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On the Interaction between Flavin and Indole Rings. Crystallographic, Spectroscopic, Polarographic and Energy Calculational Studies of a Flavinyltryptamine Peptide[1)]

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As an intramolecular model for flavin–indole interaction, the crystal structure of *N*-2-(3-indolyl)ethyl-7,8-dimethylisoalloxazine-10-propionamide, a flavinyltryptamine peptide, was determined by the X-ray diffraction method. Although the molecule took an open conformation without indole–flavin interaction, this could be a result of crystal packing effects, because ultraviolet and fluorescence data showed the existence of a prominent intramolecular stacking interaction of the rings in aqueous solution. Proton nuclear magnetic resonance analysis indicated a preferential stacking interaction between the indole ring and the pyrazinoid and pyrimidinoid portions of the flavin ring. These results were supported by the conformational analysis of this molecule based on energy calculations. Polarographic data showed that the indole ring affects the reduction state of the flavin semiquinone form to the hydroquinone form. The results suggest that a tryptophan residue of a flavin enzyme might act not only to fix the flavin coenzyme in place, but also to stimulate the reduction reaction.

Keywords——flavin–indole stacking interaction; flavinyltryptamine peptide; X-ray analysis; NMR analysis; energy calculation; polarography

Introduction

Flavin coenzymes act as electron transfer mediators in many biological redox processes. For the electron transfer, the substrate and coenzyme are bound close to each other at the active site of the enzyme, and they undergo a coupled oxidation–reduction reaction. The enzyme may act not only to bring the substrate and coenzyme together with a favorable geometry, but also to promote the subsequent electron transfer reaction.

Recent spectral and (photo) chemical studies, augmented by X-ray crystallographies, indicate that many enzymes with flavin coenzymes have tryptophanyl and/or tyrosyl residues at their coenzyme-binding sites.[2)] The possible importance of flavin associations with these aromatic amino acid residues in the enzymes led us to study the strength and nature of such

interactions by using inter- and intramolecular models.

Previously we reported on the flavin–indole and flavin–phenol complexes as intermolecular models of flavin coenzyme–tryptophan and –tyrosine interactions.[3,4] In those studies we found that the indole and phenol rings are both stacked in parallel on the pyrimidinoid and pyrazinoid portions of the isoalloxazine ring by π–π charge–transfer interaction, and have many short contacts, with a less than normal van der Waals separation distance of 3.4 Å, at the N1 and N5 atoms (reduction site) of the isoalloxazine ring. These results may imply that the tryptophan or tyrosine residue existing at the active center not only acts to ensure the tight binding of the flavin coenzyme, but also plays some role in the oxidation–reduction reaction of flavin coenzyme. Therefore it is of importance to ascertain whether the observed characteristic interaction mode is energetically stable and an intrinsic feature of flavin–indole or –phenol interaction.

As a development of these studies, we synthesized a flavinyltryptamine peptide, *N*-2-(3-indolyl)ethyl-7,8-dimethylisoallozaxine-10-propionamide (TRPDIP), as an intramolecular model for flavin–indole interaction. Generally an intramolecular model in which the interacting aromatic rings are covalently bonded to each other could be expected to show the above-mentioned behavior more prominently than an intermolecular complex. Here we report on the preferred conformation of TRPDIP and the flavin–indole interaction studied by X-ray crystallographic, spectroscopic, energy calculational and polarographic methods.

Experimental

Materials——7,8-Dimethylisoalloxazine-10-propionic acid (DIP), DIP methyl ester and TRPDIP were synthesized according to the procedure outlined by Fall and Petering[5] and Föry *et al.*[6] The purity of the synthesized materials was verified by thin-layer chromatography (chloroform–methanol–acetic acid, (95 : 5 : 3, v/v/v)), elemental analyses, mass spectroscopy and nuclear magnetic resonance (NMR) measurements.

Structure Analysis of TRPDIP Crystal——Yellowish, platelet single crystals of TRPDIP were obtained after a month from a solution in a mixture of dimethylformamide and diethyl ether (1 : 1, v/v), kept in a refrigerator (5 °C). Crystal data were determined from X-ray photographs and with a Rigaku AFC-5 diffractometer, and are listed in Table I. The crystal density was measured by a flotation method in CCl_4/C_6H_6 mixture. Reflectional intensity data were measured with the diffractometer. The X-ray source was graphite-monochromated Cu K_α radiation ($\lambda = 1.5418$ Å). The data were collected in the ω–2θ scan mode with a range of $(1.1 + 0.15 \tan\theta)°$, and a speed of $3° \cdot min^{-1}$ in 2θ. A total of 4684 independent reflections within $2\theta = 130°$ was collected ($0 \leq h \leq 16$, $0 \leq k \leq 12$, $-22 \leq l \leq 22$) and corrected for Lorentz and polarization factors, but not for absorption, because of the small size of the crystal used ($0.3 \times 0.3 \times 0.3$ mm). Finally 3119 reflections with $F_o > \sigma(F_o)$ were used, as observed data, for the structure determination and refinement. The structure was determined by the direct method, using the program MULTAN78,[7] and refined by a block-diagonal least-squares method with anisotropic temperature factors. A

TABLE I. Crystal Data for TRPDIP

Chemical formula	$C_{25}H_{24}N_6O_3 \cdot HCON(CH_3)_2$
Molecular weight	529.6
Crystal system	$P2_1/a$
Cell constants	
a (Å)	14.256 (9)
b (Å)	10.228 (6)
c (Å)	19.407 (6)
α (°)	90.0
β (°)	103.77 (6)
γ (°)	90.0
Volume (Å^3)	2748 (3)
Z	4
D_m (g·cm^{-3})	1.300 (1)
D_x (g·cm^{-3})	1.299

TABLE II. Atomic Coordinates and Isotropic Temperature Factors of Nonhydrogen Atoms

Atom	x	y	z	B_{eq} [a)]
TRPDIP				
N1D	0.3470 (3)	−0.2806 (4)	0.4909 (2)	4.3 (2)
C2D	0.3017 (4)	−0.3325 (5)	0.4264 (3)	4.7 (2)
O2D	0.2895 (3)	−0.4491 (4)	0.4173 (2)	6.4 (2)
N3D	0.2677 (3)	−0.2487 (5)	0.3690 (2)	5.1 (2)
C4D	0.2763 (4)	−0.1171 (6)	0.3703 (3)	5.1 (2)
O4D	0.2442 (4)	−0.0483 (5)	0.3176 (2)	7.2 (2)
N5D	0.3395 (3)	0.0644 (4)	0.4447 (2)	4.5 (2)
C6D	0.4008 (4)	0.2493 (5)	0.5175 (3)	4.8 (2)
C7D	0.4476 (4)	0.3050 (5)	0.5813 (3)	5.1 (2)
C8D	0.4829 (4)	0.2209 (5)	0.6395 (3)	4.9 (2)
C9D	0.4710 (4)	0.0863 (5)	0.6331 (3)	4.2 (2)
N10D	0.4096 (3)	−0.1024 (4)	0.5588 (2)	3.5 (1)
C11D	0.3603 (3)	−0.1540 (5)	0.4965 (2)	3.6 (2)
C12D	0.3268 (3)	−0.0605 (5)	0.4395 (2)	4.1 (2)
C13D	0.4231 (3)	0.0313 (4)	0.5682 (3)	3.8 (2)
C14D	0.3885 (3)	0.1133 (5)	0.5102 (3)	4.1 (2)
C15D	0.4603 (5)	0.4513 (5)	0.5886 (4)	6.4 (3)
C16D	0.5320 (5)	0.2779 (6)	0.7103 (4)	6.6 (3)
C17D	0.4480 (4)	−0.1937 (5)	0.6189 (3)	4.1 (2)
C18D	0.3755 (4)	−0.2115 (5)	0.6646 (3)	4.6 (2)
C19D	0.4282 (4)	−0.2594 (5)	0.7370 (3)	4.7 (2)
O19D	0.4929 (4)	−0.1913 (5)	0.7723 (2)	8.3 (2)
N1I	0.1595 (4)	−0.3714 (6)	0.8696 (3)	6.9 (3)
C2I	0.2134 (4)	−0.4407 (7)	0.8325 (3)	5.6 (3)
C3I	0.3036 (4)	−0.4600 (5)	0.8735 (2)	4.4 (2)
C4I	0.3762 (5)	−0.3841 (7)	1.0032 (3)	6.3 (3)
C5I	0.3540 (6)	−0.3140 (8)	1.0586 (4)	7.8 (4)
C6I	0.2631 (8)	−0.2575 (8)	1.0526 (4)	9.3 (5)
C7I	0.1906 (6)	−0.2700 (8)	0.9907 (4)	8.2 (4)
C8I	0.2146 (5)	−0.3436 (6)	0.9354 (3)	5.8 (3)
C9I	0.3044 (4)	−0.3971 (5)	0.9402 (3)	4.6 (2)
C10I	0.3858 (4)	−0.5257 (5)	0.8521 (2)	4.5 (2)
C11I	0.4518 (4)	−0.4308 (6)	0.8272 (3)	5.1 (2)
N12I	0.4024 (3)	−0.3711 (4)	0.7594 (2)	4.4 (2)
Dimethylformamide				
C1	0.254 (1)	−0.060 (2)	0.834 (1)	22. (1)
C2	0.379 (1)	−0.062 (1)	0.9108 (7)	18. (1)
N3	0.3474 (6)	0.0062 (7)	0.8506 (3)	9.4 (4)
C4	0.358 (1)	0.083 (1)	0.8086 (6)	14.6 (9)
O5	0.3335 (6)	0.1452 (7)	0.7604 (3)	11.6 (4)

a) $B_{eq}=4/3(a^2B_{11}+b^2B_{22}+c^2B_{33}+acB_{13}\cos\beta)$.

difference Fourier map showed the positions of dimethylformamide as solvent of crystallization, and they were included in the further refinements. The final least-squares refinement was computed with the following weighting scheme.

$$\omega=1.0/[\sigma(F_o)^2-0.01118\,F_o+0.01259\,F_o^2]$$

The final discrepancy value R was 0.107. The ideal positions of hydrogen atoms were calculated and included for the structure factor calculations, but kept fixed for the refinements. The final atomic coordinates for the non-hydrogen atoms are listed in Table II.[8] The standard atomic scattering factors were taken from the data of Cromer and Waber.[9] Refinement and Fourier synthesis calculations were carried out by using the programs in the UNICS system.[10]

Ultraviolet (UV) Absorption and Fluorescence Emission Spectra——The electronic absorption spectra in the

300—650 nm region were recorded at 25 °C on a Hitachi 624 spectrophotometer with dual-compartment cells (10 mm). The relative quantum efficiencies of fluorescence were determined on a Hitachi-650-40 spectrofluorometer with a Xenon lamp. The emission spectra were measured at 25 °C with 295 nm excitation, and were not corrected for monochromator efficiency and photomultiplier response. The absorption and fluorescence spectra were measured by using 2.0×10^{-5} M sample solution in 0.025 M phosphate buffer containing 30% ethanol (pH = 6.8).

^{1}H-NMR Spectra——^{1}H-NMR spectra were recorded on a Varian XL-300 (300 MHz, FT-mode) spectrometer equipped with a temperature control unit. A 3.3 mM solution dissolved in $C^2H_3O^2H$–^2H_2O (3 : 7, v/v) mixture was used. DSS (2,2-dimethyl-2-silapentane-5-sulphonate sodium salt) was used as an internal standard. The spectra were recorded from 10 to 60 °C in 5 °C increments.

Conformational Analysis of TRPDIP by Energy Calculation——For energy calculations of TRPDIP conformers, we used an empirical PPF (partitioned potential energy function) method: the total energy (E) of a conformer is obtained by summation of the nonbonded (E_{nb}), electrostatic (E_{el}) and torsional (E_t) energies.

$$E = E_{nb} + E_{el} + E_t \tag{1}$$

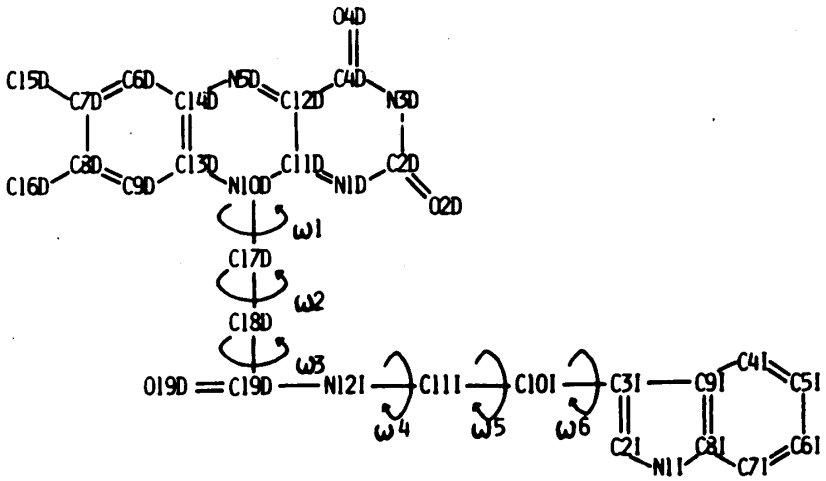
In this calculation hydrogen bond energy was not considered. These quantities, in unit of kcal/mol, can be obtained by computing the following equations.

$$E_{nb} = \sum_{i>j}\sum (-A_{ij} r_{ij}^{-6} + B_{ij} r_{ij}^{-12}) \tag{2}$$

$$E_{el} = \sum_{i>j}\sum 332.0 \times Q_i \times Q_j \times r_{ij}^{-1} \times \varepsilon^{-1} \tag{3}$$

$$E_t = \sum_{k=1}^{N} 0.5 \times V_k \times (1.0 + \cos X\Theta_k) \tag{4}$$

In Eqs. 2 and 3, r_{ij} is the distance (in Å) between atoms i and j. Parameters A_{ij} and B_{ij} used for the Lennard-Jones "6—12" potential function calculation (Eq. 2) were obtained from the literature.[11-14] The Coulombic charge[8] on atom i (Q_i) in Eq. 3 was obtained by the CNDO/2 calculation.[15] The dielectric constant (ε) of Eq. 3 was taken as 4.0, close to the experimental values for biomolecules in polar media.[12] The N in Eq. 4 is the number of the rotatable bond (=6 in this case), and V_k is the energy barrier potential for internal rotation about the k-th torsion angle (θ_k). The latter was taken as 2.5 kcal/mol for a C–C single bond, but was considered to be negligible for the C–N bond in accordance with Lakshminarayanan and Sasisekharan.[12] The symbol X is the periodicity of the barrier and was taken as 3 for ω2 and ω5, and 6 for ω1, ω3, ω4, and ω6 (see Fig. 1 for the notation of torsion angle). As starting torsion angles for the



ω1 (C11D–N10D–C17D–C18D):	±90°
ω^2 (N10D–C17D–C18D–C19D):	±60°, 180°
ω3 (C17D–C18D–C19D–N12I):	±30°, ±90°, ±150°
ω4 (C10I–C11I–N12I–C19D):	±30°, ±90°, ±150°
ω5 (C3I–C10I–C11I–N12I):	±60°, 180°
ω6 (C2I–C3I–C10I–C11I):	±30°, ±90°, ±150°, 0°

Fig. 1. Atomic Numbering Used in This Work, Notation of Torsion Angles and Possible Starting Angle Sets

Hydrogen atoms are omitted for the sake of clarity.

energy calculation, the most reasonable values were selected on the basis of the rotation barriers of sp^3–sp^3 or sp^2–sp^3 covalent bonds and of many X-ray results on flavin and indole derivatives. These starting angle sets are also shown in Fig. 1. Thus, 4536 ($2 \times 3 \times 6 \times 6 \times 3 \times 7$) different conformers of TRPDIP were calculated; however, some of them had abnormal short contacts between the neighboring atoms and were therefore excluded from the calculations. Minimization of the total energy, along with the refinement of each torsion angle, was done by using the Powell algorithm.[16] The minimization was carried out by the parabola approximation with 4° intervals and no angle was permitted to vary by more than 12° at each step.

Polarography——Polarography was done using a Yanaco P8 polarograph. Electrolytic solutions were prepared from 1 M KNO_3 (2 ml), H_2O or amine solution (1 ml), Sörensen buffer (6 ml, pH = 4.0) and 2×10^{-4} M flavin solution (1 ml in H_2O). The potentials are given with a saturated calomel electrode as the reference. Polarograms were recorded from 0.08 to −0.50 V. All measurements were made at 25 °C.

All numerical calculations were carried out on an ACOS-900 computer at the Computation Center of Osaka University.

Results and Discussion

Molecular Structure of TRPDIP

The determined molecular structure of TRPDIP is shown in Fig. 2. The bond lengths and angles appear reasonable compared with other flavin and indole compounds.[3,4] The averaged standard deviations of the bond lengths and angles for nonhydrogen atoms, except those for dimethylformamide, are 0.008 Å and 0.5°, respectively. Wang and Fritchie[17] determined "idealized" molecular dimensions for lumiflavin based on the weighted averages of three non-protonated structures. The bond distances of the flavin moiety in this structure show an average deviation of 0.002 Å from the corresponding "idealized" values. The isoalloxazine ring is almost planar as judged from the calculations of least-squares planes: the root mean square (r.m.s) deviation of the nonhydrogen atoms from the best plane is 0.004 Å, and the atoms attached to the ring are nearly on the plane (−0.063 (8) Å of C16D to 0.065 (6) Å of C17D). The angle between the planes through the atoms forming the benzenoid and pyrimidinoid rings is 0.7 (3)°. The indole ring is also planar with a maximum shift of 0.02 (1) Å for the C7I atom; r.m.s. deviation from the best plane is 0.005 Å.

Contrary to our expectation, no intramolecular interaction between the flavin and indole rings was observed. The dihedral angle of the rings is 66.0 (2)°. The conformational torsion angles are listed in Table III. The conformation of TRPDIP molecule belongs to an extended form, and each torsion angle appears to take a reasonable value. Similar values were also found in other related tryptophan and flavin derivatives.[3,4]

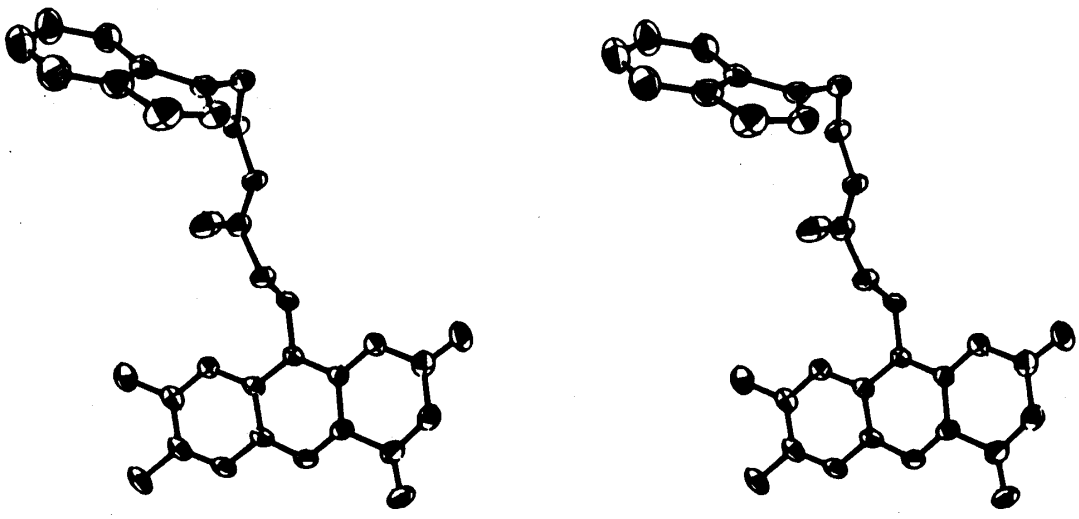


Fig. 2. ORTEP Drawing of a TRPDIP Molecule in the Crystal Structure

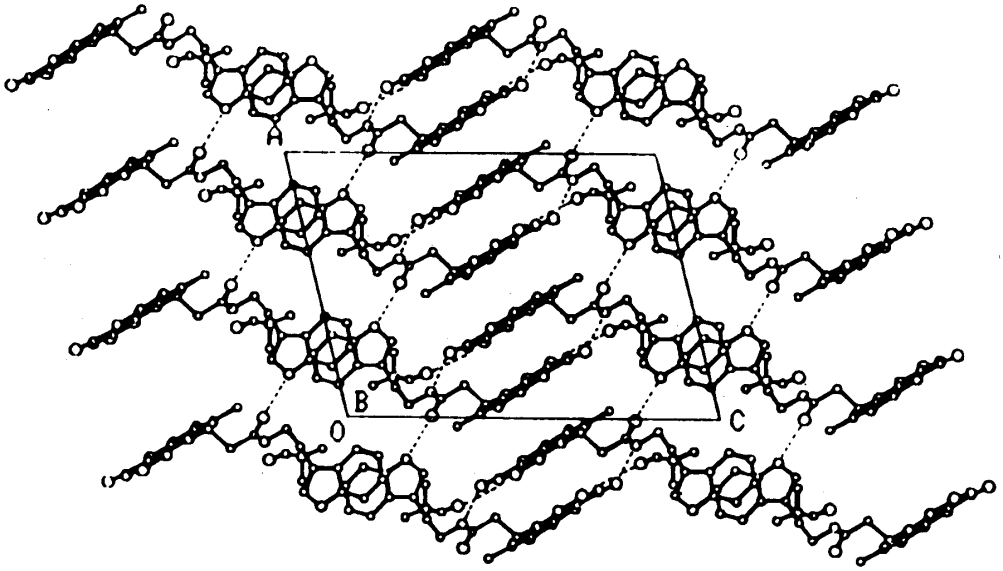


Fig. 3. Crystal Packing of the Molecule Viewed along the *b*-Axis
Dotted lines represent possible hydrogen bonds.

TABLE III. Selected Torsion Angles (°) of TRPDIP

C11D–N10D–C17D–C18D	−91.9 (5)
C13D–N10D–C17D–C18D	86.8 (5)
N10D–C17D–C18D–C19D	−158.8 (4)
C17D–C18D–C19D–N12I	−120.9 (5)
C17D–C18D–C19D–O19D	59.7 (7)
C18D–C19D–N12I–C11I	176.6 (5)
O19D–C19D–N12I–C11I	−4.0 (9)
C2I–C3I–C10I–C11I	94.7 (7)
C9I–C3I–C10I–C11I	−81.4 (7)
C3I–C10I–C11I–N12I	−68.5 (6)
C10I–C11I–N12I–C19D	157.5 (5)

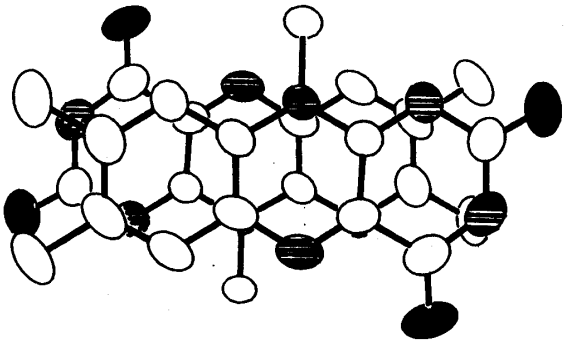


Fig. 4. Overlapping of Two Centrosymmetrically Related Isoalloxazine Rings

Crystal Packing of TRPDIP

The perspective packing diagram viewed down the *b*-axis is shown in Fig. 3. The molecules are packed as extended forms parallel to the *c*-axis direction. Two kinds of layers consisting of the flavin or indole rings run parallel to the *a*- and *b*-axes, respectively, and are nearly at right angles to each other. Although these layers are stabilized by the normal van der Waals contacts, extensive overlapping was observed in the centrosymmetrically related, and therefore parallel, flavin rings. The stacking mode is shown in Fig. 4. The averaged interplanar spacing is 3.45 Å. Since similar overlap is frequently observed in flavin derivatives,[18] this stacking mode, *i.e.*, the antiparallel alignment of the long axes of the respective isoalloxazine rings, may be a stable form for flavin–flavin interaction. The N12I and O19D atoms of the peptide moiety are hydrogen-bonded to the nearest neighboring O4D of the flavin ring and N1I of the indole ring, respectively: N12I···O4D = 2.898 (7) Å, H12I···O4D = 1.92 Å, and <N12I–H12I···O4D = 165°; N1I···O19D = 2.736 (8) Å, H1I···O19D = 1.78 Å, and <N1I–H1I···O19D = 145°. These hydrogen bonds take a role in stabilizing the molecular packing in the *a*-axis direction. Dimethylformamide existing near the tryptamino moiety appears not to be very important for the stability of TRPDIP molecular packing because of the relatively high temperature factors. Only one hydrogen bond is formed between the O5

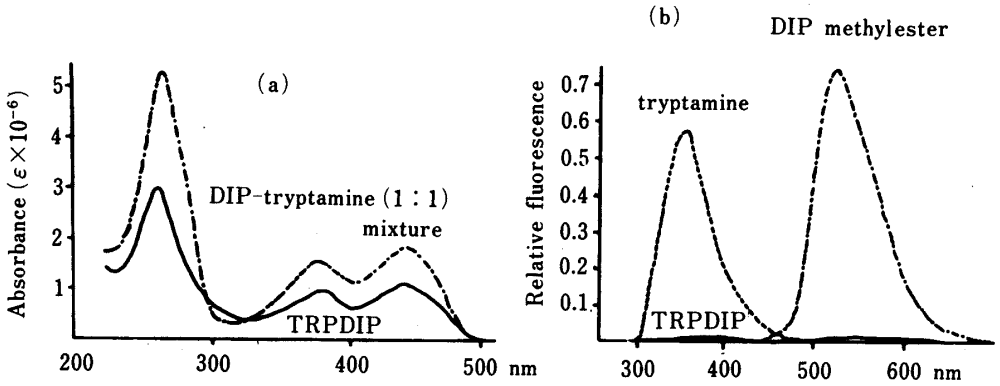


Fig. 5. UV (a) and Fluorescence (b) Spectra of TRPDIP and an Equimolar 1 : 1 Mixture of Its Components

atom of dimethylformamide and the N3 atom of the neighboring flavin ring: N3D···O5 = 2.801 (10) Å, H3D···O5 = 1.72 Å, and <N3D–H3D···O5 = 175°. This molecule could be necessary to fill the cavity caused by the molecular packing of TRPDIP into the crystal lattice. None of the polar N1D, O2D and N5D atoms of the flavin ring participated in hydrogen bond formation.

In this crystal structure TRPDIP took an extended conformation without any interaction between the flavin and indole rings. It is of interest to ascertain whether this open form is the energetically stable and intrinsic form of the TRPDIP molecule or is the result of other factors (such as packing forces) related to the crystal packing. The different colors of the crystallization solution (red) and crystals (yellow) suggest that the latter case is more likely. Therefore we carried out the following spectroscopic studies in solution, and made a conformational analysis by means of energy calculations.

UV and Fluorescence Spectra

A method for assessing ring–ring interaction is the measurement of hypochromicity (UV) or quenching (fluorescence), which depend mainly upon the degree and orientation of intra- and/or intermolecular stacking in the ground or excited state, respectively. The UV and fluorescence spectra are shown in Fig. 5. In Fig. 5 (a) prominent hypochromicity was observed in all the regions of the TRPDIP absorption spectrum, compared with a 1 : 1 mixture of its components, DIP and tryptamine: the hypochromicity was 56.4 (8)% at 268 nm, 60.0 (7)% at 374 nm and 58.5 (8)% at 450 nm. This hypochromic effect could be apparently interpreted as being due to intramolecular ring–ring interaction accompanied by folding of the TRPDIP molecule, because the sum of the DIP and tryptamine spectra was almost the same as the spectrum of their 1 : 1 mixture at this concentration. The slightly larger absorbance of TRPDIP at 295—330 nm, compared with the 1 : 1 mixture, can be assigned as the charge–transfer band resulting from the electron transition from the indole to the flavin.

Further, the fluorescence emission spectrum of TRPDIP (Fig. 5 (b)), compared with those of tryptamine and DIP methyl ester, indicated the complete quenching of indole and flavin fluorescences: the degree of quenching was 99.9 (8)% at both 358 nm (indole) and 520 nm (flavin) with reference to the components. This also indicates the existence of prominent ring–ring interaction in the excited state. A similar conclusion has also been reported for flavinyltryptophan peptides.[19,20]

^{1}H-NMR Spectra: Temperature Dependence of Chemical Shifts of Aromatic Protons

To deduce the interaction mode between the flavin and indole rings of the TRPDIP molecule, the chemical shifts of their aromatic protons were measured at various tempera-

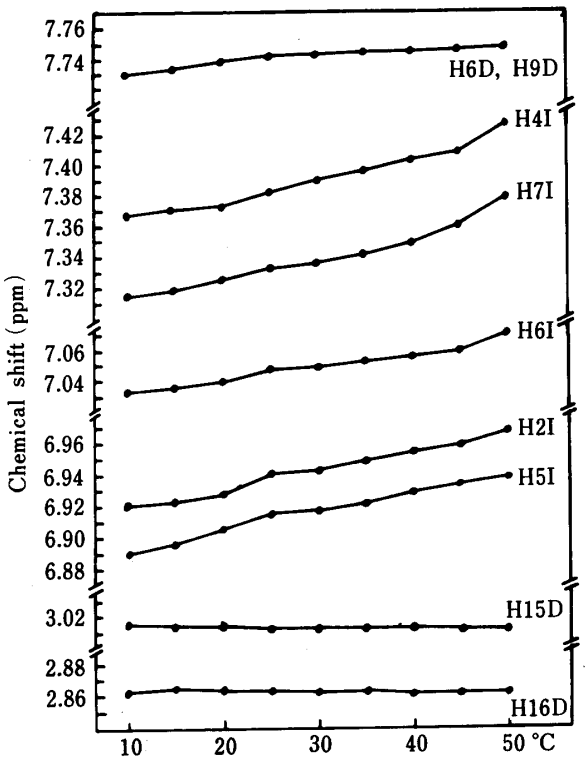


Fig. 6. Temperature Dependence of TRPDIP Chemical Shifts (pH = 7.0)

The suffixes, I and D, in the atomic designations refer to the indole and flavin moieties of the TRPDIP molecule, respectively.

tures. Although the assignment of all protons was difficult because of the low solubility of TRPDIP, and of the overlapping of solvent proton peaks, the aromatic protons could be identified. The peak assignments were based on the literature.[21-23] Figure 6 shows the temperature dependence of the chemical shifts. With increase in temperature, the proton signals of the indole ring shift to lower field. Change in temperature will affect both the inter- and intramolecular interactions and associated proton signals. The shifts to low field may be explained by a decrease in the ring-current diamagnetic shielding of the protons when molecular associations decrease owing to a partial destacking as well as partial unfolding of the TRPDIP molecule. Since the concentration used was low enough to avoid the concentration dependence of proton chemical shifts,[21] and no detectable temperature dependence was observed in the aromatic proton signals of a 1 : 1 mixture of tryptamine and DIP at the same concentration, the observed changes may be due to the intramolecular interaction of the TRPDIP molecule.

Several interesting features are evident from Fig. 6. The first is the increase in the chemical shifts of all indole protons as the temperature is increased. For example, the shielding effects decrease by 11.4 Hz at H6 to 26.1 Hz at H7 during temperature variation from 10 to 50 °C. Secondly the flavin protons did not show any noticeable diamagnetic shielding change accompanying the temperature variation. Slight changes were observed for the H6 and H9 protons (5.7 Hz).

Based on these results, we concluded that the TRPDIP molecule exists, at least to some extent, as a folded form with indole–flavin stacking interaction in aqueous solution, in accordance with the UV and fluorescence data. Furthermore, it could be considered that the whole of the indole ring probably interacts with the pyrimidinoid and pyrazinoid portions preferentially rather than the benzenoid portion of the flavin ring. Although this proposal is somewhat different from that of Föry *et al.*,[22] it is not in conflict with the X-ray results on intermolecular flavin–indole complexes.[3,4]

Conformational Analysis of the TRPDIP Molecule by Energy Calculations

To elucidate which stacking conformation that is able to explain the NMR data is

TABLE IV. List of the Starting and Refined Torsion Angles of 15 Energetically Stable TRPDIP Conformers, along with Their Energies and Conformational Types

Order	Starting angle (°)						Refined angle (°)						Energy (kcal/mol)	Type
	ω1	ω2	ω3	ω4	ω5	ω6	ω1	ω2	ω3	ω4	ω5	ω6		
1	−90	60	−90	150	60	90	−93	68	−83	157	58	86	−32.09	A
2	−90	60	−30	90	60	150	−108	83	−60	132	59	82	−26.61	A
3	−90	60	−150	−150	60	90	−99	83	−147	−128	49	89	−25.88	A
4	−90	60	−30	150	60	150	−111	92	−62	141	41	93	−25.67	A
5	90	−60	30	−150	60	150	104	−57	77	−159	64	87	−24.87	A
6	−90	−60	90	90	180	30	−83	−47	89	75	181	31	−24.32	B
7	−90	−60	90	90	180	−90	−84	−44	88	103	187	−84	−24.05	B
8	90	60	−90	−90	180	90	85	47	−92	−99	174	86	−23.57	B
9	−90	60	−90	−60	180	30	−101	50	−88	−77	176	29	−23.56	C
10	−90	60	−90	−60	180	−90	−99	55	−88	−67	193	−89	−23.50	C
11	−90	−60	90	60	180	0	−84	−44	95	91	246	25	−23.48	B
12	−90	60	−90	−60	180	−30	−102	56	−87	−74	172	−33	−23.19	C
13	−90	60	−90	−90	−60	90	−93	69	−97	−129	−61	87	−23.19	C
14	−90	−60	90	60	180	90	−84	−54	90	68	165	87	−23.13	B
15	−90	−60	90	−90	60	90	−87	−57	85	−86	56	93	−22.99	A
Crystal							−92	−159	−121	158	−69	95	−16.16	Open form

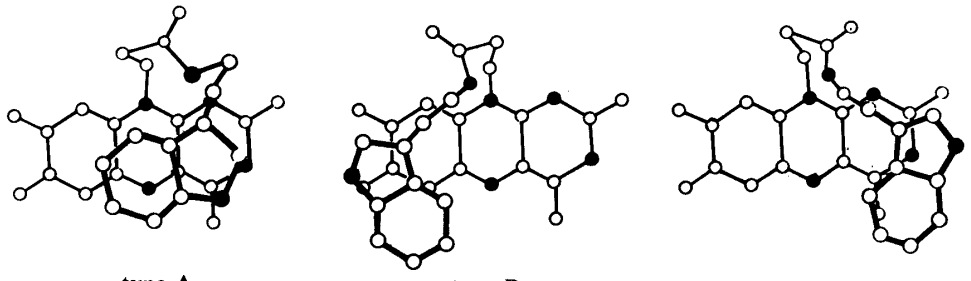


Fig. 7. Perspective Views of the Most Energetically Stable Conformers Belonging to the Various Conformational Categories

energetically most stable, we carried out energy calculations for various conformers of TRPDIP by using a minimization technique. The starting angle sets for energy calculations were shown in Fig. 1. Out of many different conformational sets, the 15 most energetically stable conformations (starting and refined torsional angles) are listed in Table IV, together with their final energies and conformational categories. As a whole, the energetically stable conformations of the TRPDIP molecule could be classified into three types (named types A, B and C) according to the stacking mode. Type A is the conformer with marked stacking of the indole ring on the pyrazinoid portion, type B on the benzenoid portion and type C on the pyrimidinoid portion of the flavin ring. The most stable conformations belonging to these three types are illustrated in Fig. 7. Among them, type A is the most stable, and the energy differences between the lowest energy in type A and types B and C are 7.8 and 8.5 kcal/mol, respectively. Therefore it would be expected that the type A stacking mode would be preferred in solution. In particular, the conformer of type A illustrated in Fig. 7 is consistent with the NMR data.

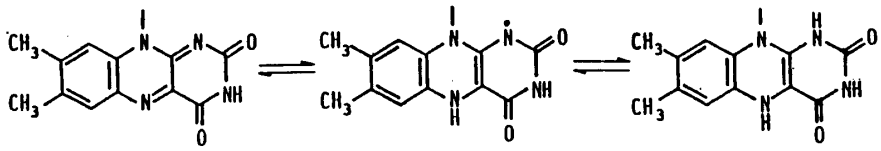
It has been apparent that TRPDIP itself preferentially takes folded conformations with intramolecular indole–flavin interactions, as has been suggested from UV, fluorescence and

^{1}H-NMR spectral studies in dilute solution. In more concentrated solution, the intermolecular interactions between both aromatic rings, in addition to the intramolecular ones, have to be taken into account, judging from preliminary experiments on the concentration dependence of the absorption and ^{1}H-NMR spectra. Although the open form without the indole–flavin interaction observed in the crystal structure is a stable conformation of TRPDIP, the inter and/or intramolecular stacking interactions might be observed in other crystals. Indeed we have prepared the dark red crystals of TRPDIP, though the crystals so far obtained are too small to analyze. In conclusion, it is clear that the indole ring can strongly bind with the flavin ring through π–π stacking interaction.

Biological Implications of the Flavin–Indole Stacking Interaction

The present spectroscopic and conformational studies, along with previous results on intermolecular indole–flavin complexes, suggest that the indole ring interacts with the pyrimidinoid and pyrazinoid moieties of the flavin ring more strongly than with the benzenoid portion. The former two portions possess the nitrogen atoms (N1 and N5) necessary for the electron transfer process in biological oxidation–reduction reactions. Since it is well known that the indole ring of tryptophan is the most π-electron rich aromatic ring among various amino acids,[24] a tryptophan residues in a flavin enzyme might act as a promotor for reduction of the flavin coenzyme as a result of the π–π electron transfer from the indole ring to N1 and N5 of flavin, because these atoms are in very close proximity to the indole ring.[2] In order to investigate the effect of the indole ring on the reduction of flavin, we have carried out the following polarographic measurements.

Reduction of the flavin ring can be described as follows:



quinone form semiquinone form hydroquinone form

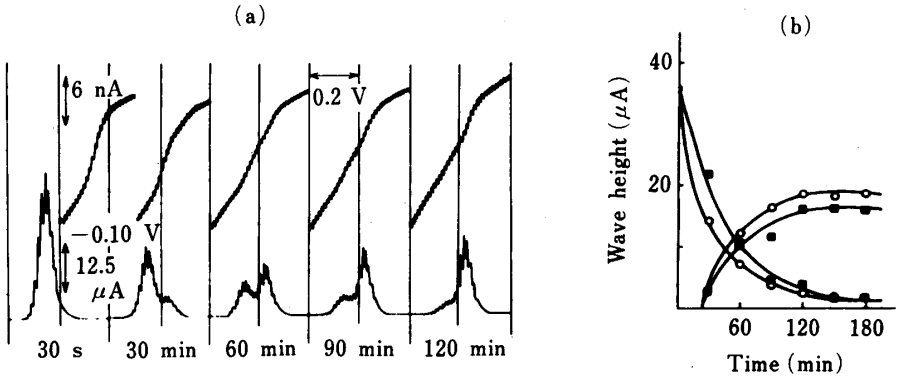


Fig. 8. (a) The Time Changes of Riboflavin Polarograms

Upper and lower curves represent the direct current (DC) and SW wave polarograms of riboflavin.

(b) The Time Changes of the SW Wave Height of Riboflavin

Alone (○—○) and in the presence of equimolar tryptamine (■—■).

In the polarograms of riboflavin, two waves can be observed: the first wave corresponds to the reduction from the flavin quinone form to the semiquinone one, and the second wave, reduction from the flavin semiquinone form to the hydroquinone one. Their time dependence

is shown in Fig. 8 (a). After 120 min, riboflavin was completely converted to the semiquinone form under the conditions used (pH = 4.0).[25] Figure 8 (b) shows the time changes of the first and the second wave heights of riboflavin alone and with equimolar tryptamine in square wave (SW) polarography. The coexistence with tryptamine, compared with riboflavin alone, delays the disappearance of the first wave of riboflavin, and decreases the second wave height.[26] The delay of the first wave could be interpreted as follows: the quinone form of riboflavin is stabilized by resonance with the indole ring due to the π–π stacking interaction. In addition, the decrease of the riboflavin second wave height would imply a tendency for the indole ring to transfer from the semiquinone form to the hydroquinone one, because the SW wave height is well known to be greatly influenced by the reversibility of the reaction, so the indole ring might shift its equilibrium between the semiquinone and hydroquinone forms toward the latter. Indeed the close proximity of an aromatic group such as tyrosine or tryptophan has been shown to raise the flavin reduction potential by 0.05 to 0.06 V for normal flavin.[27] This implies that the indole ring makes the reduction of flavin easier. The present experimental data, along with previous data on intermolecular indole–flavin complexes, suggest that a tryptophan residue in the active center of a flavin enzyme not only plays a role in the tight binding of the flavin coenzyme, but also acts as an accelerator of the reduction reaction of the flavin coenzyme.

References and Notes

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25) The conversion speed increased as the pH of the electrolytic solution increased. At pH = 7.0 (Sörensen buffer),

the riboflavin was completely converted to the semiquinone form within 60 min. In the case of TRPDIP, however, the complete conversion to the semiquinone form took more than 2 d. Therefore, the spectroscopic studies (UV and fluorescence) within 2 h after sample preparation refer to the flavin quinone form.

26) Similar polarographic features were also observed with 7,8-dimethylisoalloxazine-10-acetic acid (DIA) and DIP. On the other hand, it is of interest to note that the coexistence of another aromatic compound such as adenine or histamine completely reversed the phenomenon. Therefore it seems that the binding mode of indole or phenol ring to the flavin ring is different from that of adenine or imidazole ring. Detailed studies are in progress.

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