Heterogeneous Tryptophan Environments in the Cyclic Peptides Tyrocidines B and C

PHOSPHORESCENCE STUDIES*

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SUMMARY

Phosphorescence spectroscopy has been used to probe the microenvironments of the tryptophan residues of the cyclic antibiotic decapeptides tyrocidine B (which contains a single tryptophan residue) and tyrocidine C (which contains 2 tryptophan residues). Both peptides exhibited two distinct, overlapping tryptophan phosphorescence emission spectra under appropriate solvent conditions. The two emissions had characteristically different wavelength positions, phosphorescence lifetimes, and excitation spectra, and their relative intensities were strongly solvent-dependent. In contrast, an open chain derivative of tyrocidine B, which has no biological activity, showed only a single phosphorescence emission from tryptophan. This indicated that the environmental heterogeneity of tryptophan residues in the intact peptides arose not from their primary structures but from higher structural features. Factors which are known to increase the self-association of the tyrocidines, such as higher peptide concentrations or greater amounts of water in alcohol-water solvent mixtures, generally led to enhanced emission of the lower energy phosphorescence component relative to the higher energy one. We conclude that the higher energy component of the phosphorescence emission comes from solvent-exposed tryptophan residues of monomeric peptide molecules, and the lower energy component from tryptophan residues buried in the hydrophobic interior of peptide aggregates. These cyclic peptides reveal many of the phosphorescence complexities observed with native proteins and thus are useful as model compounds in correlating tryptophan microenvironments with emission characteristics.

The phosphorescence emission spectrum of tryptophan has two characteristics which should make it a useful probe of tryptophan environments in proteins; it is fine-structured, and the magnitude of its Stokes' shift is sensitive to aspects of the secondary and tertiary structure of proteins. Studies of indole and tryptophan phosphorescence in hydrocarbon and in highly polar glasses have shown only small changes in Stokes' shift are caused by solvent (1). Chemical substitutions of the amino or carboxyl group of tryptophan also have little effect on phosphorescence emission (2). Nevertheless, the tryptophan phosphorescence of native proteins is typically found to be red-shifted by 5 to 7 nm relative to that of free tryptophan. Upon denaturation, the phosphorescence moves to shorter wavelengths and approaches the position of free tryptophan. The origin of the red shift of native protein phosphorescence is not known, but presumably results from the microenvironment of tryptophan residues within the protein (for a review of peptide and protein luminescence, see Longworth (3)).

Because of the sharp vibrational bands of the tryptophan phosphorescence emission, and the apparent sensitivity of Stokes' shift to protein conformation, multiple tryptophan phosphorescence emissions should be detectable. For example, if a protein contains several tryptophan residues in different micro-environments, and the emissions from them are not quenched, then several overlapping but partially resolved tryptophan emissions might be observed. This would enable one to study selectively individual residues or classes of residues. Despite this potential usefulness, there are few examples of multiple tryptophan phosphorescence from proteins. Purkey and Galley (4) first reported the phenomenon with horse liver alcohol dehydrogenase, which contains 2 tryptophan residues per monomer and emits two overlapping tryptophan phosphorescence spectra. They showed that one of the emissions was quenched to a greater extent when the cofactor NADH was bound to the enzyme, and that the excitation spectra of the two emissions could be distinguished. They also reported multiple tryptophan phosphorescence emissions from papain and trypsin. Shaklai and Daniel (5), in a study of the phosphorescence of hemocyanin, observed that binding of copper ions to the apoprotein caused a second tryptophan emission to appear. Neither the apoprotein nor oxygenated hemocyanin showed the second emission. It is not yet clear whether the small number of proteins known to...
possess multiple tryptophan phosphorescence emissions is simply
due to insufficient experimental studies of protein phospho-
rescence or to a limited occurrence of the phenomenon.

Thus, although protein phosphorescence studies have con-
siderable promise as a means of studying the microenvironment
of selected tryptophan residues, several unresolved questions
must be approached before the applicability of the method is
demonstrated. Investigation of appropriate model compounds
will be important in this regard. In our studies of the lumini-
scence properties of several peptide hormones and antibiotics,
we have observed a dramatic dual tryptophan phosphorescence
emission from the cyclic decapeptide antibiotics tyrocidine B
and tyrocidine C, which is described in this paper. An abstract
of this work has appeared (6).

The tyrocidines have been used as models for studying im-
portant physicochemical properties of peptides and proteins.
In particular, their self-association behavior has been studied
by ultracentrifugation (7, 8), nuclear magnetic resonance spec-
troscopy (9), thin film dialysis (10), and by the use of a fluores-
cent probe molecule (11). The tyrocidines form an interesting
series of peptides for luminescence studies, since the differences
among them involve only the substitution of tryptophan for
phenylalanine at specific points in the sequence. Their primary
structures are cyclic(-Val-Orn-Leu-2Phc-Pro-X2-2Tyrr-Asn-
Gln-TyrGln), where X is tryptophan and Y is phenylalanine in
tyrocidine B, and both X and Y are tryptophan in tyrocidine C.
(Tyrocidine A contains phenylalanine at positions X and Y.)

Tyrocidine B, therefore, contains 1 tryptophan and tyrocidine C
contains 2. As well as defined primary structures, the secondary
structure of tyrocidine A determined by magnetic resonance
spectroscopy has been reported (12).1 Like the related peptide
gramicidin S-A (tyrocidine S) whose secondary structure was
determined previously (13), tyrocidine A is a flat β-pleated sheet
molecule having four intramolecular hydrogen bonds.

In this publication, we report the phosphorescence properties
of these peptides. Both tyrocidine B and tyrocidine C exhibit
two tryptophan phosphorescence emissions characterized by
different excitation and emission wavelengths, lifetimes, and
solvent behavior. The two emissions cover the extremes of
Stokes' shifts found in native proteins, and are attributed to
different microenvironments for the tryptophan residues. The
nature of these microenvironments has been correlated with
the known secondary structure and aggregation properties of the
peptides.

EXPERIMENTAL PROCEDURE

The tyrocidines were isolated from crude tyrocidine by counter-
current distribution (14) and were further purified by chromatog-
raphy on Sephadex G-25 in 10% acetic acid (14). The tyrocidines
adsorb to the Sephadex matrix with different affinities depending
upon their tryptophan contents, and they may be selectively
eluted by 10% acetic acid solution. Linear tyrocidine B was
obtained by reductive cleavage of the phenylalanine-proline bond
of tyrocidine B (15). This derivative has been shown to have no
antibiotic activity (16).

Phosphorescence emission spectra were obtained on an instru-
ment previously described (17), which utilizes phase-sensitive de-
tection of light modulated by electromechanical tuning forks (18).
Samples, ranging in concentration from 10-4 to 10-5 M, were pre-
pared in spectroquality solvents. Quartz tubes, 3-mm in internal
diameter, were used as cuvettes for phosphorescence measure-
ments. A Dewar with an unsilvered tail section was used to
maintain the samples at liquid nitrogen temperature. Excitation
was at 280 nm unless otherwise stated, and both excitation and
emission bandwidths were less than 5 nm.

To obtain the phosphorescence decay of a sample, the output of
the phase-sensitive detector was applied to a logarithmic amplifier,
and the logarithmic output was stored in a 512-channel analyzer
which used a stable averaging algorithm to obtain a mean value
(19). The signal to noise ratio was enhanced by accumulating
several replicate sweeps of the decay of each sample. The pre-
cision of the lifetime measurements was about ±0.05 s.

Fluorescence absorption polarization spectra were obtained on
the instrument described by Knopp et al. (20). Values of I/Io
for each excitation wavelength are given directly, where I0 and I
are the emission intensities parallel and perpendicular to the polar-
ized excitation, respectively. Anisotropies were then calculated
from the equation, A = (I/Io) - 1/(I/Io) + 2. The influ-
ence of residual polarization in the monochromatic exciting light
was minimized by determining I/Io for horizontally polarized
exciting light at each excitation wavelength. Fluorescence was
isolated by filters having a low wavelength cut-off at 335 nm.
Samples were contained in standard 1-cm cuvettes, and had an
absorbance at 280 nm of approximately 0.75. The temperature of
the sample for polarization measurements was 223 ± 3° K. The
anisotropy values were precise to ±0.01, and the accuracy was
estimated to be of the same order of magnitude.

RESULTS

Dual Tryptophan Phosphorescence Emission: Solvent and Con-
centration Effects—The phosphorescence spectrum of tyrocidine
C dissolved in ethanol clearly has two tryptophan emission
components (Fig. 1, Curve A). The 0-0 transitions of the two
emissions are centered at 409 and 416 nm. In glycerol-water
(1:1) and trifluoroethanol, two tryptophan emissions are also
seen, but their relative intensities are quite different (Fig. 1,
Curves B and C). In trifluoroethanol the 0-0 transitions are
centered at 413 and 405 nm, with the latter having greater in-
tenicity, while in glycerol-water (1:1) the emission at 416 predom-
inate over the one at 408 nm. Dual tryptophan phosphorescence
is also seen with tyrocidine B, in spite of the fact that tyrocidine B
contains only a single tryptophan. In ethanol and glycerol-water
(1:1), predominantly one tryptophan emission is observed, the 0-0 transitions occurring
at 408 and 415 nm, respectively (Fig. 1, Curves D and E).
In trifluoroethanol, two emissions are distinctly present, located at
403 and 412 nm (Fig. 1, Curve F).

The phosphorescence spectra of a model compound, N-acetyl-
L-tryptophanamide, were obtained for comparison. This
compound has a single tryptophan emission under all solvent condi-
tions employed, and the Stokes' shift of its emission varies with
solvent by at most 1 or 2 nm except in trifluoroethanol. The
wavelength in nanometers of the peak of the 0-0 transition was
405.5 in ethanol, 405.5 in ethanol-water (1:1), 405.5 in methanol,
405.0 in methanol-water (1:1), 406.0 in glycerol-water (1:1), and
410 in trifluoroethanol.

The resolution of the phosphorescence emission spectrum of
tyrocidine B in methanol-water (1:1) into its two components is
shown in Fig. 2. The spectrum was resolved by subtracting the
emission of tyrocidine B in pure methanol, in which only a single
tryptophan emission is present, from the spectrum in methanol
water (1:1). This figure reveals that both tryptophan emission
components are normal in shape, and differ only in their Stokes' shift.

These peptides, therefore, have overlapping "blue" and "red"
tryptophan emissions. The 0-0 transition of the blue
component is found between 403 and 409 nm, and that of the red
component between 412 and 416 nm. By comparison, the range
of values for the position of this peak in the spectra of several
native proteins is 406 to 416 nm (3), with typical values of 410

1 W. A. Gibbons, H. R. Wyssbrod, M. Fein, C. F. Beyer, L. C.
Craig, J. Dadok, and R. F. Sprecher, manuscripts in preparation.

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Fig. 1. Phosphorescence emission spectra of tyrocidine C (Curves A, B, and C) and tyrocidine B (Curves D, E, and F). Solvents were ethanol (A and D), glycerol-water, 1:1 (B and E), and trifluoroethanol (C and F). Peptide concentrations were 0.2 to 0.4 mg per ml.

Fig. 2 (left). Resolution of the observed phosphorescence spectrum of tyrocidine B in methanol-water (1:1) into its blue and red components. Curve A, observed spectrum; Curve B, resolved spectrum of blue component; Curve C, resolved spectrum of red component.

Fig. 3 (center). Effect of tyrocidine concentration on the fractional intensity of the blue phosphorescence emission component. The solvent was ethanol. •, tyrocidine B; ■, tyrocidine C.

Fig. 4 (right). Effect of solvent composition on the fractional intensity of the blue phosphorescence emission component of tyrocidines B and C. Peptide concentrations were 0.3 mg per ml. •, tyrocidine B in methanol-water mixtures; ○, tyrocidine B in ethanol-water mixtures; ■, tyrocidine C in methanol-water mixtures.
to 413 nm (21). It should be noted that, although the exact wavelength positions as well as the relative intensities of the two components are solvent-dependent, the separation between them in a given solvent is constant at 8 ± 1 nm (50 nm⁻¹). This separation is quite large. Purkey and Galley (4) observed a separation of 30 nm⁻¹ between the tryptophan emission components of horse liver alcohol dehydrogenase.

In addition to the sharp 0-0 peak at the blue edge of the tryptophan spectrum, there is another fairly sharp peak at 433 nm in N-acetyl-L-tryptophanamide. The dual tryptophan emission from tyrocidines B and C can also be discerned by observation of this region of the spectrum (Fig. 1). The blue component is found between 431 and 438 nm and the red component between 440 and 445 nm. As with the 0-0 transitions, the wavelength positions vary with the solvent, but the separation between the two components is 8 ± 1 nm, and the separation between the peak in the 440-nm region and the 0-0 transition for each individual tryptophan component is 28 ± 1.

If tyrocidine B is cleaved between the phenylalanine-proline bond to give a linear decapeptide with the same amino acid sequence as tyrocidine B itself, dual tryptophan phosphorescence is no longer observed. Linear tyrocidine B has a single emission when dissolved in ethanol or 50% glycerol, and its 0-0 transition is at 408 nm in both solvents. The cyclic structure of tyrocidine B, and presumably also of tyrocidine C, is therefore a requirement for dual tryptophan emission. Thus, the emission heterogeneity arises not from the primary structure of the peptides but from higher structural features.

Since the tyrocidines are known to self-associate extensively under appropriate conditions, we performed studies to determine the effect of peptide concentration on phosphorescence. Fig. 3 shows the fractional intensity of the blue component of the emission of tyrocidines B and C as a function of peptide concentration in ethanol. Fractional intensities were calculated by first subtracting the tryptophan phosphorescence contribution, if any, from the observed phosphorescence spectrum, then resolving the spectrum into its two tryptophan components, and then determining the ratio of the intensity of the blue component, measured at the wavelength maximum of the 0-0 transition, to the sum of the intensities of the blue and red components. At the low end of the concentration range, the fractional intensity of the blue component decreases as concentration increases. This change is more noticeable for tyrocidine C than for tyrocidine B. At higher concentrations, the fractional intensity plateaus at about 0.6 for tyrocidine C and 0.8 for tyrocidine B.

The extent of aggregation of these peptides may also be influenced by changing the composition of the solvent while keeping the peptide concentration constant. In general, organic solvents inhibit aggregation, while water promotes it. The fractional intensity of the blue component of the tyrocidines in alcohol-water mixtures is shown in Fig. 4. In pure methanol, both peptides have only a single tryptophan emission, which is located at the typical blue position. As water is added to the methanol, a second component progressively emerges in an approximately linear manner up to 40 volume% water. A more rapid increase of the second component occurs at higher water concentrations. The results for both peptides are similar. In contrast, the phosphorescence emission of tyrocidine B is completely independent of solvent composition in ethanol-water mixtures (Fig. 4). Thus, while the phosphorescence spectra of both tyrocidines are very sensitive to solvent composition in methanol-water mixtures, the phosphorescence of tyrocidine B is independent of solvent composition when the alcohol is ethanol.

The effect of peptide concentration on phosphorescence emission was also studied by adding tyrocidine A to either B or C in ethanol. Tyrocidine A contains no tryptophan but it co-aggregates with the other tyrocidines. Total peptide concentration can be increased in this manner without increasing the concentration of tryptophan residues. At concentration ratios of tyrocidine A to B or C of up to 8, no change in the tryptophan phosphorescence of B or C is observed.

**Phosphorescence Lifetimes**—The phosphorescence decay curves of tyrocidine C in glycerol-water (1:1) are shown in Fig. 5. They reveal that the two emission components have distinct lifetimes of phosphorescence decay. The blue component can be observed independently of the red by setting the emission monochromator to the wavelength maximum of the blue 0-0 transition (see Fig. 2). The red component, however, is always overlapped by the blue. Decay observed at the red 0-0 maximum therefore contains two components. But because of the closeness of the two decay times, the curve appears linear over the approximately 2 logs of intensity. Despite the fact that the red component is contaminated to some extent by the blue component, it is clear that the lifetimes of the two components are different by about 1 s.

The phosphorescence lifetimes of the tryptophan emissions of tyrocidines B and C in selected solvents are collected in Table I. In all cases where two components in the emission spectra are found, the components have different lifetimes. The blue component always has a longer lifetime than the red component, although the absolute magnitudes of the lifetimes differ from one solvent to another.
Table I

Phosphorescence lifetimes of tryptophan emissions from tyrocidines B and C

| Peptide   | Solvent             | Wavelength of observation | Phosphorescence lifetime |
|-----------|---------------------|---------------------------|--------------------------|
| Tyrocidine C | Ethanol             | 400                       | 6.30                     |
|           |                     | 416                       | 5.40                     |
|           | Glycerol-water, 1:1 | 407                       | 6.15                     |
|           |                     | 415                       | 5.20                     |
|           | Trifluoroethanol    | 405                       | 6.95                     |
|           |                     | 414                       | 6.30                     |
| Tyrocidine B | Ethanol             | 435                       | 6.20                     |
|           |                     | 415                       | 5.25                     |
|           | Glycerol-water, 1:1 | 415                       | 6.65                     |
|           | Trifluoroethanol    | 405                       | 6.65                     |
| Linear tyrocidine B | Ethanol | 455                       | 5.85                     |
|           | Glycerol-water, 1:1 | 491                       | 5.80                     |

There is a correlation between the Stokes' shift of the phosphorescence component and its lifetime. This is seen most clearly for tyrocidine B in ethanol and glycerol-water (1:1), in comparison with tyrocidine C. On going from ethanol to glycerol-water, the predominant emission of tyrocidine B shifts from 405 to 415 nm. These positions correspond to those of the blue component of tyrocidine C in ethanol, and the red component in glycerol-water. The data in Table I indicate that the lifetime of tyrocidine B in ethanol corresponds closely to the lifetime of the blue component of tyrocidine C, while in glycerol-water tyrocidine B has a lifetime similar to that of the red component of tyrocidine C.

The linear derivative of tyrocidine B, which does not exhibit a dual phosphorescence emission, has the same phosphorescence lifetime in two solvents in which tyrocidine B itself has quite different lifetimes (Table I), again indicating the importance of the covalent cyclic structure.

Tyrosine phosphorescence emission is also evident in the spectra of tyrocidine B, although its intensity is weak (Fig. 1). Tyrosine emission peaks at about 390 nm and has a broad, faintly structured appearance. It is clearly distinguishable from tryptophan phosphorescence both by its wavelength position and by its much shorter lifetime (22). The phosphorescence lifetime of tyrocidine B in glycerol-water (1:1) observed at 390 nm (where tryptophan does not emit) was 2.4 s.

Phosphorescence Excitation—As the wavelength of the exciting light is increased from 280 to 300 nm, the blue phosphor component of tyrocidine C in ethanol becomes smaller relative to the red component (Fig. 6). This indicates that the red component is preferentially excited by longer wavelength light and implies that the absorption spectrum of the tryptophan population responsible for the red component is shifted to the red relative to the absorption spectrum of the blue tryptophan population. Similar results were obtained for both tyrocidines B and C in all solvents in which two emission components are present. Preferential excitation of the red component by red light could also be directly demonstrated by comparison of the phosphorescence excitation spectra of the blue and red components.

Fluorescence Absorption Polarization Spectra—The existence of two tryptophan phosphorescence emissions from these peptides implies that energy transfer between tryptophan residues, both at the singlet and triplet levels, cannot be very efficient.

This is surprising in the case of tyrocidine C, because the two contiguous tryptophans would be expected to transfer efficiently. Orientation factors cannot be invoked to explain the lack of transfer since it is very unlikely that the 2 tryptophan residues could be unfavorably oriented for both singlet and triplet transfer. To determine experimentally whether singlet energy transfer between tryptophan residues occurs, the fluorescence absorption polarization spectra of tyrocidines B and C and of N-acetyl-L-tryptophanamide were determined (Fig. 7). The polarization anisotropy of tyrocidine B is less than that of the reference monomer at wavelengths between 250 and 295 nm. This depolarization is due to tyrosine to tryptophan singlet energy transfer and not to tryptophan-tryptophan transfer, since, at wavelengths above 295 nm, where tyrosine absorbance is negligible, the anisotropy values of both compounds are essentially the same. Tyrocidine C, on the other hand, has considerably lower values than both tyrocidine B and the monomer at all wavelengths between 310 and 250 nm, suggesting strongly that tryptophan-tryptophan energy transfer is taking place. We are thus faced with an apparent contradiction, energy transfer at the singlet level between tryptophan residues of tyrocidine C, and at the same time two distinct phosphorescence emissions from these tryptophans. This apparent contradiction will be resolved in the discussion section in terms of two, spatially separated pools or collections of peptide molecules.

**DISCUSSION**

We have shown that two overlapping tryptophan phosphorescence emissions can sometimes be observed from tyroci-
phoresce at the position of the red component of the tyrocidines and which are perhaps complexed with amide residues which are shielded from solvent, are in a less polar solvent. The solvent responsible for the blue emission was more exposed to the rigid, polar solvent molecules that favor the latter explanation. The blue component, which is closest to the position of N-acetyl-n-tryptophanamide (410 nm), was found by many workers to have its blue edge maxima at about 415 nm (1, 21, 23, 24). Also, Purkey and Galley (4) found that the blue emission from horse liver alcohol dehydrogenase was quenched by sodium bromide to a greater extent than the red emission, suggesting that the tryptophan population responsible for the blue emission was more exposed to the solvent.

The red phosphorescence component may be assigned to residues which are shielded from solvent, are in a less polar environment, and which are perhaps complexed with an amide carbonyl or polar side chain. There appear to be no simple tryptophan derivatives which have been observed to phosphoresce at the position of the red component of the tyrocidines (about 415 nm). Even most proteins have phosphorescence spectra which are not as red-shifted relative to the monomer as these peptides. When proteins are denatured, their tryptophan phosphorescence generally shifts somewhat to the blue, which has been interpreted as due to the exposure of previously buried residues. The reason why the phosphorescence of native proteins is red-shifted in the first place has not been firmly established. Purkey and Galley (4) suggest that it is a result of the greater polarizability of the interior of the protein compared to the rigid polar matrix seen by exposed residues. According to their argument, the polarizable protein interior is able to adjust to changes in the dipolar character of the chromophore upon excitation, whereas the polar molecules of the solvent matrix (rigid at 77° K), favorably oriented about the exposed chromophore in its ground state, are not able to reorient about the excited chromophore. This model leads to the expectation that both the absorption and phosphorescence of buried residues should be red-shifted relative to exposed residues, which is compatible with what is observed for the tyrocidines.

However, there are two lines of evidence which complicate these assignments. The first is our own observation that, in trifluoroethanol, it is the red component (at 412 or 413 nm) which is closest to the position of N-acetyl-L-tryptophanamide (410 nm). The blue component in this case is at higher energy than the monomer phosphorescence. Secondly, several studies of the fluorescence of the single tryptophan residue of ribonuclease T1 have led to the conclusion that it is buried in the hydrophobic interior of the native protein, has no neighboring polar groups, and does not form an exciplex within the lifetime of the excited state (25-27). This tryptophan residue has its 0-0 maximum in phosphorescence at 406 nm (25). A somewhat different situation is found with Staphylococcus aureus endonuclease, whose 0-0 phosphorescence peak comes at 408 nm (25). Its single tryptophan residue is in an environment with a low dielectric constant (28, 29), even though the residue is sufficiently exposed to solvent molecules to form an exciplex during the excited state lifetime, as judged by the fluorescence characteristics of the protein (29). The tryptophan residues of these two proteins are buried to different degrees but both have phosphorescence spectra near the blue end of the range. These examples point out the oversimplification of the exposed versus buried dichotomy, which has been noted by others with reference to fluorescence (3) and electronic absorption (30). There are without doubt degrees of exposure to solvent and degrees of solvent mobility among tryptophan residues in proteins. The effects of various combinations of polarity, polarizability, mobility, and complex formation on the excited state properties of chromophores are complicated and difficult to evaluate separately. In particular it seems likely that interactions between the amide backbone or polar side chains and the heterocyclic ring of tryptophan are of importance in peptides and proteins.

Correlation of Tryptophan Environments with Peptide Structure and Aggregation—If we accept the conclusion that the blue phosphorescence component comes from exposed residues and the red component from buried residues, the next question to consider is how such distinct environments could arise in these peptides. Two explanations may be proposed, the peptides exist in two or more conformational forms which place the tryptophan side chain in different environments, or peptide aggregation results in a change in the environment of the tryptophan residues. As detailed below, the bulk of the evidence favors the latter explanation.

**Fig. 7.** Fluorescence absorption polarization spectra of N-acetyl-L-tryptophanamide (Curve A), tyrocidine B (Curve B), and tyrocidine C (Curve C). The solvent was ethanol-glycerol (1:1).

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**Natures of Two Tryptophan Environments**—From a comparison with simpler tryptophan derivatives and literature data, we suggest that the two environments may be characterized generally as "exposed" and "buried." The blue component, which is emitted by the exposed tryptophan population, has its 0-0 maximum at about 408 nm in all solvents studied except trifluoroethanol. This is near the position of the corresponding peak in the spectra of N-acetyl-L-tryptophanamide (406 nm), which is fully exposed to the rigid, polar solvent molecules that compose the matrix of the low temperature glass. The phosphorescence emission spectra of tryptophan and simple derivatives of it, including synthetic oligopeptides, have consistently been found by many workers to have their blue edge maxima at about 406 nm (1, 21, 23, 24). Also, Purkey and Galley (4) found that the blue emission from horse liver alcohol dehydrogenase was quenched by sodium bromide to a greater extent than the red emission, suggesting that the tryptophan population responsible for the blue emission was more exposed to the solvent.
From previous studies using several techniques, the tyrocidines are known to aggregate extensively in solution (7-10, 16, 31, 32). The aggregation is driven primarily by hydrophobic forces and the aggregate itself is thought to have a micelle-like structure, with a polar exterior and hydrophobic interior. This behavior is consistent with what is known of the primary and secondary structures of these peptides. Recent proton magnetic resonance results have led to a postulated structure of the amide backbone ring of tyrocidine A (12) and, in addition, the orientations of several amino acid side chains have been deduced from C$_{6}$H-C$_{5}$H proton coupling constants and other lines of evidence (12). The peptide backbone of tyrocidine A in dimethyl sulfoxide forms a β sheet structure, very similar to the structure of gramicidin S-A (13). The molecule has four intramolecular hydrogen bonds involving the amide protons of Val$_{1}$, Leu$_{3}$, Phe$_{6}$, and Asn$_{8}$. The conformation of the backbone ring and the presence of a β-amino acid at position 7 in the sequence result in a clustering of most of the hydrophobic side chains on one side of the plane formed by the backbone ring. On this side are found the side chains of Val$_{1}$, Leu$_{3}$, Pro$_{9}$, Phe$_{6}$, and Phe$_{7}$, as well as those of Asn$_{8}$, Gln$_{9}$, and Tyr$_{10}$. On the other side of the backbone ring are found the side chains of only 2 residues, Orn$_{2}$ containing the only charged group in the peptide, and Phe$_{7}$.

Thus, the individual peptide molecules possess a polar “head group” (the ornithine β-amino group) and a hydrophobic “tail” (the cluster of nonpolar side chains on the opposite side of the peptide ring). In view of this structure, the similarities between detergent aggregation and tyrocidine aggregation (8, 11) are not surprising. The peptide aggregate most likely possesses a polar surface and a hydrophobic interior. Studies of fluorescent probe binding to tyrocidine aggregates have shown that the aggregates do contain hydrophobic regions (11). From these structural considerations, it is likely that a tryptophan residue would be more exposed to the solvent in a monomeric peptide molecule than in a molecule which was part of an aggregate. The blue phosphorescence component would therefore be assigned to tryptophan residues in peptide monomers, and the red component to residues in peptide aggregates.

This conclusion is supported by the solvent dependence of tyrocidine phosphorescence in methanol-water mixtures. In pure methanol, a solvent in which the peptides are expected to be monomeric at the concentrations used, only the blue component is present. With increasing percentages of water, which leads to increasing aggregation, the red component appears and grows in intensity. Both peptides behave essentially identically, as would be expected if the two components arise simply from aggregated and monomeric forms.

In ethanol solutions, the relative intensities of the two phosphorescence components are dependent on peptide concentration at the low end of the concentration range studied. This suggests that peptide-peptide interactions are involved in producing the environment responsible for the red component. However, at higher peptide concentrations, the relative intensities remain nearly constant, and the plateau values for tyrocidines B and C are different. Moreover, the addition of water to ethanol solutions of tyrocidine B at concentration levels in the plateau region produces no enhancement of the red component, in striking contrast to the results in methanol-water mixtures. The addition of tyrocidine A to B or C in ethanol also does not change the relative intensities of the two components. These differences between methanol and ethanol solutions are puzzling. Considering just tyrocidine B for the moment, two characteris-

tics of the phosphorescence emission must be distinguished; these are the relative intensities and the wavelength positions of the two components in ethanol and methanol solutions. The relative intensities of the components depend on solvent, but their wavelength positions do not. Thus, it appears that, although the two environments in which the tryptophan residue of tyrocidine B can find itself are the same in ethanol and methanol solutions, the number of residues in each environment, and hence the intensity of each emission component, is different. This could be due either to different extents of aggregation in the two solvents, or to different structures of the aggregate, such that the number of tryptophan residues brought into the second environment for a given extent of aggregation was dependent upon the solvent milieu.

Perhaps the strongest argument in favor of aggregation as the cause of the shift of tryptophan environment is the finding of extensive energy transfer at the singlet level between the two tryptophan residues of tyrocidine C. This would be expected to lead to a single phosphorescence emission from the residue with the lowest triplet energy. Since two emissions are in fact observed, the tryptophan pools responsible for each emission must be sufficiently separated spatially to prevent energy transfer. This condition is satisfied by aggregated and monomeric forms of the peptide. Intra- or intermolecular energy transfer, or both, could occur between tryptophan residues in aggregated peptide molecules, and intramolecular transfer could occur between tryptophan residues in monomeric peptide molecules, but intermolecular transfer between residues of monomeric and aggregated peptide molecules could not occur.

It should also be noted that the open chain derivative of tyrocidine B, which does not possess the phosphorescence heterogeneity of the intact peptide, does not self-associate (16). Its single tryptophan emission has a spectral distribution characteristic of the blue component of tyrocidine B. Thus, the red phosphorescence component, the ability to self-associate, and antibiotic activity all appear to be linked in the tyrocidine series of peptides.

A second possibility to account for two tryptophan environments in the tyrocidines is the existence of multiple conforma-
tional forms. Williams et al. (7) have suggested that multiple forms may account for the time dependence of peptide aggregation in aqueous salt solutions. It is plausible that the tryptophan side chain could experience quite different environments depending on whether it extended into solution or folded back over the hydrophobic side of the peptide ring (in terms of the model discussed earlier). While it is possible that conformational effects are combined with aggregation to modify tryptophan environments, it seems unlikely, in view of the evidence discussed above, that conformational effects could be of primary importance.

In conclusion, we have presented evidence from phosphorescence studies that the tryptophan residues of the cyclic peptides tyrocidines B and C can experience two distinct environments, either exposed to the polar solvent in monomeric peptide molecules, or buried in the nonpolar interior of peptide aggregates. Thus, the concept of environmental heterogeneity is not limited to proteins, but can also be applied to these relatively small peptides. Although the environmental heterogeneity arises from quaternary rather than secondary or tertiary aspects of peptide structure, nevertheless it remains a useful concept when applied in this way. As far as the nature of the microenvironment itself is concerned, it makes no difference whether it arises from the aggregation of a peptide or the folding of a protein.
Thus, the tyrocidines emerge as especially interesting model systems for luminescence studies in that their tryptophan residues can be placed in environments like those found in proteins, and the environments can be manipulated by changing the state of aggregation of the peptide by, for instance, changing solvent composition.

Furthermore, the results presented here suggest that phosphorescence heterogeneity may be more common in proteins than is currently suspected. Given that many proteins possess tryptophan residues in different environments, then phosphorescence heterogeneity from them should be limited only by energy transfer or nonradiative decay of the excited state energy. More work on protein phosphorescence would seem justified in order to use tryptophan environmental heterogeneity to probe the structure and function of proteins.

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