# Unusual Emission Properties of the Tryptophans at the Surface of Short Ragweed Allergen Ra5†

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ABSTRACT: The fluorescence and low-temperature phosphorescence of short ragweed pollen allergen Ra5 have been examined. Both the fluorescence of Ra5 in aqueous solution at room temperature and the phosphorescence of Ra5 in rigid glycol–water solvents at low temperature result entirely from the tryptophan residues in the molecule. The heterogeneity in the low-temperature phosphorescence spectra indicates that the two tryptophans in Ra5 are located in distinct environments and can be studied independently. The fluorescence wavelength maximum and large solvent-relaxation shift in the fluorescence of the allergen in 1:1 ethylene glycol–water reveal that at least one of the residues is exposed to the polar solvent. Studies of the iodide and cesium ion quenching of the fluorescence of Ra5 are consistent with this assignment but suggest that one of the tryptophans may be shielded by a negative charge in the protein. Phosphorescence data obtained in the presence of iodide and with increasing temperature confirm that both aromatic residues are readily subject to perturbation but identify the more susceptible one with one of the two spectral components that manifest themselves in the phosphorescence of Ra5. The phosphorescence wavelength maxima and the triplet-state lifetimes of the distinct tryptophans reveal that both residues lie in unusual local environment at the protein surface. While one aromatic side chain appears to be stabilized by an intramolecular interaction with a polar function, the other is perturbed quite possibly through an interaction with a disulfide linkage in the molecule.

The relatively low molecular weight ($M_r$ 5000) and knowledge of the complete amino acid sequence (Mole et al., 1974, 1975) of the allergen Ra5 of the pollen of *Ambrosia elatior*, commonly known as short ragweed, suggest that it should be possible to use this protein to explore structure–function relationships in the field of immediate hypersensitivity. The allergen consists of a single polypeptide chain of 45 amino acid residues of which 8 are cysteines in the form of 4 Cys–Cys bridges of as yet undetermined pairing. As with other protein allergens (Marsh, 1975), there are no distinguishing chemical features which suggest themselves as being at the root of its sensitizing property. However, some though possibly not all (Goodfriend, 1976) the relevant features may well be associated with the overall three-dimensional structure of the molecule or more likely with particular topographical loci which occur as a result of protein folding. We have initiated a study of the secondary structure of Ra5 and are examining distinctive groups on the surface of the molecule in an attempt to find correlations with its allergenic activity.

Tryptophan residues, of which threre are two in the allergen, while often buried in the polypeptide folds of globular proteins, are also frequently found at the surface of proteins and on occasion in local environments which give rise to unusual emission properties (Galley, 1976; Hershberger et al., 1980). Under conditions where these aromatic residues are experiencing particular perturbations at the surface of proteins, they may form part of loci which are readily recognized. The significance of the lone tryptophan in myelin basic protein as an antigenic site for the induction of antibodies which lead to experimental encephalomyelitis (Patterson, 1966) and of aromatic groups as haptens (Pressman & Grossberg, 1968) suggests that residues of this kind may well be distinguished as antigenic determinants.

The location of tryptophan residues in proteins can be assessed by taking advantage of the emission properties of these intrinsic aromatic chromophores. In addition to the fluorescence properties of globular proteins at ambient temperatures which can be used in understanding the location of these residues, phosphorescence experiments for the proteins both in rigid media at low temperatures (Konev, 1967; Longworth, 1971; Ross et al., 1979; Hershberger et al., 1980), and in fluid solution (Saviotti & Galley, 1974; Imakubo & Kai, 1977) provide additional information on the perturbations affecting particular intrinsic chromophores.

In the present work the emission properties of ragweed allergen Ra5 have been examined both in a preliminary search for distinguishing physical features which might be related to allergenic activity and as an aid in characterizing the structure and following structural modifications in the protein in general. The data indicate that the allergen provides an example of a protein in which distinct spectral contributions from the two tryptophans in the molecule readily appear in the low-temperature phosphorescence spectra. Quenching and spectral data derived from both fluorescence and phosphorescence experiments reveal that both residues in the allergen lie at the surface of the protein and are experiencing unusual perturbations from their local environments.

## Materials and Methods

Water used in the preparation of buffer was deionized and distilled from glass. Reagent grade chemicals were used as received from commercial sources. Tris(hydroxymethyl)-aminomethane (Tris) was obtained from Fisher Scientific, Canada. Cesium chloride, optical grade, was obtained from Schwarz/Mann, Orangeburg, NY. L-Tryptophan, N.R.C. grade, was obtained from Anjinomoto, New York. Glycerol and spectrophotometric grade glycerol were from Aldrich while ethylene glycol was chromatoquality from Matheson, Coleman & Bell. Solutions with ethylene glycol were formed by adding

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the glycol to a buffer solution of Ra5 by volume while solutions with glycerol were formed by weight due to the high viscosity of the glycerol.

Ragweed allergen Ra5 was isolated and purified to size and charge homogeneity as previously described (Lapkoff & Goodfriend, 1974). Protein concentrations were obtained by using $E^{1\%}_{1cm}$ (280) = 2.65 (Lapkoff & Goodfriend, 1974).

Fluorescence spectra of aqueous solutions of the allergen were measured in quartz cells (0.3- or 1.0-cm paths) on a Farrand Model Mark 1 spectrofluorometer equipped with an analogue recorder, Texas Instruments Servo/riter II (10-in. chart). Slits were used such that both excitation and emission monochromators had a band-pass of 5 nm. Quenching constants were calculated from Stern-Volmer plots and from Lehrer plots (Lehrer, 1971) of the data. Least-squares calculations were done by using a Wang programmable calculator.

Phosphorescence and fluorescence spectra of the allergen in the presence of glycols were obtained with an apparatus that has been described elsewhere (Purkey & Galley, 1970). Measurements were carried out with the samples contained in 2–3-mm i.d. Spectrosil tubes. The temperature of the sample was controlled by the flow of cold nitrogen through an optical Dewar housing the sample tube and was monitored with a copper–constantan thermocouple.

The nonexponential decay of the phosphorescence of the allergen was decomposed into a sum of two exponential components. The decay after 6 s, which contained only the slow component, was analyzed on a logarithmic scale with a linear least-squares fit. the difference between the experimental decay and this least-squares line was taken for times less than 6 s and was analyzed with a second linear least-squares fit.

## Results

*Fluorescence of Ra5.* Excitation of an aqueous solution of Ra5 at 270 and 290 nm yields essentially identical fluorescence spectra characteristic of tryptophan (Figure 1). The fluorescence band maximum is located at 342 nm (bandwidth at half-maximum 58 nm). This spectrum is not as far as to the red as that observed for solvent-exposed tryptophans in many proteins or peptides (Konev, 1967; Longworth, 1971; Burstein et al., 1973) or as the 350–353-nm emission maximum of free tryptophan. However, the Ra5 spectrum is influenced by the addition of glycols to the solvent. The fluorescence maximum in 1:1 ethylene glycol–phosphate buffer is at 339 nm. Furthermore the spectrum undergoes a large retreat to 318 nm in this solvent as the temperature is lowered. The 21-nm blue shift shown in Figure 1 occurs as a consequence of the inability of the solvent at high viscosities to effectively reorganize about the chromophore during the excited-state lifetime (Eisinger & Navon, 1969). This is supported by observations with glycerol–buffer (70:30 w/w) as a solvent. When the solutions are either quite rigid at low temperatures or very fluid at room temperature, the fluorescence wavelength maxima are the same in either EG–buffer or glycerol-buffer. However, the transition between these wavelength extrema is observed at a higher temperature range in the more viscous glycerol–buffer solvent. At −70 °C, the Ra5 fluorescence spectrum can be seen in Figure 1b to have almost completely relaxed to its room-temperature limit with EG-buffer as a solvent but still remains closer to the low-temperature unrelaxed limit with the glycerol–buffer. This solvent-dependent relaxation as well as the solvent perturbation to the room-temperature spectrum indicates that one or both tryptophans in the allergen must be at least partially exposed to the polar solvent.

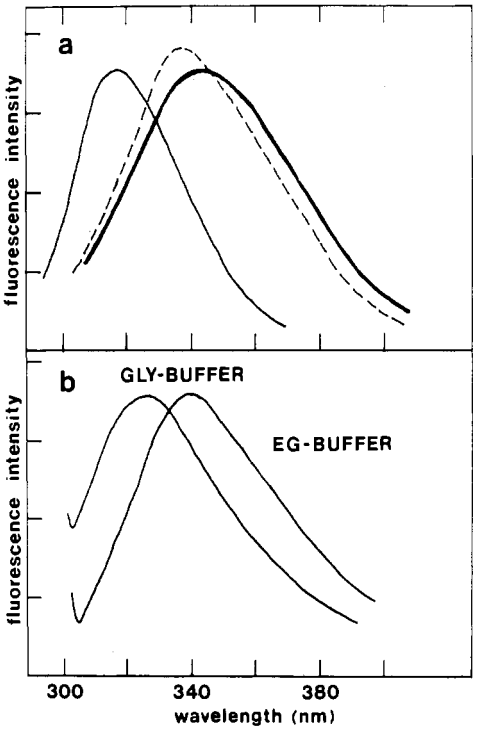


FIGURE 1: (a) Fluorescence spectra of ragweed allergen Ra5 excited at 290 nm. (—) At room temperature in Tris-HCl buffer (0.01 M, pH 7.0). (---) At room temperature in 1:1 ethylene glycol–phosphate buffer (EG–buffer, 0.01 M, pH 7.0). (—) At −130 °C in 1:1 EG–buffer. (b) Fluorescence spectra at a temperature at which the difference in solvent relaxation with EG–buffer as a solvent and the more viscous 2:1 glycerol–buffer is apparent. Temperature = −70 °C.

Since no change in intensity or shift in the band maximum of the spectrum occurred upon excitation at the two different wavelengths, it may be suggested either that efficient tyrosine to tryptophan energy transfer takes place or that the tyrosines are being efficiently quenched, perhaps by the disulfides present. In view of the size of the allergen, distances between groups within the molecule are likely to be small and lead to efficient transfer or quenching.

The fluorescence quantum yield ($q$) was determined by comparing the area under the fluorescence spectrum of Ra5 in aqueous solution (295-nm excitation) with the area under the fluorescence spectrum of an L-tryptophan solution having the same absorption at 295 nm. The $q$ (Ra5) was found to be 0.08 ± 0.01 if $q$ (Trp) is assumed to be 0.13 (Chen, 1967).

From the above data, it can be seen that the tryptophan fluorescence of Ra5 does not conveniently fit into any of the three classes proposed by Burstein et al. (1973) as it falls between classes II and III with respect to the position of the band maximum and the bandwidth at the half-maximum, whereas with respect to quantum yield, it seems to fit into class I. The fluorescence, however, may well be occurring from both tryptophans in the molecule as suggested by the phosphorescence data below, so that it would not be surprising that the emission cannot be readily classified into one form or another.

The fluorescence excitation spectrum, when monitored at 350 nm, had a peak intensity at 280 nm which directly corresponded with the $\lambda_{max}$ of the ultraviolet absorption spectrum of the protein.

*Phosphorescence of Ra5.* The phosphorescence spectrum of the allergen in rigid medium (1:1 ethylene glycol–buffer at −165 °C) appears in Figure 2. The phosphorescence excited at 280 nm is entirely due to tryptophan. The absence of a tyrosine contribution to the phosphorescence as well as the fluorescence indicates that quenching of the latter does not

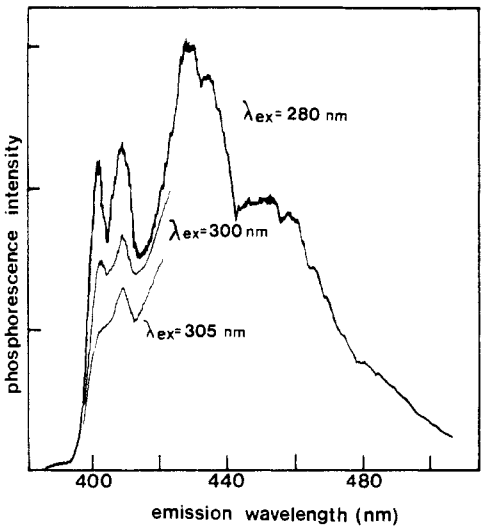


FIGURE 2: Phosphorescence spectrum of $10^{-4}$ M Ra5 in rigid 1:1 EG–buffer at −165 °C. The emission bandwidth was 2 nm. The composite nature of the spectrum is apparent in the 400–420-nm region in particular.

occur as a result of intersystem crossing to the triplet state. The tryptophan phosphorescence spectrum is clearly heterogeneous, reflecting the independent emission of two residues in distinctly different environments in the protein (Purkey & Galley, 1970). The 0–0 tryptophan phosphorescence transition is a doublet with maxima at 403 and 409.5 nm. An additional pair of wavelength maxima appear at 430 and 437 nm. Thus the overall spectrum of the protein is a superposition of a spectrum with maxima at 403 and 430 nm and a second tryptophan component possessing maxima at 409.5 and 437 nm. These wavelength maxima are essentially unchanged with glycerol–buffer (70:30 w/w) as a solvent. This evidence for contributions from distinct tryptophans in the same molecule requires that these residues be sufficiently well separated to preclude efficient triplet–triplet energy transfer between them which would tend to localize the excitation on the residue of lower energy.

Excitation at various wavelengths does not result in a high degree of selectivity of one component in the phosphorescence spectrum over the other. This is consistent with the absence of an exciting-wavelength dependence for the room-temperature fluorescence and indicates that either (a) the absorption spectra of the two tryptophan residues strongly overlap even at the red edge or (b) there is some exchange of excitation energy between the two residues at the excited singlet level. It is apparent from Figure 2 that while excitation at longer wavelengths results in preferential excitation of the 409.5-nm species, the long wavelength component cannot be selectively excited free of the other as with liver alcohol dehydrogenase (Purkey & Galley, 1970).

Typically, tryptophan residues buried in globular proteins show phosphorescence maxima at wavelengths > 410 and 438 nm for the 0–0 transition and overall wavelength maximum, respectively (Konev, 1967; Ross et al., 1980; Hershberger et al., 1980). Solvent-exposed tryptophans on the other hand display emission maxima in the 406–409-nm and 434–437-nm regions. Free tryptophan displays a 0–0 transition at 407 nm and overall wavelength maximum at 435 nm. The tryptophan residue displaying a 0–0 transition at 409.5 nm, henceforth referred to as the 409.5-nm component, lies at the upper end of the range of values observed for solvent-exposed residues and suggests the presence of protein as well as solvent interactions with the indole side chain. This tryptophan likely contributes to the solvent-relaxation red shift observed in the

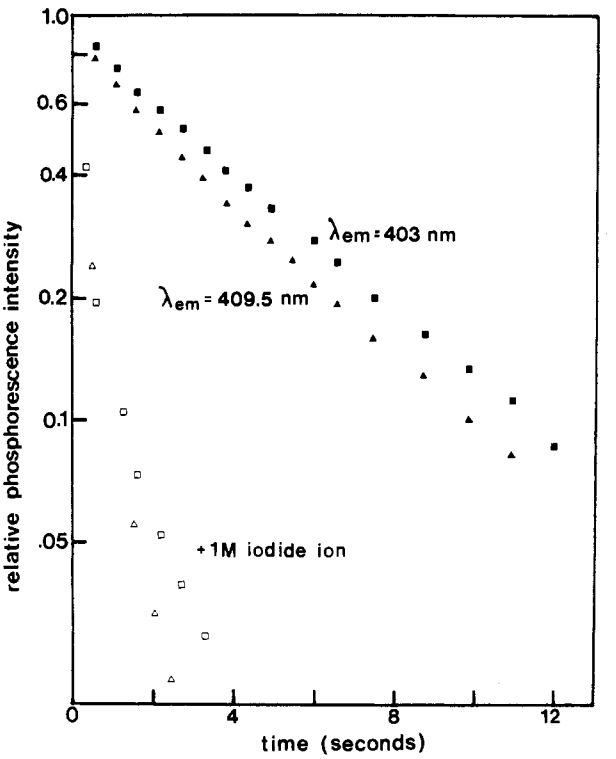


FIGURE 3: Decay in the tryptophan phosphorescence intensity of $10^{-4}$ M Ra5 in EG–buffer at −170 °C. The nonexponential decay contains a larger proportion of the short-lived species when the measurement is made at 409.5 nm. In the presence of 1 M iodide, the decay at the same two emission wavelengths is indicated with the corresponding open data points.

Table I: Components in the Nonexponential Decay of the Tryptophan Phosphorescence of Ra5 [a]

| $\lambda_{emission}$ (nm) | $P_1$ [b] | $\tau_1$ (s) | $P_2$ [b] | $\tau_2$ (s) |
|---|---|---|---|---|
| 403 | 0.86 ± 0.02 | 5.37 ± 0.06 | 0.14 ± 0.04 | 1.0 ± 0.1 |
| 409.5 | 0.67 ± 0.02 | 5.30 ± 0.07 | 0.33 ± 0.05 | 1.2 ± 0.2 |

[a] Experimental conditions: 1:1 (v/v) ethylene glycol–phosphate buffer, $\lambda_{excitation}$ 290 nm, and $T = -170$ °C. The values were determined by a least-squares fit. [b] Relative initial intensities of the slow and fast components with lifetimes $\tau_1$ and $\tau_2$, respectively.

fluorescence. The 403-nm component would appear to represent an example of an anomalous class of tryptophan residues in proteins (Galley, 1976; Hershberger et al., 1980) which due to stabilizing polar interactions with the protein, emit even more to the blue than solvent-exposed residues. In addition to the very blue emission, residues of this type have a more structured phosphorescence spectrum. It can be seen in Figure 2 that the 403-nm 0–0 transition is distinctly sharper than the one occurring at 409.5 nm.

The decay in the steady-state intensity monitored at both the 403- and 409.5-nm peaks appears in Figure 3. The intensity at 409.5 nm decays more rapidly than that at 403 nm, indicating that the lifetimes of the triplet state of the two tryptophans that make up the spectrum are not the same. The decay at each wavelength can be represented as a sum of two exponential components due to the overlapping contributions of the two tryptophans at both wavelengths. This decomposition, the results of which appear in Table I, indicates that while the 403-nm component displays a low-temperature triplet lifetime (5.2 s) which is more typical of tryptophan residues exposed to the polar solvent or buried within the polypeptide folds of globular proteins (Purkey & Galley, 1970; Longworth,

Table II: Quenching Constants and Fraction of Maximally Accessible Fluorescence of L-Tryptophan and Allergen Ra5

| | quencher[a] | | | | | |
|---|---|---|---|---|---|---|
| | cesium ion | | | iodide ion | | |
| | $K_q^{sv}$ [b] | $f_a$ (eff) [c,d] | $K_q$ (eff) [c,d] | $K_q^{sv}$ [b] | $f_a$ (eff) [c,d] | $K_q$ (eff) [c,e] |
| L-tryptophan | 2.4 ± 0.2 [f] | 0.91 ± 0.09 | 2.5 ± 0.3 | 14 ± 2 [g] | 1.03 ± 0.1 | 11 ± 1 |
| Ra5 | 1.5 ± 0.2 | 1.0 ± 0.09 | 1.5 ± 0.2 | 5.9 ± 0.6 | 0.97 ± 0.1 | 6.64 ± 0.6 |

[a] Experimental conditions: 0.01 M Tris-HCl buffer, pH 7.0, 25 ± 2 °C. Ionic strength: iodide ion experiments, 0.3 M (0–0.3 M $I^-$); cesium ion experiments, 0.6 M (0–0.6 M $Cs^+$). Ionic strength maintained by addition of sodium chloride. $\lambda_{ex}$ = 350 or 360 nm. [b] Determined from Stern-Volmer plot, $F_0/F$ vs. [$I^-$]. [c] Determined from Lehrer plot, $F_0/(F_0 - F)$ vs. 1/[quencher] (Lehrer, 1971). [d] $f_a$ (eff) = fraction of maximally accessible fluorescence. [e] $K_q$ (eff) = quenching constant of accessible fluorophores. [f] Literature: 2.2 ± 0.3 (Ostashevskii et al., 1973). [g] Literature: 12.0 (Lehrer, 1971) and 13.0 (Pownall & Smith, 1974).

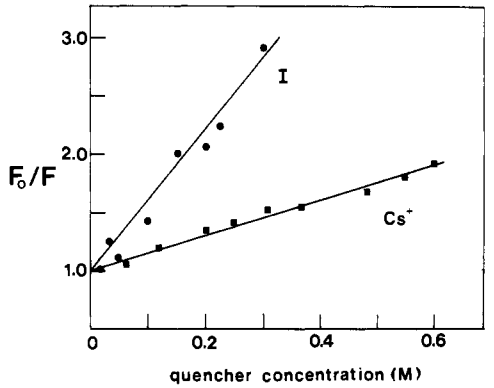


FIGURE 4: Stern-Volmer plot of iodide and cesium ion quenching of Ra5 fluorescence. Tris-HCl buffer (0.01 M), pH 7.0. Salt concentration 0.3 M. $\lambda_{ex}$ = 295 nm; $\lambda_{em}$ = 350 nm. Solid lines are those obtained from a least-squares fit of the data.

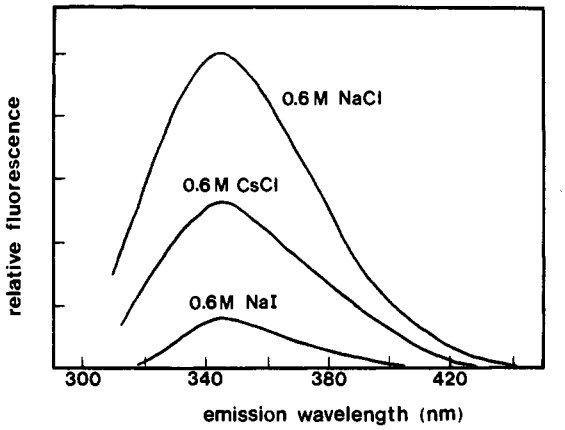


FIGURE 5: Fluorescence spectrum of Ra5 in the absence and presence of ionic quenching agents. Tris-HCl buffer (0.001 M), pH 7.0. Salt concentrations are indicated. $\lambda_{ex}$ = 295 nm.

1971), the 1.3-s lifetime of the 409.5-nm species is anomalously short, with lysozyme being one of the few proteins possessing tryptophan phosphorescence lifetimes in rigid media which are less than 3.5 s (Konev, 1967; Longworth, 1971; Domanus et al., 1980). Energy transfer or quenching due to a particular interaction of the indole side chain could give rise to shortened triplet lifetimes of this type. Triplet energy transfer can be ruled out in that it is the 409.5-nm component, which is lower in energy and would behave as the acceptor not the donor, that is short-lived. The suggestion that the anomalous tryptophan phosphorescence lifetime of lysozyme arises from the proximity of aromatic side chains to disulfide linkages (Longworth, 1971) appears attractive in the present situation in view of the four disulfide linkages in the allergen. However, no model studies exist to date to indicate that the proximity of a disulfide would result in a 1.3-s tryptophan phosphorescence lifetime.

*Tryptophan Fluorescence Quenching.* The effect on the intrinsic emitting groups within protein molecules of quenchers added to the external medium can be used to confirm the degree of accessibility of these groups to solvent. When inorganic ion quenchers having the same ionic size and yet different electrostatic charges are used, some idea of the electrostatic environment surrounding emitting groups can be obtained. Two such quenching agents are iodide ($I^-$) and cesium ions ($Cs^+$). The effect of both these ions on the intrinsic fluorescence of the allergen has been assessed and related to their effect on the fluorescence of L-tryptophan (pH 7.0). Quenching constants ($K_q$'s) were obtained from the experiments in which the concentration of the various quenching ions was varied. The results were plotted according to the method of Stern-Volmer and the results are shown in Figure 4 and Table II. Stern-Volmer plots of the data obtained with both ions were linear over the range of concentration of quencher used, indicating that quenching by specifically bound ions was not likely occurring and that there was no marked heterogeneity in the susceptibility of the tryptophan fluorescence to quenching. This was confirmed with Lehrer (1971) plots of the data, the results of which are included in Table II. With iodide ion the $K_q$ obtained from the Stern-Volmer plot was approximately 6 $M^{-1}$, indicating that iodide is about 50% as effective in quenching Ra5 fluorescence as it was in quenching L-tryptophan. The results with cesium (Table II) indicate that it was at least 70% as effective in quenching the allergen fluorescence as it was in quenching L-tryptophan. Considering the fact that due to the slow diffusion of the protein the quenching constants for the allergen are expected to be somewhat smaller than those for free tryptophan, the results are consistent with a high degree of accessibility of the tryptophans in Ra5 to the solvent. The difference in the ability of iodide and cesium to quench the fluorescence of the allergen relative to that of free tryptophan suggests that one or both of the intrinsic fluorescent groups in the allergen are surrounded by negative charges which tend to repel the iodide ion, thus decreasing its effectiveness as a quencher.

When the fluorescence emission spectrum was measured in the presence of both quenchers (Figure 5) no shift in the wavelength of maximum fluorescence occurred. This in itself would suggest that the fluorescing groups (the tryptophans) were in similar environments.

*Quenching and Enhancement of the Tryptophan Phosphorescence.* The resolution of the Ra5 phosphorescence into contributions from the individual tryptophan residues allows one to establish whether perturbations to the emission do influence both residues in the protein to the same extent or whether one is affected in preference to the other.

The phosphorescence from both tryptophans in the allergen were found to be very sensitive to thermally induced quenching. When the glycol–buffer solvents were no longer rigid at temperatures above −115 °C, the phosphorescence was quenched as with free tryptophan or solvent-exposed residues in proteins.

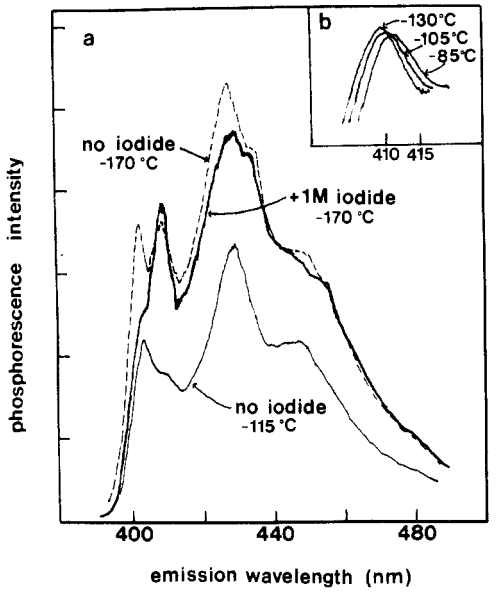


FIGURE 6: (a) Perturbations to the phosphorescence spectra of Ra5 [2:1 (w/w) glycerol–phosphate buffer] in the presence of iodide ion and with increasing temperature. (b) The solvent-relaxation red shift of the 0–0 phosphorescence maximum in the presence of 1 M iodide.

This is in contrast to the phosphorescence of buried tryptophan residues in globular proteins (Domanus et al., 1980), some of which display long-lived emission at room temperature (Saviotti & Galley, 1974; Imakubo & Kai, 1977; Kai & Imakubo, 1979). Despite the sensitivity of both tryptophans in Ra5 to triplet quenching, the two components in the spectrum were not influenced to the same extent. With increasing temperature the phosphorescence spectrum depicted in Figure 6a is altered. The 409.5-nm component is seen to be more sensitive to thermally induced quenching than the component at 403 nm. This is again in contrast to the situation in liver alcohol dehydrogenase in which it is the buried tryptophan which emits to the red, which is much more resistant to quenching (Savioetti & Galley, 1974).

For determination of whether the quenching of the fluorescence which was observed with iodide ion arose preferentially from an effect on one of the two tryptophans in the allergen, the influence of iodide on the phosphorescence spectrum of Ra5 was examined. The phosphorescence spectrum for Ra5 in 1:1 ethylene glycol–buffer at −170 °C in the presence of 1 M $I^-$ appears in Figure 6a. A high concentration of iodide was required because of the absence of diffusion in the rigid media. The peak at 409.5 nm is seen to be much larger now than the 403-nm component which appears only as a shoulder. This indicates that the 409.5-nm component is influenced by $I^-$ to a greater extent than the 403-nm component, bearing in mind that the influence of external heavy-atom perturbations on tryptophan or indole (Lessard & Durocher, 1978; Monsigny et al., 1978) as well as on aromatic hydrocarbons (McGlynn et al., 1962) is to enhance rather than to quench triplet emission, primarily by increasing intersystem crossing from the excited-singlet state. The specific enhancement in the phosphorescence of tryptophan in the presence of iodide is by a factor of 3 (W. E. Lee and W. C. Galley, unpublished results). This enhanced emission, however, is accompanied by a marked reduction in triplet lifetime. The decay of the phosphorescence at 409.5 and 403 nm was monitored, and the data appear in Figure 3. The nonexponential decays at both wavelengths are very rapid ($1/e$ times $< 0.3$ s), indicating that both tryptophans in Ra5 are perturbed by $I^-$. However, the relatively higher proportion of slow unperturbed component in the 403-nm decay confirms the spectral evidence which indicates a greater effect on the tryptophan emitting at 409.5 nm. This same pattern was observed with 2:1 (w/w) glycerol–buffer as the solvent.

The selective enhancement and shortened lifetime of the 409.5-nm component in the presence of $I^-$ allowed one to follow the temperature dependence of this spectral component in the absence of interference from the other species. The data in Figure 6b indicate that the phosphorescence maximum in 2:1 (w/w) glycerol–buffer undergoes a 2.5-nm red shift over a narrow temperature range at about −110 °C. This is the temperature region in which given the shortened lifetime of the $I^-$ perturbed emission and the solvent employed, solvent relaxation about the chromophore in the triplet state would be expected to manifest itself in the phosphorescence spectrum (Milton et al., 1978). These data confirm that the tryptophan giving rise to the 409.5-nm spectral component is indeed, in part at least, exposed to the polar solvent.

In the absence of iodide ion, the phosphorescence spectrum which at higher temperatures is dominated by the 403-nm species also appears to red shift with temperature. The spectrum, however, became very broad in this region with increasing temperature, partly due to inclusion of the 409.5-nm component, so that the evidence for solvent relaxation about this tryptophan was much less convincing.

## Discussion

The emission data in the present work indicate that the two tryptophan residues in the Ra5 ragweed are at the surface of the molecule and that both reside in unusual local environments. Whether either of these residues form an integral part of distinguishing topographical features on the surface of the protein which serve as immunodominant groups can only be answered on examination of a number of allergens and with tryptophan-modification experiments. On the basis of the present work, neither residue can be ruled out by virtue of it being hidden within the protein structure.

The surface nature of the tryptophans was established through a combination of emission properties from fluorescence and low-temperature phosphorescence experiments. The most reliable parameter in establishing solvent exposure for the tryptophans in a situation of this type was the large solvent-dependent relaxation red shift observed in the fluorescence spectrum of the allergen. The wavelength maximum per se of the fluorescence spectrum of Ra5 obtained in aqueous solution at room temperature does not occur sufficiently far to the red, and the bandwidth is not wide enough to clearly establish the emitting groups as largely solvent exposed or as Burstein's type III residues (Burstein et al., 1973). Similarly both the 403- and 409.5-nm 0–0 transitions observed in the low-temperature phosphorescence spectra are not truly characteristic of solvent-exposed tryptophan residues; the former occurs too far to the blue while the latter falls on the borderline between exposed and buried residues (Hershberger et al., 1980). On the other hand, the characteristic large blue shift that occurs in the fluorescence of the allergen as the viscosity of the solvent is increased (Eisinger & Navon, 1969) provides an unmistakable indication of an interaction of at least one of the two emitting residues with the polar solvent. By virtue of the correspondingly small shifts that occur in tryptophan phosphorescence spectra in response to solvent relaxation (Purkey & Galley, 1970) coupled with the overlapping contributions of the two components, it was difficult to confirm this assignment in the Ra5 phosphorescence. Only in the presence of iodide where the 409.5-nm component was selectively enhanced was it possible to clearly demonstrate a

solvent-relaxation red shift which confirmed the exposed nature of the tryptophan responsible for this emission.

The quenching data provide less direct evidence for the surface nature of aromatic residues in that their accessibility to solvent and solvent-mediated quenching agents is a function of protein flexibility as well as location in the protein (Weber & Lakowicz, 1973; Saviotti & Galley, 1974; Eftink & Ghiron, 1975). Nevertheless the susceptibility of the room-temperature fluorescence to iodide and cesium quenching is consistent with exposure of the residues to the solvent, and the response of the 409.5-nm component in the phosphorescence to thermally induced quenching and iodide perturbation confirm this assignment for this tryptophan. While the phosphorescence data indicate that the 403-nm emission is less susceptible to thermally induced quenching and iodide perturbation, the response to these perturbations in comparison with those of buried tryptophan residues suggests that this second residue lies essentially at the protein surface if not actually exposed to the polar solvent.

The anomalous nature of these "surface" residues in Ra5 is evident in the protein phosphorescence. While the tryptophan responsible for the 409.5-nm emission is undoubtedly exposed, the wavelength of the emission and the short triplet lifetime reveal that solvent interactions alone are not responsible for the emission properties. The proximity of this residue to a disulfide in the allergen may well be responsible both for the short-lived triplet emission and for the low quantum yield of the room-temperature fluorescence of Ra5. Similarly the very blue emission (403 nm) from the second tryptophan is inconsistent with an environment in which the residue is largely solvent exposed and suggests that this residue is stabilized by a stronger polar interaction involving perhaps a charged group within the allergen. The appropriate location of an aspartic acid or glutamic acid side chain with respect to the aromatic ring could be responsible not only for the anomalous phosphorescence spectrum but also for the selective resistance of the room-temperature fluorescence to iodide quenching as well.

An unusual feature of the present work is that the heterogeneity that is so evident in the phosphorescence of the allergen is absent in the singlet properties of the tryptophans in the molecule. The fluorescence appears homogeneous both with respect to excitation wavelength and on quenching with iodide. In addition, the relative insensitivity of the phosphorescence spectrum to exciting wavelength suggests that the absorption spectra of the two spectral species overlap much more than they do with other systems that display heterogeneous phosphorescence spectra (Purkey & Galley, 1970; Galley, 1976). The difference in local environment about the two tryptophans in the allergen appears to have a greater influence on the chromophores in their triplet rather than excited-singlet states.

The particularly characteristic emissions associated with each of the two tryptophans in ragweed allergen Ra5 serve as intrinsic probes which may be used to monitor structural modifications in the molecule. This is apparent from the data on the reduced and alkylated form of the allergen (unpublished observations). In addition, the selective modification of one of the two residues in the molecule might permit the association of the individual spectral components not only with particular residues in the amino acid sequence but also with perturbations to its allergenic activity.

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