (w/w), at 25 °C. Compounds are: FMN (or RF) (×); AcCysRF (○); and AcCysTyrRF (△).

flavinyl pentapeptide isolated from the active site of mitochondrial monoamine oxidase and further substantiates the structure proposed for the latter.

Solvent Effects. In general, flavins exhibit an increase in fluorescence as solvent polarity decreases (Koziol and Knobloch, 1965; Yagi et al., 1972). In Figure 2, the fluorescence of tetraacetylated flavinyl peptides are compared with tetraacetylriboflavin in varying ethanol–water concentrations. The thioether derivatives exhibit the greatest relative increase of fluorescence with increase in ethanol, while the fluorescence of sulfone derivatives increases less extensively than tetraacetylriboflavin.

Tyrosine-containing flavinyl peptides have a lower fluorescence intensity in phosphate buffer but approach the fluorescence intensity of their respective nontyrosine-containing counterparts as the ethanol concentration is increased. A comparison of the behaviors of deacetylated flavinyl peptides and riboflavin (or FMN) is given in Figure 3. Again, one sees relatively large increases in the fluorescence of the thioether-substituted flavins as the concentration of ethanol is increased. Although only relative fluorescence intensity is measured, comparisons between flavins are essentially quantitative, since the fluorescence emission spectra of all of the 8α-substituted flavins in this study are nearly the same.

In the even less-polar and aprotic dioxane solutions (Figures 4 and 5), virtually all of the same trends in fluorescence behavior are observed as with the ethanol solutions, but the effects are greater. The log of fluorescence intensity is plotted in Figure 5, as the fluorescence of 8α-thioether-substituted tetraacetylriboflavins increases over two orders of magnitude from 0 to 100% dioxane. Although the curve for $AcGlyCysTyrAc_4RF$ is not as simple as the others, the tyrosine-containing flavinyl peptides exhibit, in all cases, a somewhat greater quenching at low dioxane concentrations than the nontyrosine-containing ones. As the dioxane concentration increases, the fluorescence intensities of all of the related flavinyl peptides approach the same value. Fluorescence intensities of the deacetylated flavinyl peptides (i.e., AcCysRF and AcCysTyrRF) approach each other at a considerably lower percentage of dioxane than their respective acetylated analogues but, similarly, increase to a maximal value in the pure solvent (Figure 5).

The data presented in Table I document the large effects of various solvents on the fluorescence of the 8α-S-linked flavinyl peptides. While the fluorescence intensity of Ac_4RF increases only about twofold in solvents of low polarity

Table II: Effects of Inter- and Intramolecular Methionyl Residues on the Flavin Fluorescence in Varying Ethanol–Water Solutions at 25 °C.

Compound	M × 10^5	Ethanol–Water[a] (%, v/v)	Relative Fluorescence	Change from Riboflavin in Water (%)
N^{10}-Flavinyl-L-methionine	2	0	40	−60
(n = 5)	2	20	52	−48
	2	50	77	−23
Riboflavin +	2	80	90	−10
L-Methionine	2 + 200	0	97	−3
DL-Methionine sulfoxide	2 + 200	0	100	0
DL-Methionine sulfone	2 + 200	0	100	0
Riboflavin	2	0	100	0
	2	20	109	+9
	2	50	116	+16
	2	80	126	+26

[a] All solutions were 0.01 M in sodium phosphate (pH 7).

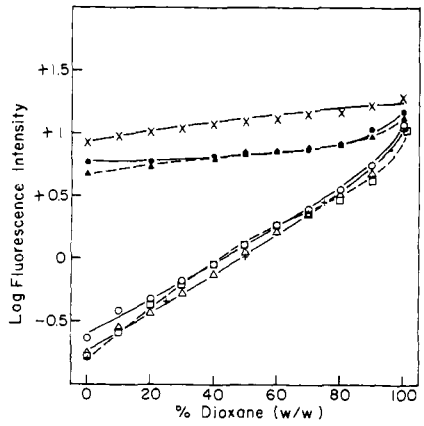

FIGURE 4: Log fluorescence of tetraacetylated flavinyl peptides, 10^{-5} M in dioxane–water solutions (w/w), at 25 °C. Compounds are: Ac_4RF (×); $AcCys(O_2)Ac_4RF$ (●); $AcCys(O_2)TyrAc_4RF$ and $AcGlyCys(O_2)TyrAc_4RF$ (▲); $AcCysAc_4RF$ (○); $AcCysTyrAc_4RF$ (△); $AcCysTyr(O\text{-Me})Ac_4RF$ (+ − +); and $AcGlyCysTyrAc_4RF$ (□).

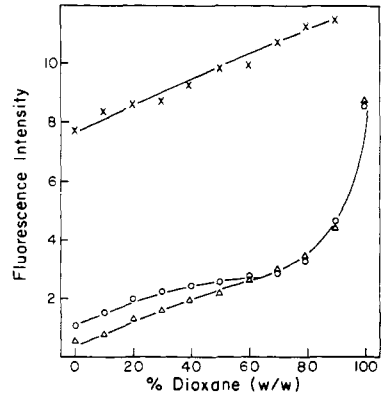

FIGURE 5: Fluorescence of deacetylated flavinyl peptides, 10^{-5} M in dioxane–water solutions (w/w), at 25 °C. Compounds are: FMN (or RF) (×); AcCysRF (○); and AcCysTyrRF (△).

compared to aqueous medium, the increase with 8α-substituted thioethers is as much as 75-fold. All of the 8α-substituted flavinyl peptides (sulfones and thioethers) generally exhibit larger changes in fluorescence intensity with change in solvent than does Ac_4RF. In dimethyl sulfoxide, the fluorescence intensity of Ac_4RF is less than in water, whereas the fluorescence intensities of the 8α-substituted thioethers increase about tenfold. It is apparent that both decreasing solvent polarity and hydrogen bonding capacity have at least three effects on the fluorescence intensity. The dominant factor is the partial restoration of fluorescence that was lost, largely due to 8α substitution by a thioether. Of lesser magnitude is an increase in intrinsic fluorescence, which is realized by all flavins, including typical 8-methyl-substituted cases, such as Ac_4RF and FMN. Simultaneously, an additional restoration of intrinsic fluorescence is observed in those flavinyl peptides that contain a vicinal tyrosyl residue, which is less quenched in the less-polar solvents than in water, where compacting interactions are favored.

Although the values of λ_{max}^{emis} in Table I are uncorrected and only approximate (±2 nm), it is clearly evident that the λ_{max}^{emis} of the 8α-substituted flavinyl peptides varies in a manner parallel to the 8-methylflavins, both in direction and magnitude. All of the flavins measured undergo a bathochromic shift of approximately 10–15 nm (0.2–0.6 × 10^3 cm^{-1}) as the solvent varies from nonpolar to polar.

N^{10}-Flavinyl-L-methionine. As seen in Table II, methionyl residue, covalently attached by an alkyl chain (five methylenes) to the N^{10} position of a 7,8-dimethylisoalloxazine, which is isoelectronic with such typical flavins as lumiflavin or riboflavin (viz., N^{10}-flavinyl-L-methionine, n = 5), has a sizable quenching effect on the flavin fluorescence. This is clearly due to the partially constrained proximity of the thioether function, since methionine added intermolecularly to flavin solutions effects slight quenching, whereas none is seen with comparable concentrations of the sulfoxides or sulfones of methionine. The quenching by the covalently attached thioether function is considerably less intense than the quenching effect of the 8α-substituted thioethers but nearly comparable to the quenching effects of such an aromatic amino acid as tyrosine covalently attached in a similar manner through an electronically insulating side chain in the flavin N^{10} position (Föry et al., 1968). Earlier studies on the effect of organic solvents on the fluorescence intensity of N^{10}-flavinyl amino acids point to the commonality of the similar effects of solvents of N^{10}-flavinyl-L-methionine. This suggests that the large increase in fluorescence intensity, with respect to free flavin as solvent polarity decreases, may be partially caused by opening of a "dark" (nonfluorescent), ground-state complex(es) between the methionine and isoalloxazine ring portions of the molecule.

Effects of Temperature on Fluorescence. Flavins, in general, exhibit a decrease in fluorescence intensity as the temperature increases. The data in Figures 6 and 7 illustrate the effect of change in temperature on fluorescence of 8α-S-linked flavins and are plotted as a percentage of the fluorescence intensity of free flavin to correct for the contribution of the latter. When the side chain hydroxyls are protected (Figure 6), fluorescence of the flavinyl peptides decreases linearly as temperature increases. There is no additional temperature effect on $AcCysAc_4RF$ over that exhibited by Ac_4RF. The fluorescence of $AcCysTyrAc_4RF$ decreases somewhat more markedly as the temperature increases and must reflect additional, intramolecular quenching by the tyrosyl moiety. As the temperature increases, the solvent viscosity decreases, and intermolecular Brownian motion increases. This leads to an increase in collisions and a resulting decrease in fluorescence intensity. If opening of

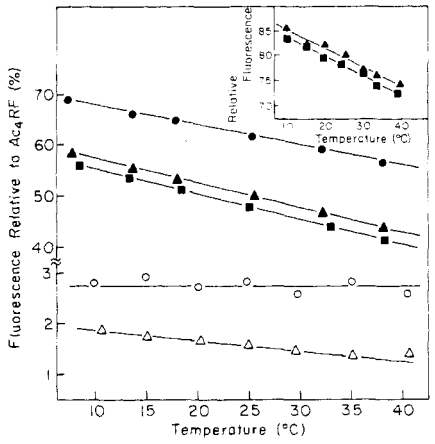

FIGURE 6: Fluorescence-temperature profile of tetraacetylated flavinyl peptides, 2×10^{-5} M in 0.05 M potassium phosphate buffer (pH 7). Fluorescence is expressed as a percentage of the fluorescence of Ac_4RF, measured under the same conditions. Compounds are: $AcCys(O_2)Ac_4RF$ (●); $AcCys(O_2)TyrAc_4RF$ (▲); $AcGlyCys(O_2)TyrAc_4RF$ (■); $AcCysAc_4RF$ (○); and $AcCysTyrAc_4RF$ (△). The insert shows the effect of temperature on tetraacetylated flavinyl peptide sulfones, expressed as a percentage of the fluorescence of $AcCys(O_2)Ac_4RF$, measured under the same conditions ((▲) $AcCys(O_2)TyrAc_4RF$, and (■) $AcGlyCys(O_2)TyrAc_4RF$).

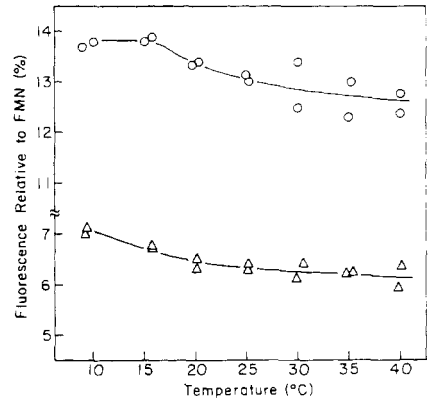

FIGURE 7: Fluorescence-temperature profile of deacetylated flavinyl peptides, 2×10^{-5} M in 0.05 M potassium phosphate buffer (pH 7). Fluorescence is expressed as a percentage of the fluorescence of FMN (or RF), measured under the same conditions. Compounds are: AcCysRF (○); and AcCysTyrRF (△).

"dark", ground-state complexes between the flavin and tyrosine were involved to a significant extent, there should be an increase in fluorescence intensity in competition with the collisional effects. However, it is clear from Figure 6 that collisional effects are more pronounced as the temperature is increased.

All of the sulfones have a more extensively decreasing fluorescence intensity with increasing temperatures than does Ac_4RF. This is not surprising, as different substituents have different temperature coefficients in many systems, usually depending upon vibrational modes of restraints. The fluorescence changes of tyrosine-containing, 8α-substituted sulfones are presented in the insert in Figure 6 as a percentage of the fluorescence intensity of $AcCys(O_2)Ac_4RF$ to emphasize that they, too, show an additional decrease in fluorescence intensity as the temperature increases. Again, this decrease reflects the collisional quenching by tyrosyl residues.

In Figure 7, the effect of temperature on the fluorescence of the riboflavin derivatives is compared to FMN (the temperature profiles of FMN and RF are quite similar to Ac_4RF). The fluorescence intensities no longer exhibit a simple linear decrease with increasing temperature in these cases. This may be due to the additional interaction from the free-hydroxyl function of the side chain, as could also be related to the lower fluorescence of such flavins in more-aqueous solvents. Nevertheless, the effect of the tyrosyl residue can be detected in the slightly greater decrease in the fluorescence of AcCysTyrRF, compared to AcCysRF, with increase from lower temperatures.

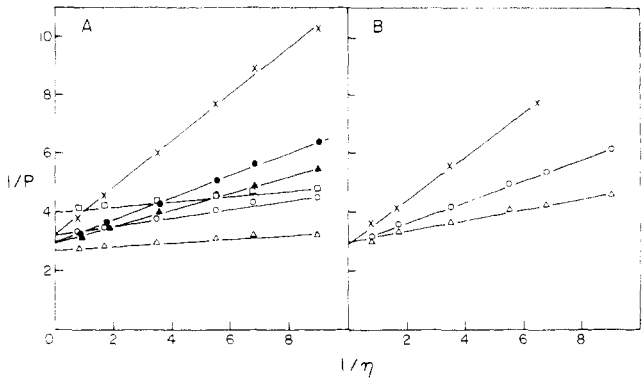

FIGURE 8: Double-reciprocal plot of the polarization of fluorescence of tetraacetylated (A) and deacetylated (B) flavinyl peptides, 10^{-5} M in glycerol-water solutions of varying viscosity, at 20 °C. Symbols are: P, polarization of fluorescence; and η, viscosity (poise). Compounds in A are: Ac_4RF (×); $AcCys(O_2)Ac_4RF$ (●); $AcCys(O_2)TyrAc_4RF$ (▲); $AcGlyCysTyrAc_4RF$ (□); $AcCysAc_4RF$ (○); and $AcCysTyrAc_4RF$ (△). Compounds in B are: FMN (or RF) (×); AcCysRF (○); and AcCysTyrRF (△).

Effect of Flavin Concentration on Fluorescence. To determine the extent to which intermolecular, as well as intramolecular, interactions affect the fluorescence intensity, measurements were made over a 1000-fold concentration range in various solvents. To minimize errors due to inner-filter effects, the data are also presented as a percentage of the fluorescence intensity of free flavin (FMN or Ac_4RF) subjected to the same procedures. In phosphate buffer, over the range of 10^{-3} to 10^{-6} M, the relative fluorescence of $AcCysAc_4RF$ and $AcCysTyrAc_4RF$ (as percent of FMN) is invariant. Therefore, there is no additional intramolecular interaction, measurable by fluorescence, for the 8α-substituted flavins over those normally observed for 8-methylflavins in aqueous solution. In chloroform and ethanol, over the concentration range employed for all of the other fluorescence measurements, the relative fluorescence of $AcCysAc_4RF$ and $AcCysTyrAc_4RF$ (as percent of Ac_4RF) is again invariant. However, at 10^{-4} M and above, in chloroform and ethanol the relative fluorescence of both 8α-substituted flavins decreases about 40% below that observed at lower concentrations. As is not usual for compounds with highly polar functional groups, at the higher concentrations examined in the nonaqueous solvents there appears to be a significant, additional intramolecular quenching interaction for the 8α-substituted flavins.

Fluorescence Polarization. Fluorescence lifetimes can be used to discriminate between the two general modes of quenching (static or dynamic). Dynamic quenching is entirely reflected by a proportional decrease in the mean lifetime of fluorescence, whereas static quenching, in general, has no effect on lifetime (Weber, 1948).

Fluorescence polarization can be used to obtain an estimate of the fluorescence lifetime. Perrin (1926) derived the

Table III: Estimates of Fluorescence Lifetimes and Quenching of Flavins.

Flavin	Mol Wt ≈ V	$1/P_0 - 0.33$[a]	Slope[a]	Ib	I_0/Ib	τ[c]	τ_0/τ[c]	% Static Quenching Relative to Ac_4RF[c]
Ac_4RF	545	2.91	0.80	100		5		
$AcCysAc_4RF$	692	2.89	0.15	2.8	36	1.2	4.17	91
$AcCysTyrAc_4RF$	869	2.44	0.06	1.8	56	0.7	7.14	89
$AcGlyCysTyrAc_4RF$	926	3.71	0.08	2.6	38	0.7	7.14	89
$AcCys(O_2)Ac_4RF$	724	2.67	0.38	72	1.4	3.4	1.45	0
$AcCys(O_2)TyrAc_4RF$	901	2.68	0.28	63	1.6	3.2	1.59	0
FMN or RF	457	2.63	0.74	88	1.14	4.3	1.16	0
AcCysRF	524	2.67	0.36	7.8	12.8	2.4	2.13	90
AcCysTyrRF	701	2.74	0.17	5.8	17.2	1.4	3.45	85

[a] Obtained from Figure 8. [b] Obtained from Table I; I = fluorescence intensity in phosphate buffer; I_0 = fluorescence intensity of Ac_4RF in phosphate buffer. [c] Calculated from $[(I_0/I - 1) - (\tau_0/\tau - 1)]/(I_0/I - 1) \times 100$, assuming that $I_0/I = \tau_0/\tau$ for dynamic quenching, and where τ = mean lifetime of fluorescence, and τ_0 = mean lifetime of fluorescence of Ac_4RF.

following equation for the polarization of fluorescence in media of varying viscosity,

$$\frac{1}{P} = \frac{1}{P_0} + \left(\frac{1}{P_0} - \frac{1}{3}\right)\frac{RT}{\eta V}\tau_0$$

where P is the measured polarization of fluorescence of a substance with a fundamental polarization of P_0; τ_0, the mean lifetime of the excited state; V, the molar volume; R, the gas constant; η, the viscosity of the medium; and T, the absolute temperature. Figure 8 presents double-reciprocal plots of the effect of viscosity of a series of glycerol–water solutions on the polarization of flavin fluorescence. The slope, S, of the lines is given by

$$S = \left(\frac{1}{P_0} - \frac{1}{3}\right)\frac{RT}{V}\tau_0$$

Weber (1966) showed that the polarization of flavin fluorescence is proportional to molecular weight. Assuming V to be proportional to molecular weight, and obtaining S and $1/P_0$ from the plots in Figure 8, one can calculate values for τ_0 for all of the flavins. Using the instrument described in the Experimental Section, a value of $\tau_0 \simeq 5 \times 10^{-9}$ sec was directly obtained for Ac_4RF in aqueous media; the other values of τ_0 in Table III are calculated using this as a standard. In contrast to the behavior of certain of the N^{10}-flavinyl-L-tyrosine series studied by MacKenzie et al. (1969), all of the 8α-substituted compounds examined in the present work exhibited a linear correspondence of $1/P$ to $1/\eta$. From the data in Table III on the percent static quenching relative to Ac_4RF, it is evident that most of the fluorescence quenching of the flavinyl peptides can be attributed to a static, rather than dynamic, mechanism. A comparison of the values for τ and I for the tyrosine-containing flavinyl peptides to the nontyrosine-containing ones leads to an assignment of a dynamic mechanism as the predominant mode of quenching of fluorescence due to the vicinal tyrosyl residue. Similarly evident from Table III is that fluorescence quenching due to the ribityl side chain in FMN (and RF) is primarily a dynamic process.

Fluorescence Lifetimes. By direct measurement (L. S. Forster et al., personal communication), the mean lifetime of fluorescence of FMN was found to be within 10% of the literature value of 4.65 ± 0.5 ns (Spencer and Weber, 1968), and τ for Ac_4RF varied, in 10–90% (v/v) aqueous ethanol solutions, from 5.0 to 6.1 ns, in good agreement with the similar magnitude of change in the fluorescence intensity presented in Figure 3. It would appear that the effect of solvent, then, is primarily on the lifetime of the flavin excited state.

Discussion

The close correspondence in chemical and spectral properties now established between the synthetic flavinyl peptides and the naturally obtained flavinyl pentapeptide from monoamine oxidase provides a firm basis for identification of these 8α-substituted flavins. As expected, acetylation of the amino terminus of the synthetic flavinyl peptides has no significant effect on the spectral properties of such compounds and, in fact, provides derivatives that (more) closely approximate natural N-acetylation by additional amino acyl residues within the intact, native enzyme. In some respects, the tetraacetylated flavinyl peptides are probably better models for the actual milieu of the intact, active site of the FAD-dependent monoamine oxidase than their deacetylated counterparts, since the hydroxyl functions of the 8α-riboflavinyl peptides in solution probably interact with the thioether function to bring about an increase in fluorescence. Within the protein envelope of the enzyme, however, such hydroxyls are likely to be masked.

Little definitive work has been reported on the quenching of flavins by sulfur-containing amino acids. Yet, the importance of such interactions is underscored by the large number of flavoproteins with sulfur-containing amino acids implicated near the flavin-binding site. Not only is the flavin portion of FAD within monoamine oxidase convalently bound to a thioether sulfur from a cysteinyl residue (Walker et al., 1971), but a thiohemiacetal linkage between the covalently bound FAD and a cysteinyl residue occurs within the *Chromatium* flavocytochrome c_{552} (Walker et al., 1974). The "active-site disulfides" of FAD-dependent lipoamide dehydrogenase, glutathione reductase, and thioredoxin reductase (Williams et al., 1971), and the methionyl residue in the FMN-dependent *Clostridia* MP flavodoxin (Burnett et al., 1974) are a few examples of the interactions between sulfur-containing amino acids and flavins in some noncovalently bound flavoproteins. While there has been some work on the flavin fluorescence in these enzymes (Visser et al., 1974; Wahl et al., 1975), the mechanism of quenching and nature and extent of perturbation of the flavin electronic system, as a result of the nearby sulfur-containing amino acids, have never been examined.

Although there is some precedence for weak complex for-

mation between FMN and various disulfides in solution (Draper and Ingraham, 1970), none has been demonstrated for methionine, or other thioethers and thiols. There have been some preliminary studies on the quenching of flavins in solution by sulfur-containing compounds; these have been reviewed by Penzer and Radda (1967), and, in general, little or no data have been presented as to the mode of quenching (static or dynamic), let alone the explicit mechanism involved.

The quenching of tryptophan, and other indoles, and tyrosine by sulfur-containing compounds has been well documented (Cowgill, 1967, 1970; Steiner and Kirby, 1969). The quenching seems to be collisional, with the sulfur-containing compounds perhaps accepting electrons directly from the excited state of the indole or phenol. However, the mechanism is only tentative and can become markedly more complex as a result of such factors as the presence or absence of molecular oxygen and ensuing photochemical events.

In this study, it has been shown that the quenching of flavin fluorescence by sulfur-containing compounds is under fairly strict steric requirements, paralleling the sulfur-dependent quenching of indoles and phenols. As the intramolecular complex in the N^{10}-flavinyl-L-methionine compound is opened in ethanol by removing the hydrophobic compaction allowed in water, the fluorescence quenching decreases rather dramatically. The 8α-thioether-substituted flavins have an even more rigid and intimate interaction between the sulfur orbitals and the π system of the isoalloxazine ring and, therefore, exhibit a markedly greater fluorescence quenching. That this quenching is dependent on solvent interactions with the nonbonding orbitals of the thioether is amply demonstrated by comparing the effect of increasing ethanol concentration with AcCysAc$_4$RF and AcCys(O$_2$)Ac$_4$RF. The substantial increases in fluorescence intensity of the 8α-thioethers in ethanol and, even more strikingly, in the aprotic solvent, dioxane, may implicate hydrogen bonding as the predominant mode of solvent interaction. The large decrease in fluorescence lifetime for AcCysAc$_4$RF with respect to Ac$_4$RF indicates that the quenching is due to an increased dissipation of the singlet (S_1) state. This is accompanied by a large decrease in the triplet and radical yields for the 8α-substituted flavinyl peptides (McCormick et al., 1975) and the N^{10}-flavinyl-L-methionine (Gillard and Tollin, 1974). Therefore, it is unlikely that the quenching of flavin S_1 is due to an increased intersystem crossing. The fact that the fluorescence intensities of AcCysAc$_4$RF and Ac$_4$RF vary with temperature in a parallel manner, and that there are serious steric restraints of the system, rules out a quenching mechanism due mainly to a collisional event between the thioether and isoalloxazine ring, whereby the light-absorbed energy is dissipated only kinetically. Hyperconjugation, unlikely in any event for the ground state, is also unreasonable, in view of the quenching of flavin fluorescence caused by the thioether sulfur within the N^{10}-flavinyl-L-methionine.

Intramolecular charge transfer of the type described by Kasha and Rawls (1968), or mixing of different excited states, as may occur with 1,5-substituted naphthalenes (Li et al., 1975), is unlikely to be a factor in the present system, since the energies of fluorescence of the 8-methylflavin and 8α-substituted flavinyl peptides are similar and exhibit similar, relatively invariant, solvent dependencies.

The position of three of the four absorption bands of 8-methylflavins is insensitive to the composition of the solvent, while the near-uv absorption band shifts bathochromically as the solvent varies from hydrocarbon-like solvents to hydrogen-bonding, polar solvents (Koziol and Knobloch, 1965). Kasha and Rawls (1968) report a hypsochromic shift for charge-transfer bands under similar solvent variations. Hence, the cause for thioether sulfur quenching of the 8α-substituted flavins in hydrogen-bonding solvents, such as water, must be due to an interaction of the nonbonding electrons of the sulfur with the isoalloxazine ring and the solvent. A possible mechanism for nonradiative transition is an increase in vibrational modes for dissipation of the excited-state energy due to these solute–solvent interactions.

Interactions between flavins and aromatic amino acids, both in solution and in flavoproteins, were briefly discussed in the preceding paper (Falk et al., 1976). There is strong evidence for this type of interaction in many covalently bound flavoproteins. The sequences of the flavinyl peptides of monoamine oxidase (Kearney et al., 1971), D-6-hydroxynicotine oxidase (Brühmüller and Decker, 1973), and cytochrome c_{552} (Kenney et al., 1974) have all revealed tyrosyl residues vicinal to the residue that is covalently attached to FAD. The interaction of a second tyrosyl residue with the flavin in cytochrome c_{552} has been demonstrated by CD and increased chemical stability of the flavin (Kenney et al., 1974). There is the further suggestion for a weaker interaction of the carboxy-terminal tyrosyl residue with the flavin in monoamine oxidase and cytochrome c_{552}, on the basis of relative stabilities of the flavinyl peptides before and after removal of the carboxy-terminal tyrosine (Kenney et al., 1974).

The additional fluorescence quenching of tyrosine-containing 8α-flavinyl peptides over the nontyrosine-containing 8α derivatives indicates an interaction between the aromatic amino acid and isoalloxazine ring system. Comparison of fluorescence lifetimes and the effect of temperature on fluorescence of AcCysAc$_4$RF and AcCysTyrAc$_4$RF indicates that a collisional quenching mode is predominantly responsible for this tyrosine-dependent fluorescence quenching in aqueous solutions, although the negation of this additional tyrosine-dependent quenching by solvents of decreasing polarity and hydrogen-bonding capability indicates that some dark complexing may by occurring. Previous work on the N^{10}-flavinyl-L-tyrosine compounds (MacKenzie et al., 1969; Föry et al., 1970) indicated that the tyrosyl moiety can fold over to complex with the isoalloxazine ring in a planar ring-stacking configuration. This complex was opened by increasing the ethanol concentration of the solvent. A similar effect was observed for the 8α-substituted flavinyl peptides in aqueous organic solvent mixtures. Hydrophobic forces were implicated in the N^{10}-flavinyl-L-tyrosine study (MacKenzie et al., 1969) as being primarily responsible for the intramolecular complex. Hydrophobic interactions are probably contributory to an intramolecular complex in the 8α-substituted tyrosyl-containing flavinyl peptides in aqueous solutions. The close correspondence in fluorescence intensity of AcCysTyr(*O*-Me)Ac$_4$RF and AcCysTyrAc$_4$RF, as the organic solvent concentration and temperature were varied, rules out any significant contribution of hydrogen bonding between the tyrosine hydroxyl and pyrimidine ring of the flavin in aqueous solutions, although such bonding may become important in nonpolar, aprotic media. The significant differences between the CD spectra of these flavinyl peptides in $CHCl_3$ may be considered further evidence for hydrogen bonding in aprotic media (Falk et al., 1976). The small, but significant, fluorescence

quenching of the tyrosine-containing flavinyl peptides in some nonaqueous solutions clearly indicates that forces other than hydrophobic are contributory in these solvents. The more-pronounced interaction between the tyrosine and isoalloxazine moieties of the tetraacetylated derivatives with respect to the deacetylated derivatives in the dioxane solutions emphasizes this point.

The absence of any new absorption bands (Falk et al., 1976) would seem to rule out charge-transfer interactions, which are unlikely to contribute significantly to the stacking energy. Dipole–dipole and dipole-induced dipole interactions have been suggested for the N^{10}-flavinyl aromatic amino acid series (MacKenzie et al., 1969) and may well be extrapolated to the 8α-substituted flavinyl peptide series. Additional evidence for intramolecular complex formation in aqueous solvents was provided by flash-photolysis techniques (McCormick et al., 1975), which showed a decreased rate of decay of flavin radical, both aerobically and anaerobically, for the tyrosyl-containing cases.

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