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DISULFIDE LUMINESCENCE. EMISSION CHARACTERISTICS OF CYCLIC TETRAPEPTIDE DISULFIDES

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SUMMARY Luminescence has been detected in cyclic tetrapeptide disulfides containing only nonaromatic residues. Excitation of the -S-S- $n-\sigma^*$ transition between 280 and 290 nm leads to emission in the region 300-340 nm. The position and intensity of the emission band depends on the stereochemistry of the peptide and polarity of the solvent. Quantum yields ranging from 0.002 to 0.026 have been determined. Disulfide luminescence is quenched by oxygen and enhanced in solutions saturated with nitrogen. Contributions from disulfide linkages should be considered, when analysing the emission spectra of proteins, lacking tryptophan but having a high cystine content.

INTRODUCTION

The luminescence properties of proteins have been widely investigated and ascribed exclusively to aromatic residues (1,2). Fluorescence, arising from tyrosine (3) and tryptophan (4), has proved extremely useful in studies of protein structure and interactions. Tyr emission is observed between 305 and 310 nm while Trp emission occurs around 330 to 350 nm. Low quantum yields have precluded the use of phenylalanine fluorescence (observed at ~270 nm), in protein chemistry (5). All these aromatic residues have excitation (absorption) bands in the range 250-290 nm. Disulfides, which are an important structural feature in proteins, also absorb in this region, with the wavelength of absorption varying as a function of the

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Abbreviations : Boc, t-butyloxycarbonyl ; NHMe, N-methylamide ; Aib, α-aminoisobutyric acid.

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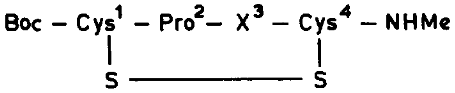


$$\begin{array}{l} \text{Boc}-\underset{\underset{\displaystyle \text{S}}{|}}{\text{Cys}^1}-\text{Pro}^2-\text{X}^3-\underset{\underset{\displaystyle \text{S}}{|}}{\text{Cys}^4}-\text{NHMe} \end{array}$$

-S-S- dihedral angle (6). We wish to describe disulfide luminescence in cyclic tetrapeptides of the type where X = Aib, L-Ala, D-Ala or Gly.

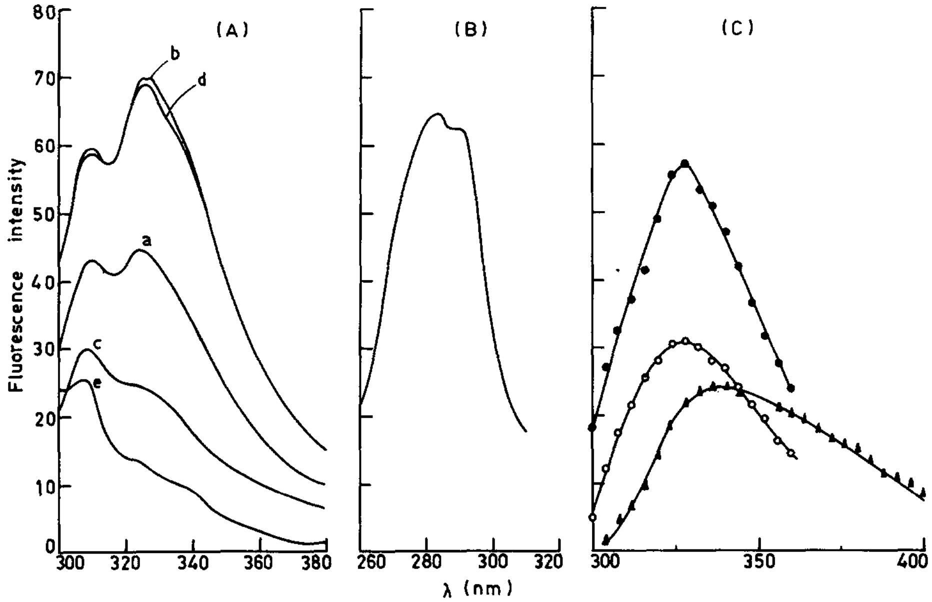
MATERIALS AND METHODS

Peptide disulfides were synthesized by solution phase procedures using dicyclohexylcarbodiimide-1-hydroxybenzotriazole mediated couplings. The benzyl group was used for sulfhydryl protection and its removal was effected by reduction in Na/liquid NH_3. Oxidation to the disulfide was carried out in dilute aqueous solution, using $K_3Fe(CN)_6$. All peptides were purified by column chromatography on silica gel and checked for homogeneity in multiple solvent systems on TLC. Peptides were characterized by 270 MHz ^1H NMR, elemental analysis and molecular weights were determined by mass spectrometry for X = L-Ala, D-Ala and Gly. For X = Aib the structure of the peptide disulfide has been established by single crystal X-ray diffraction (B.V.V. Prasad, unpublished).

Luminescence experiments were carried out on a Perkin Elmer MPF-44A fluorescence spectrometer operated in the AC mode for solution phase studies and DC mode for low temperature, glass phase experiments. Room temperature (298°K) and 278° K measurements were carried out using 1 cm pathlength quartz cells. Studies at 77°K were performed by dissolving the peptide in an ethanol:methanol:diethylether (8:2:1) mixture, which forms a transparent glass at low temperatures. The solution in the glass forming mixture was placed in a cylindrical quartz cell and surrounded by liquid nitrogen in a quartz jacket. All emission and excitation spectra are uncorrected.

RESULTS AND DISCUSSION

The disulfides chosen in this study have compact solution conformations with a transannular 4→1 hydrogen bond, stabilizing a Pro-X β-turn in the 14 membered disulfide ring. This has been established by 270 MHz ^1H NMR studies. Further the Aib^3-disulfide has a highly folded incipient 3_{10} helical conformation, with an S-S bridge dihedral angle of ~80° in the solid state (un-



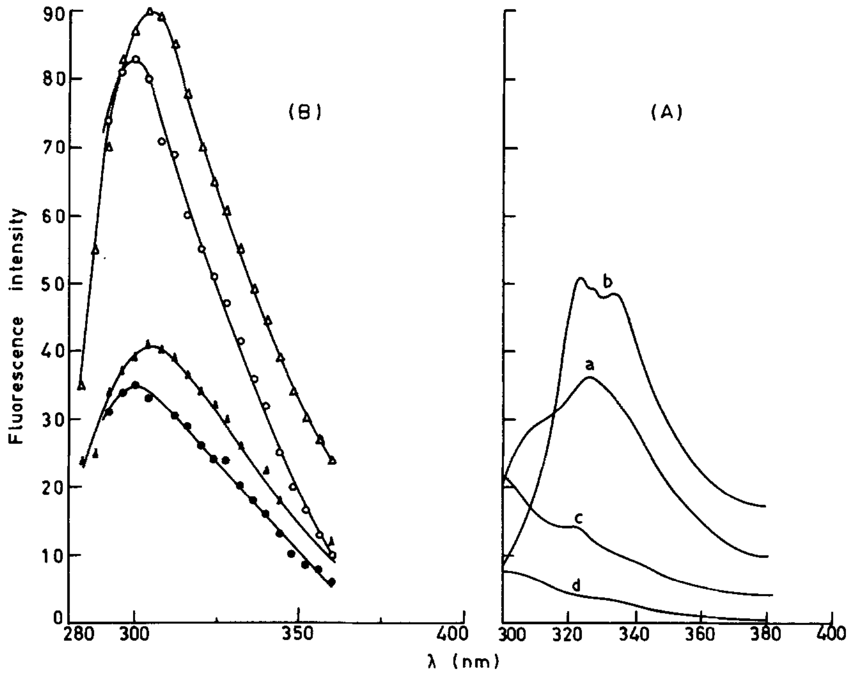
1. A. Emission spectra of Aib^3-disulfide (0.58 mM) in methanol, excited at 290 nm. a) 298°K ; b) 278°K after bubbling N$_2$; c) sample b) after bubbling O$_2$; d) sample c) after bubbling N$_2$; e) methanol at 298°K.

B. **Excitation spectrum of Aib^3-disulfide (0.58 mM) in methanol at 278°K after bubbling N$_2$.**

C. Emission spectra of Aib^3-disulfide (0.58 mM) corrected for solvent Raman band and scatter. o-o, 298°K in CH$_3$OH ; ●-● , 278°K in CH$_3$OH after bubbling N$_2$; ▲-▲ , 278°K in CHCl$_3$ after bubbling N$_2$.

published results). These conformationally restricted disulfides should therefore prove useful in exploring further spectroscopic characteristics of S-S linkages.

Figure 1A shows the emission spectra of the Aib^3-disulfide in methanol, excited at 290 nm. At room temperature a broad emission is detected at 327 nm, superimposed on the Raman band of the solvent. The appropriate blank spectrum of the solvent is also shown. The intensity of the emission peak is dramatically enhanced on bubbling nitrogen through the solution and by cooling. When oxygen is passed through the solution the emission band is effectively quenched (Figure 1A). The passage of a stream of nitrogen through the oxygenated solution restores the emission band to its original intensity. This demonstrates that oxygen quenching is reversible and does not involve any chemical transformation of the disulfide. The excitation spectrum of the Aib^3-disulfide is shown in Figure 1B. The compound shows an electronic absorption band at 279 nm due to the $n-\sigma^*$ transition of the S-S group, which is in agreement with the observed position of the excitation maximum. Figure 1C shows the emission spectra of the Aib^3-disulfide, corrected for solvent background under different conditions. The position of the emission maximum is at 327 nm in CH_3OH and at 338 nm in $CHCl_3$. There is also a fall in emission intensity in $CHCl_3$. Figure 2A compares the spectrum of Aib^3-disulfide at 298° K and 77° K, in a glass forming solvent mixture. Enhanced emission intensities are observed at low temperature. Of the other compounds tested, D-Ala^3 and L-Ala^3 disulfides show similar emission characteristics (Figure 2B). The band positions are blue shifted by about 20-30 nm as compared to the Aib^3-disulfide. The Gly^3-disulfide, cystine and dibenzyl disulfide had no detectable emission. Quantum yields relative to quinine sulfate in 1N H_2SO_4 (ϕ = 0.55) were determined for the various peptides. Values of ϕ = 0.026 (λ_{em} = 327 nm, CH_3OH) and 0.002 (λ_{em} = 330 nm, glass forming mixture) were determined for Aib^3-disulfide, at room temperature. The values obtained for L-Ala^3 and D-Ala^3 disulfides are



2. A. Emission spectra of Aib^3-disulfide (1.16 mM) in glass forming solvent mixture. a) 298°K with CH$_3$OH surrounding cell ; b) 77°K with liquid N$_2$ surrounding cell ; c) liquid N$_2$ blank ; d) CH$_3$OH blank.

B. Emission spectra of disulfides in CH$_3$OH corrected for solvent Raman band and scatter. o-o, D Ala^3-disulfide (1 mM) ; ●-●, D Ala^3-disulfide (0.6 mM) ; Δ-Δ , L Ala^3-disulfide (1.4 mM) ; ▲-▲ , L Ala^3-disulfide (0.7 mM).

0.002 (λ_{em} = 305 nm) and 0.004 (λ_{em} = 300 nm), respectively. No emission could be detected for the L-Ala^3 and D-Ala^3 disulfides in the glass forming mixture. This is not surprising since even in the Aib^3 disulfide there was a drastic reduction in the quantum yield on going from methanol to this mixture. Suitable choice of experimental conditions is necessary for optimal detection of disulfide emission. At low concentrations ($<$ 200 μM) the contributions from the Raman band of the solvent are dominant, while at high concentrations (>1 mM) quenching effects are observed.

The above results establish that disulfide luminescence is detectable. Further, the quantum yield of emission depends upon solvent polarity, with increasing intensities observed in more polar media. The position and quantum yield of emission also appear to be a function of peptide disulfide geometry. In the more sterically constrained Aib^3-disulfide, the quantum yields are an order of magnitude higher than the Ala^3 derivatives and the band position is also shifted to longer wavelength. The quantum yield of Tyr in peptides is in the range 0.01 to 0.03 (7) while Trp has a considerably higher value of 0.2 (4). Thus, in proteins lacking Trp, but containing geometrically constrained disulfide bridges, emission in the region 300-340 nm may be observed. The assignment of such bands to Tyr residues must then be made with caution. It is noteworthy that in the case of cytotoxins lacking Trp, but containing 4 Tyr and 4 disulfide bridges, anomalous 'Trp like' fluorescence has been observed. This has been ascribed to tyrosinate emission (8). The results of our present study suggest that disulfide luminescence warrants further detailed investigation, with respect to its applicability in studies of proteins. The observed oxygen sensitivity of this luminescence, even at atmospheric pressures, suggest that emission from triplet excited states cannot be ruled out. It is pertinent to note that room temperature phosphorescence has been recently reported for benzophenone bound to chymotrypsin (9).

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