

Volume Changes in the Formation of Internal Complexes of Flavinylyltryptophan Peptides[†]

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ABSTRACT: The effect of pressure, up to 10 kbar, on the fluorescence yield and lifetime of two flavinylyltryptophan peptides was investigated. These peptides differed only in the number of methylene groups, respectively three and five, separating the chromophores. At atmospheric pressure the closed non-fluorescent form predominated in both compounds constituting 94% of the total in the short-linked peptide and 80% in the long-linked one. The fluorescence of both peptides decreased at high pressure and the volume change upon formation of the

nonfluorescent complex in the short peptide (−1.8 mL/mol) was less than half of the change in the long peptide (−4.8 mL/mol) or the value for FAD (−4.3 mL/mol). The much smaller compressibility of the short peptide is attributed to the mechanical constraint to the approach of the interacting rings, imposed by the short link. Mechanical constraints of similar nature may be expected to be operative in proteins. Their importance in pressure denaturation is discussed.

The effect of pressure upon the equilibrium between the isoalloxazine and adenine moieties in flavin adenine dinucleotide (FAD)¹ reveals that the volume decreases upon formation of the intramolecular complex, while the activated quenching complex is expanded (Weber et al., 1974). In FAD the multiple linkages between the aromatic rings conceivably facilitate formation of an internal complex with minimum inter-ring distance, and therefore maximum interaction energy. The question arises as to whether compounds with fewer intervening bonds between the rings would permit the same decrease in volume upon complex formation and therefore show a similar pressure dependence of the equilibrium between open and complexed forms. To examine this possibility experimentally we selected two flavinylyltryptophan compounds, in which the aromatic residues are linked via a peptide bond and an alkyl chain of different length, namely three or five methylene groups (Figure 1). This type of compound is also an appropriate model system for the tight association of flavin with aromatic residues in flavoproteins, demonstrated in flavodoxins by x-ray crystallography (Burnett et al., 1974). The connecting chain in these peptides is much shorter than in FAD. Their synthesis and some spectral properties have already been reported (Föry et al., 1968; MacKenzie et al., 1969).

Materials and Methods

The compounds were a gift of Dr. Franz Müller, Agricultural University, Wageningen, The Netherlands. Prior to use, concentrated stock solutions of the compounds in dimethylformamide (Matheson Coleman and Bell, spectroquality, re-

distilled) were diluted in 0.05 M Tris buffer (pH 7.5). The final concentration of dimethylformamide was in all cases less than 0.5%. The choice of buffer was dictated by the relative insensitivity of Tris pK to pressure (Neuman et al., 1973). The very small fluorescence efficiencies of the flavinylyl peptides—on the order of 4 to 1%—required the use of solutions of 0.3 absorbancy at the exciting wavelength (375 nm). The molar concentrations were less than 5×10^{-5} M, so that intermolecular interactions may be confidently excluded. Fluorescence spectra and yields at high pressure were determined as described previously (Li et al., 1976). Lifetimes as a function of pressure were obtained by the phase shift method as described by Spencer and Weber (1969) and Weber et al. (1974). Usually this phase shift is obtained by comparing the modulated signals from fluorescent and scattering solutions under identical conditions. Since the high-pressure bomb could not be replaced rapidly for the scattering solution cell, the reference signal was obtained from a scattering solution placed in an alternate light bypass system, as done by Lakowicz and Weber (1973). The exciting light was modulated at 30 MHz, a frequency high enough to permit measurements of lifetimes below 2 ns with an accuracy of a few parts percent. Because of the weak signals from our materials, about 1000 observations were averaged to achieve this precision. This took 10 min at each particular pressure. For excitation of the fluorescence the nominally monochromatic 375-nm exciting light was additionally filtered through a Corning 7-59 filter and the emission was viewed through a Corning 3-71 and a liquid NaNO₂ filter.

Results

Fluorescence lifetimes and relative yield measurements can be combined to give the proportion of folded forms in the "dark" complex, that is in the absence of illumination (Spencer and Weber, 1972). At any given pressure, p , the degree of dissociation of the complex $\gamma(p)$ is given by:

$$\gamma(p) = \frac{q(p)}{q_0(p)} \frac{\tau_0(p)}{\tau(p)} \quad (1)$$

where q and τ designate relative fluorescence yields and lifetimes and the zero subscript refers to these quantities in free (unquenched) flavin. The compound used for comparison as "free flavin" is the methyl ester shown in Figure 1.

The equilibrium constant is:

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¹ Abbreviations used are: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

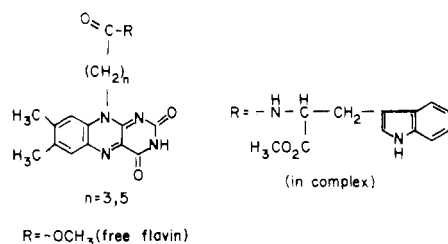


FIGURE 1: Structure of flavin derivatives investigated.

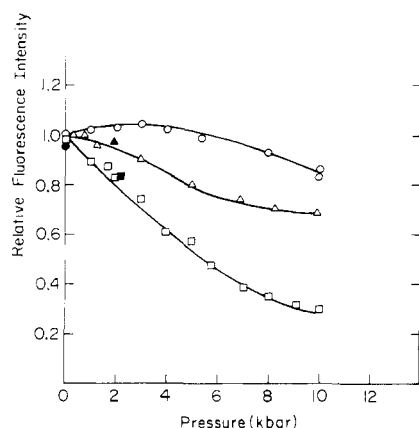
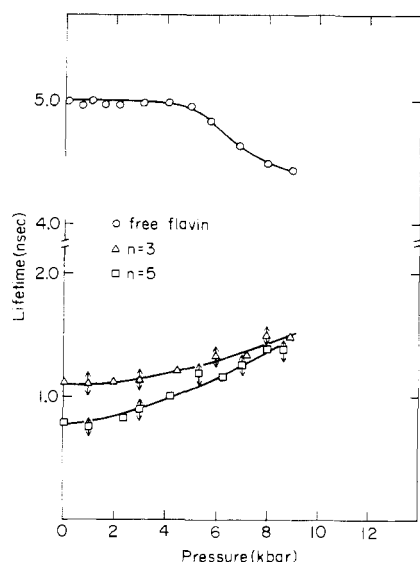
FIGURE 2: Fluorescence intensities: (Δ) $n = 3$; (\square) $n = 5$; (\circ) free flavin.

FIGURE 3: Fluorescence lifetimes.

$$K(p) = 1/\gamma(p) - 1 \quad (2)$$

and the volume change upon complex formation follows from the equation:

$$\Delta V^\circ = RT d \ln K(p) dp \quad (3)$$

The rate of quenching encounters k_+^* is given by:

$$k_+^* = 1/\tau(p) - 1/\tau_0(p) \quad (4)$$

and the activation volume change on formation of the quenching complex is:

$$\Delta V^* = -RT d \ln k_+^* / dp \quad (5)$$

At atmospheric pressure the fluorescence yield of the tri-

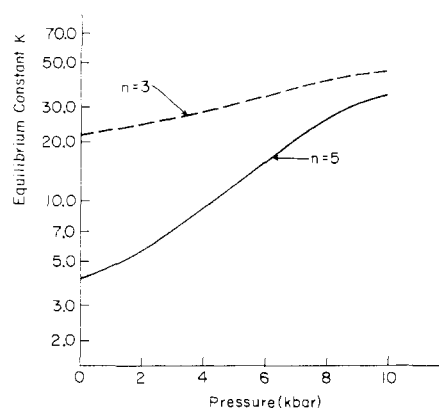


FIGURE 4: Equilibrium constants (eq 2) of the dark complexes, determined from the data of Figures 2 and 3.

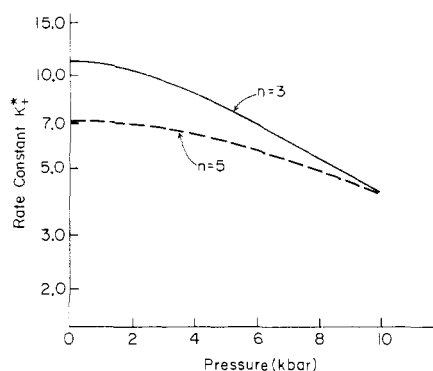
FIGURE 5: Rate of quenching encounters in the excited state k_+^* from the data of Figure 3.

TABLE I: Volume Change upon Compression of Some Flavinyltrypophan Peptides.

Compd	$\Delta V^\ddagger(0-4 \text{ kbar})$ (mL/mol)	$\Delta V^\ddagger(6-10 \text{ kbar})$ (mL/mol)	ΔV° (mL/mol)
$n = 3$	0.6	2.2	-1.8
$n = 5$	1.5	3.0	-4.8

methylene peptide was 1% and that of the pentamethylene peptide 3% of free flavin. From the respective lifetimes and eq 1 and 2 it follows that the former compound ($n = 3$) exists as a mixture of 96% of folded forms and 4% of open forms. For $n = 5$ the closed forms are 80%, quite comparable to FAD (Spencer and Weber, 1972). The respective free energies of formation are -1.9 and -1 kcal/mol. The pressure dependences of yields and lifetimes of the complexes and of free flavin are shown in Figures 2 and 3. Figure 4 shows semilog plots of $K(p)$ against pressure for the two peptides and Figure 5 a similar plot of $k_+^*(p)$.

Figure 4 indicates that the change in volume upon formation of the complex is -4.8 mL/mol for the compound with $n = 5$ but only less than half for the compound with $n = 3$ (see also Table I). The former value is quite close to that observed for FAD; the latter is significantly smaller and also smaller than the value of -3.1 mL/mol, of the change in volume upon formation of the FMN-riboflavin binding protein complex (Li et al., 1976). The change in k_+^* with pressure (Figure 4) may be ascribed to a change in viscosity of the solvent as well as to a change in volume upon formation of the quenching complex. In the latter case the changes would be small and positive, and larger for $n = 5$ than $n = 3$.

TABLE II: Thermodynamic Constants of Tryptophan-Flavin Complexes at 20 °C.^a

Compd	γ	K_a^d	ΔH°	$T\Delta S^\circ$	ΔG°	$k_+^* \times 10^8$ s ⁻¹ mol ⁻¹
$n = 3$	0.046	20.7	-3.7	-1.9	-1.8	7.3
$n = 5$	0.192	4.2	-4.7	-3.9	-0.8	10.9
FMN + 53 mM L-Trp ^b	0.111	202	-4.0	-0.9	-3.1	36.5
FAD ^c	0.165	5.1	-4.3	-3.3	-1.0	1.9

^a Determined as described by Spencer and Weber (1972). The compounds were dissolved in 0.05 M sodium phosphate (pH 7.2). ΔH° , $T\Delta S^\circ$, and ΔG° in kcal/mol. ^b Notice the small entropy change and large k_+^* value when there are no links restricting the motions of the partners.

^c From Spencer and Weber (1972). ^d K_a , equilibrium constant at atmospheric pressure.

TABLE III: Relative Fluorescence Efficiencies, Lifetimes, and Degree of Dissociation of the Complexes of Flavinyltrypthophanyl Peptides at 20 °C in Different Solvents.^a

Compd	Solvent	F/F_0	τ (ns) ^d		γ
			30 MHz	10 MHz	
$n = 3$	H ₂ O	0.010	1.09	1.73	0.05
$n = 5$	H ₂ O	0.030	0.77	0.81	0.20
Ref.	H ₂ O	1.0	5.00	5.10	
$n = 3$	FOR	0.041	1.05	1.50	0.22
$n = 5$	FOR	0.133	1.36	1.48	0.54
Ref.	FOR	1.0	5.56	5.72	
$n = 3$	DMF	0.085	0.95	1.25	0.57
$n = 5$	DMF	0.180	1.42	1.62	0.82
Ref.	DMF	1.0	6.40	6.70	
$n = 3$	Me ₂ SO	0.331	0.62	0.65	0.82
$n = 5$	Me ₂ SO	0.369	1.17	1.17	0.73
Ref.	Me ₂ SO	1.0	2.30	2.26	

^a H₂O, 0.04 M sodium phosphate (pH 7.2); FOR, formamide, MCB, reagent grade, distilled, stored on molecular sieve; DMF, dimethylformamide, MCB, spectroquality, distilled; Me₂SO, dimethyl sulfoxide, MCB, spectroquality, stored on molecular sieve. The reference compound was the one with $n = 4$ and $R = CH_3$ (Figure 1). All optical densities in solvents other than H₂O were <0.1 at excitation wavelength 375 nm. γ , degree of dissociation of complexes.

The enthalpy change in the formation of the complexes at atmospheric pressure, ΔH° , was determined from the fluorescence yields and lifetimes at a series of temperatures. The lifetimes and relative yields of the complexes were measured at small intervals in the range of 1–35 °C. The lifetimes of the complexes, measured at 10- and 30-MHz modulation, were reasonably close but some heterogeneity was observed in the water solutions of the trimethylene-bridged compound. This showed at 20 °C, $\tau(30 \text{ MHz}) = 1.09 \text{ ns}$, $\tau(10 \text{ MHz}) = 1.73 \text{ ns}$,² while the compound with $n = 5$ gave values of 0.77 and 0.81 ns, respectively. The measurements at 30 MHz are considered the most significant as they would give less weight to any longer-lived component if the emission is heterogeneous. For this reason the lifetimes at 30 MHz were employed in the calculation of γ and $K(p)$ by eq 1 and 2 from which:

$$\frac{\Delta H^\circ}{R} = \frac{d \ln K(p)}{d(1/T)} \quad (6)$$

The experimental results and the derived thermodynamic constants are summarized in Table II. For comparison the table includes similar values for FMN in the presence of 53 mM L-tryptophan, in which the value of γ for the flavin fluorescence is comparable to the covalently linked derivatives. The results already published by Spencer and Weber (1972) for

² Notice (Table III) that this heterogeneity does not exist in the good solvent, dimethylformamide or Me₂SO, thus excluding a long-lived fluorescent impurity as the cause.

FAD are also included. The thermodynamic parameters of all these flavin complexes show evident similarities.

Table III compares the fluorescence of the complexes in different solvents at the single temperature of 20 °C. The ground-state complexes are most prevalent in water and next in formamide, solvents which are extensively hydrogen bonded. In contrast, the open forms prevail in dimethylformamide and dimethyl sulfoxide. The "solvophobic" nature of the complexes as well as the superior position of water in allowing their formation are points worthy of note.

Discussion

The equilibrium between open and closed forms in each of the two compounds will depend upon both enthalpy and entropy contributions, including those from changes in solvent interaction. However, the *difference* between the two cases may be reasonably expected to arise from contributions owing entirely to the peptides, since the solvent contributions are likely to be much the same in both cases. Because of the larger freedom of rotation of the compound with five methylene links (n_5) as compared to n_3 , the decrease in entropy upon complex formation would be greater in the former derivative, that is: $|\Delta S^\circ(5)| > |\Delta S^\circ(3)|$.

The contribution of ring interactions to the enthalpy ΔH° may likewise be expected to be larger in n_5 than in n_3 because the rigid constraint from the short covalent link of the latter would not allow the rings to reach an equilibrium distance as small as in n_5 . This should reflect itself in a value of ΔV° larger for the compound with the longer links, as is experimentally observed. Thus, we expect $|\Delta H^\circ(5)| > |\Delta H^\circ(3)|$. While the decrease in entropy upon complex formation will monotonically increase with the number of links n about which free rotation is possible, we expect ΔH° to reach a limiting value at a number n that permits closest ring approach and to keep this value at larger n .

On increasing n from its minimum value the free energy of formation ΔG° would exhibit a region of approximate compensation in which it changes little in comparison with ΔH° and ΔS° followed by a region in which it increases steadily with n , favoring the open form. These qualitative expectations are in agreement with the thermodynamic quantities collected in Table II.

These considerations are probably relevant to the case of protein folding and to the denaturing effects of pressure upon the protein (Li et al., 1976). In the native protein many amino acid residues will be prevented from reaching the closest equilibrium approach by the spatial constraints imposed by the covalent peptide backbone. Equilibrium between isomers will involve compensating changes in ΔS° and ΔH° resulting in appreciable conformational differences but with a small free-energy change. Moreover, one can visualize the pressure denaturation reaction in proteins as arising out of the decrease

in volume that results from replacing nearby, spatially constrained amino acid residues by freely compressible solvent. The native protein conformation would not undergo appreciable decrease in volume with pressure because the rigidity of the backbone prevents nearby residues from coming together. On the other hand, these residues will contribute to the decrease in volume as they come in contact with solvent upon unfolding. A quenched derivative with a very short link could be expected, in the limit, to behave like the protein, increasing its fluorescence as the pressure is raised beyond a certain value. The short-linked flavinyl peptide investigated does not have that behavior, but its much poorer compressibility as compared with the larger peptide, or with FAD, indicates that the linking covalent frame produces the expected effects. The synthesis of appropriate molecular models, which would undergo unfolding under pressure like the proteins, seems therefore a distinct possibility.

Acknowledgments

We thank Drs. F. Müller and J. Santema for making available the model compounds.

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Antiproteolytic Aldehydes and Ketones: Substituent and Secondary Deuterium Isotope Effects on Equilibrium Addition of Water and Other Nucleophiles[†]

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ABSTRACT: Equilibrium constants for hydration of ketones, in dilute D₂O solution at 34 °C, observed by proton magnetic resonance under conditions of slow exchange, were acetone 0.002, chloroacetone 0.08, 1,3-dichloroacetone 4.17, bromoacetone 0.07, and 1,3-dibromoacetone (an inhibitor of papain) 1.85. Neither acetamidoacetone nor *N,N*-diacetylaminacetone showed evidence of appreciable hydration in dilute aqueous solution, nor was any hydrate detectable in solutions of tosylglycine chloromethyl ketone. Substitution of acetaldehyde with acylamido substituents, as in several potent reversible inhibitors of papain, was found to enhance its equi-

librium constant for covalent hydration by an order of magnitude; these inhibitors are about 90% hydrated in dilute aqueous solution, and their affinity for proteases may have been underestimated accordingly. The effects of deuterium substitution at C-1 of acetaldehyde, on equilibrium addition of oxygen and sulfur nucleophiles, are substantial and vary with the nature of the nucleophile. These isotope effects may be useful as a means of distinguishing between alternative structures of complexes formed between enzymes and aldehydes.

Carbonyl compounds bearing appropriate specificity determinants are strong inhibitors of proteolytic enzymes and amidases. Halomethyl ketones inactivate proteases of the chymotrypsin type by alkylating an active site nucleophile (Schoellman and Shaw, 1962). Further reaction may ensue, as in alkylation of subtilisin BPN, which is accompanied by the formation of an internal hemiacetal involving a serine residue at the active site (Poulos et al, 1976). Halomethyl ketones are sometimes found to act as very strong reversible in-

hibitors, as in the case of certain aminopeptidases (Birch et al., 1972; Kettner et al., 1974). Several explanations could be offered for these latter observations, but it appears likely that they may be related to the ability of such compounds to undergo reversible addition of nucleophiles. A strong inhibitor of cholinesterase has recently been designed on this basis (Dafforn and Kerr, 1976).

Aldehydes, related in structure to carboxylic acids released by substrate hydrolysis, have also been found to serve as highly effective inhibitors of several hydrolytic enzymes, including papain (Westerik and Wolfenden, 1972), elastase (Thompson, 1973), L-asparaginase (Westerik and Wolfenden, 1974), and a bacterial amidase (Findlater and Orsi, 1973). These aldehydes were designed to form adducts that might resemble intermediates in substrate transformation. Umezawa and his

[†] From the Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514. Received May 25, 1977. Supported by Grant No. GM-18325 from the National Institutes of Health, U.S. Public Health Service.

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