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# Fluorescence of peptide *N*-terminal 2-oxoacyl and quinoxaline derivatives

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A peptide reacts with glyoxalic acid resulting in transamination of the *N*-terminal residue to form a 2-oxoacyl group. This further reacts with *o*-phenylenediamine, leading to a quinoxaline derivative of the original *N*-terminal amino acid, which is cleavable in mild acid [Dixon & Fields (1972) Methods Enzymol. **25**, 409–419]. The 2-oxoacyl peptides are weakly fluorescent with emission maxima around 410 nm and excitation maxima at about 320 nm, depending on the nature and length of the peptide. Formation of the quinoxaline derivative results in a marked increase of fluorescence, with emission maximum of 363 nm when excited at 303 nm. The fluorescence properties of these derivatives change with the nature and length of the peptides and are affected by the presence of organic solvents, NaCl and denaturants. It is suggested that such fluorescent derivatives could be used as probes for the study of the conformation of the *N*-terminal region of peptides and proteins.

## INTRODUCTION

Dixon and colleagues reported some years ago (Dixon, 1964; Dixon & Moret, 1965) that treatment of an oligopeptide having a free *N*-terminal amino group with glyoxalic acid in the presence of pyridine and $Cu^{2+}$ results in a transamination reaction leading to the formation of a derivative which has the first amino acid changed into a 2-oxoacyl residue. Further reaction with *o*-phenylenediamine in acid leads to the peptide being shortened by one residue from the *N*-terminus, a reaction used for structure–function studies of the *N*-terminal sequences of proteins (e.g. Van Heyningen *et al.*, 1968; Dijkstra *et al.*, 1984). We present evidence in this paper that an intermediate is formed at neutral or alkaline pH, which is probably the *N*-quinoxalin-2-yl derivative of the shortened peptide, and that this degrades in acid to release the shortened peptide.

Li *et al.* (1990) reported that a 2-oxoacyl derivative of peptides, as described by Dixon *et al.*, is weakly fluorescent with an emission peak at 410 nm. We have now shown that the quinoxaline derivatives are highly fluorescent with emission maxima around 363 nm, and it is the purpose of the present study to characterize the fluorescence properties of both derivatives and to explore the possibility that these compounds could be used as probes of the *N*-terminal region of proteins. A preliminary report of the results obtained has appeared (He & Tsou, 1991).

## MATERIALS AND METHODS

### Reagents

Glyoxalic acid was obtained from Serva. Insulin and all the oligopeptides used were obtained from Sigma. Quinine sulphate was a local guaranteed reagent. Guanidine hydrochloride was also from Sigma but was purified according to the method of Nozaki (1971) before use. KI was a local reagent of guaranteed grade and all other reagents were local products of analytical grade. These were used without further purification.

### Formation of the 2-oxoacyl derivatives

For the peptide derivatives, the method used was that described by Li *et al.* (1990). The reaction mixture contained 1 $\mu$M-peptide, 20 $\mu$M-$CaCl_2$ and 20 $\mu$M-glyoxalate and was heated in a boiling water bath for 20 min to allow the reaction to reach completion as shown by the quantitative yield of glycine from the added peptide. The unheated mixture was used as a control for fluorescence measurements. For insulin, the method used was essentially that of Dixon & Fields (1972). To 1 ml of a solution of 50 mg of insulin in dilute NaOH with a pH of 9.0 was slowly added 2 ml of a buffer containing 2 M-sodium acetate, 10 mM-acetic acid, 0.1 M-sodium glyoxalate, 2 mM-$NiSO_4$, pH 5.5, at 20 °C. The mixture was left to stand at this temperature for 30 min, being occasionally shaken, and the transaminated product formed from insulin was then freed from the reagents by passing through a Sephadex G-25 column.

### Formation of the quinoxaline derivative

To 2 ml of 0.1 M-Tris buffer, pH 9.0, containing 25 $\mu$M-2-oxoacyl peptide and 1 mM-EDTA was added 5 $\mu$l of *o*-phenylenediamine at 20 °C and the mixture was allowed to stand for 24 h in order for the reaction to reach completion. The reaction product was then directly used for fluorescence measurements with *o*-phenylenediamine as control. The quinoxaline derivative formed from insulin was isolated from the reaction mixture by column chromatography with Sephadex G-25. The quinoxaline derivative of the original *N*-terminal residue could be removed in acid as described by Dixon & Fields (1972). The reaction product of glyoxal with *o*-phenylenediamine showed the same absorption spectrum as that reported for quinoxaline (Cheeseman, 1958). For quantitative measurements of the insulin derivatives, the protein concentration was determined according to Lowry *et al.* (1951).

### Fluorescence measurements

Fluorescence measurements were made either with Hitachi MPF-4 or Hitachi 850 spectrofluorimeters. Determinations of the fluorescence quantum yields were carried out at 25 °C with quinine sulphate as a standard (Parker & Rees, 1960; Chen, 1967; Scott *et al.*, 1970). Quenching with KI was measured at 25 °C in phosphate buffer, pH 7.0, containing 1 mM-EDTA. In all cases, the absorbance of the sample at the excitation wavelength was kept at less than 0.03.

## RESULTS

### Fluorescence of the 2-oxoacyl derivative

Li *et al.* (1990) reported that the transaminated derivatives

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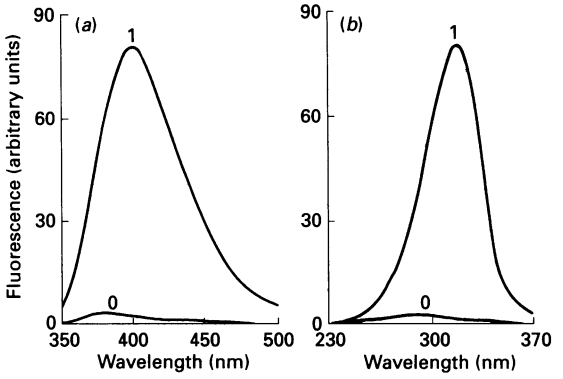


**Fig. 1. Fluorescence spectra of glyoxyloyl glycine**

Experimental conditions were as follows: 25 $\mu$M-glyoxyloylglycine in 0.1 M-phosphate buffer, pH 7.0, at 20 °C. (*a*) Emission spectrum with excitation at 312 nm and (*b*) excitation spectrum with emission at 405 nm. The control sample contained the same concentration of glyoxalic acid and diglycine before transamination. Sample, line 1; control, line 0.

**Table 1. Fluorescence properties of the 2-oxoacyl derivatives of oligopeptides**

Experimental conditions were as for Fig. 1. For the measurement of the 2-oxoacyl derivative of insulin, excess reagents were removed by gel filtration through Sephadex G-25.

| Parent compound | Excitation maximum (nm) | Emission maximum (nm) | Quantum yield |
|---|---|---|---|
| Gly | 345 | 420 | < 0.001 |
| $Gly_2$ | 312 | 396 | 0.025 |
| $Gly_3$ | 314 | 403 | 0.024 |
| $Gly_4$ | 318 | 407 | 0.022 |
| $Gly_5$ | 318 | 408 | 0.022 |
| $Gly_6$ | 318 | 408 | 0.022 |
| $Ala_2$ | 333 | 420 | 0.028 |
| $Ala_3$ | 330 | 418 | 0.028 |
| $Ala_4$ | 320 | 410 | 0.026 |
| $Ala_5$ | 318 | 405 | 0.024 |
| $Ala_6$ | 318 | 405 | 0.024 |
| Insulin | 318 | 405 | 0.025 |

**Table 2. Fluorescence properties of the quinoxaline derivatives of oligopeptides**

Derivatives were measured directly in 0.1 M-$NaHCO_3$ buffer, pH 9.0, containing 0.02 M-NaCl, at 20 °C, except for the quinoxalinyl derivative of insulin from which reagents were removed in 0.1 M-Tris buffer, pH 9.0, by gel filtration through Sephadex G-25.

| Parent compound | Excitation maximum (nm) | Emission maximum (nm) | Quantum yield |
|---|---|---|---|
| Glycine | 293 | 362 | 0.14 |
| Insulin | 318 | 353 | 0.12 |
| Glycine oligopeptides | 293 | 362 | 0.14 |
| Alanine oligopeptides | 305 | 360 | 0.14 |

formed from insulin and ribonuclease are weakly fluorescent. Fig. 1 shows the excitation and emission spectra of $Gly_2$ after transamination. The unheated control shows no fluorescence. Table 1 lists the excitation and emission maxima and the quantum

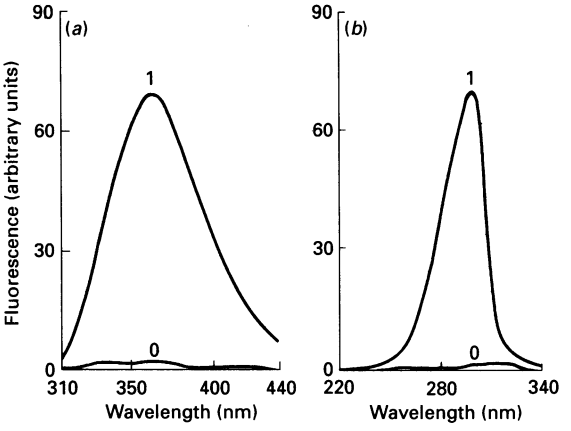


**Fig. 2. Fluorescence spectra of the quinoxaline derivative formed from tetraglycine**

Experimental conditions were as follows: 1 $\mu$M-quinoxaline derivative, in 0.1 M-$NaHCO_3$ buffer, pH 9.0, containing 0.02 M-NaCl at 20 °C. (*a*) Emission spectrum with excitation at 293 nm and (*b*) excitation spectrum with emission at 362 nm. The control sample contained the same concentration of *o*-phenylenediamine and diglycine before transamination. Sample, line 1; control, line 0.

yields of a number of 2-oxoacyl derivatives of homo-Gly and homo-Ala peptides examined. Glyoxalate itself is very weakly fluorescent, with a quantum yield less than one-twentieth that of the 2-oxoacyl derivatives of the peptides, showing that the presence of the peptide bond with the second residue has a profound effect on the properties of the fluorophore, whereas from the third residue on, a further increase in the chain length has only a minor effect on the quantum yield and the excitation as well as the emission maxima. Insulin reacted similarly with glyoxalate to give a fluorescent derivative and the relevant results are also included in Table 1 for comparison. The 2-oxoacyl derivatives are stable at 4 °C for at least 3 months.

### Formation of the quinoxaline derivative

It is known that *o*-phenylenediamine reacts with 2-oxo acids to form 2-hydroxyquinoxalines (Hickinbottom, 1954). In the present study, it was found that reaction products of 2-oxoacyl peptides with *o*-phenylenediamine have absorption spectra very similar to those of the corresponding quinoxaline derivatives (Cheeseman, 1958). The quinoxalinyl peptides are strongly fluorescent compared with the 2-oxoacyl peptides with markedly greater quantum yields (Table 2). The fluorescence excitation and emission spectra are shown in Fig. 2 and the respective maxima are around 300 nm and 360 nm. Unlike the 2-oxoacyl derivatives, the fluorescence properties of the quinoxaline derivatives of oligopeptides show little change with the length and nature of the peptides, except those for the di-2-oxoacyl derivative of insulin which are slightly different. The emission maxima and the fluorescence quantum yields of 3-hydroxyquinoxaline and the quinoxalinyl peptides are summarized in Table 2. Treatment of the quinoxaline derivative with acid leads to the removal of the quinoxalinyl group from the peptide and the removal of one residue from the *N*-terminus of the original peptide, as shown by Dixon & Fields (1972). This has been shown in the present study for insulin by the separation of the rest of the insulin molecule from the fluorescent quinoxaline derivative on Sephadex G-25 (results not shown).

### Energy transfer with an aromatic residue

Energy transfer has been used frequently as a spectroscopic measure for the study of distances between fluorophores within

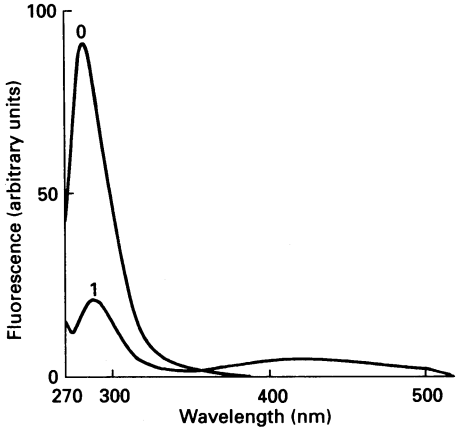


**Fig. 3. Energy transfer between the *N*-terminal glyoxyloyl group with an internal phenylalanine residue**

A solution of Gly-Gly-L-Phe (0.5 mM), glyoxalate (5 mM) and $CaCl_2$ (2.5 mM) was adjusted to pH 9.0 with KOH and heated for 25 min in a boiling water bath to form the fluorescent glyoxyloyl derivative. An unheated sample was used as the control and the fluorescence emission spectrum (1) was compared with the emission spectrum of the transaminated sample (2) with an excitation wavelength of 265 nm.

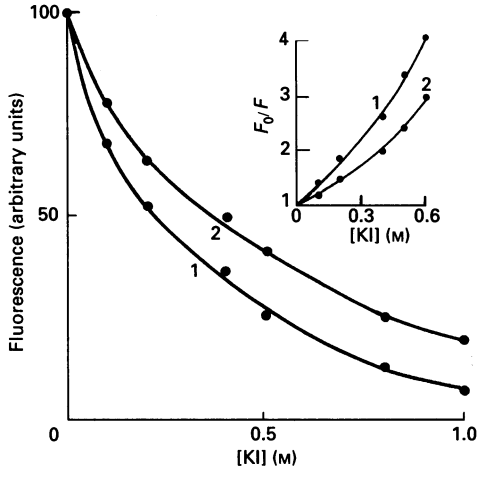


**Fig. 4. Fluorescence quenching of glyoxyloyl-Gly-Gly and glyoxyloyl-Gly-L-Leu by KI**

Experimental conditions were as follows: 25 $\mu$M-glyoxyloyl-peptides in 0.1 M-phosphate buffer, pH 7.0, containing the required concentrations of KI at 20 °C. Fluorescence emission spectra were measured after 30 min incubation with excitation at 312 nm. The control sample contained the same concentration of glyoxalic acid and the peptides before transamination. The inset shows the corresponding Stern–Vollmer plots. Curves 1 and 2 are for glyoxyloyl Gly-L-Leu and Gly-Gly respectively.

a molecule (Stryer, 1978; Searcy *et al.*, 1989). The fluorescence emission spectra of Gly-Gly-L-Phe before and after the formation of the fluorescent 2-oxoacyl derivative are shown in Fig. 3. Fluorescence of the phenylalanine residue has an emission spectrum which overlaps with the excitation spectrum (Fig. 1*b*) of the 2-oxoacyl derivative. As is evident from Fig. 3, the emission intensity of 2-oxoacyl-Gly-L-Phe decreases to a very marked extent compared with that of Gly-Gly-L-Phe, due undoubtedly to energy transfer between the phenylalanine residue and the 2-oxoacyl fluorophore, which are in close proximity in

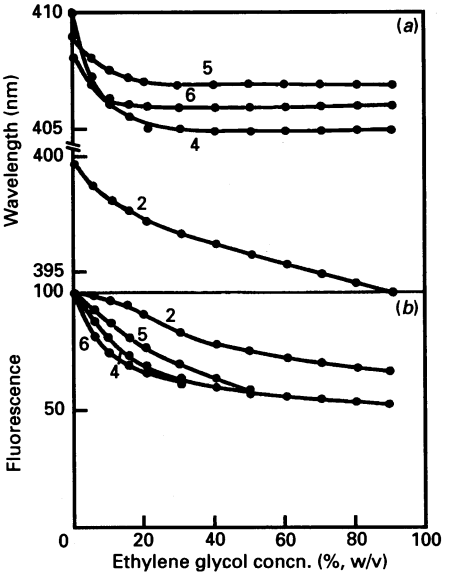


**Fig. 5. Effect of ethylene glycol on the fluorescence properties of glyoxyloyl oligoglycines**

Experimental conditions were as follows: 25 $\mu$M glyoxyloyl peptides in 0.1 M-phosphate buffer, pH 7.0, containing the indicated amounts of ethylene glycol. The control sample contained the same concentration of glyoxalic acid and the peptides before transamination. The number indicates the number of Gly residues in the peptides before transamination with glyoxalic acid. (*a*) Emission maxima and (*b*) emission intensities. The excitation wavelength for each 2-oxaloyl peptide was selected according to the excitation maxima listed in Table 1.

this tripeptide. Re-absorption by the 2-oxoacyl chromophore could account for only a minor proportion of the fluorescence emission of the phenylalanine residue.

### Quenching by iodide

KI is well known as a fluorescence quenching reagent (Lakowicz, 1983). Fig. 4 shows the quenching of the fluorescence of 2-oxoacyl-Gly-Gly, and 2-oxoacyl-Gly-L-Leu by increasing concentrations of iodide, and the inset shows the Stern–Vollmer plots. It is to be noted that for the above peptides (and others; results not shown) the Stern–Vollmer plots tend to bend upwards at the higher KI concentrations, indicating the presence of both dynamic and static types of quenching (Lakowicz, 1983). The two 2-oxoacyl-peptides are similarly affected, with the emission of the peptide containing L-leucine being slightly more sensitive to the quenching of KI. The quenching is accompanied by red shifts of the emission maxima from 402 nm to 415 mm for the peptide containing L-leucine. Comparison of 2-oxoacyl-glycine peptides of different lengths shows that the fluorescence emission intensities of the oligomers of chain lengths from 2 to 6 residues are similarly affected by KI. On the other hand, although the shorter peptides have emission maxima at shorter wavelengths, the emission maxima tend to increase more markedly with increasing concentrations of KI, so that at high KI concentration all the oligo-Gly peptides have similar emission maxima at about 415 nm (results not shown).

### Effect of ethylene glycol

As with most fluorophores (Brand & Witholt, 1967; Lakowicz, 1983) the fluorescence properties of both the 2-oxoacyl and the quinoxalinyl derivatives are sensitive to organic solvents. Fig. 5 shows the decrease in emission intensities together with a blue shift of 5 nm in the emission maximum of 2-oxoacylglycine at

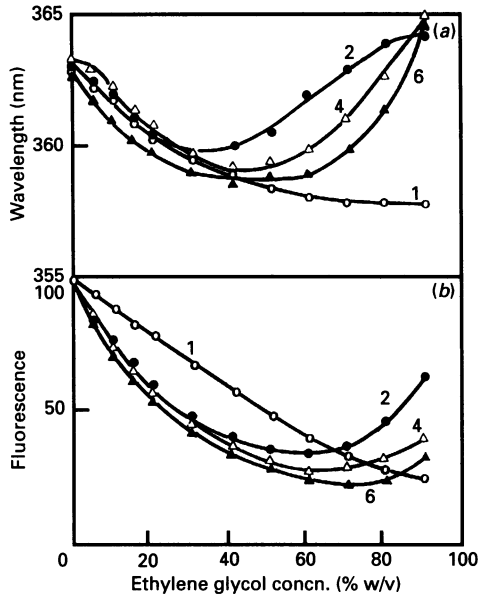


**Fig. 6. Effects of ethylene glycol on the fluorescence properties of the quinoxaline derivatives formed from oligoglycines**

Experimental conditions were as follows: 2.5 $\mu$M-quinoxaline peptides in 0.1 M-phosphate buffer, pH 7.0, containing the required concentrations of ethylene glycol at 20 °C. Fluorescence emission spectra were measured with excitation at 293 nm. Curves are numbered with the number of Gly residues in the peptides used before transamination. (*a*) Emission maxima and (*b*) emission intensities.

increasing ethylene glycol concentrations. Similar results were obtained for other peptides although the extents of emission decrease and the degree of blue shift were different for different peptides. The effect of ethylene glycol on the fluorescence properties of the quinoxaline derivatives is more variable in its dependence on the length and nature of the peptides involved. Fig. 6 shows the effects of ethylene glycol on the emission intensities and maxima of the derivatives formed from glycine and di-, tetra- and hexa-glycine. It can be seen that both the emission intensities and maxima decrease with increasing concentrations of ethylene glycol. However, the presence of a peptide chain has a marked effect, as both the emission intensities and maxima increase again, after an initial decrease, at higher ethylene glycol concentrations. On the other hand, red shift of the emission maxima of 2-oxoacylalanine with increasing ethylene glycol concentrations may be due to the side groups of the peptide being different from those of 2-oxoacylglycine.

### Effects of NaCl and denaturants

The fluorescence properties of both the 2-oxoacyl and the quinoxaline fluorophores are also sensitive to NaCl as well as denaturants, as are most fluorophores (Lakowicz, 1983; Yao *et al.*, 1984). For 2-oxoacyl derivatives, an increase in NaCl concentrations leads to an increase in emission intensity and to a slight blue shift; whereas for the quinoxalines a marked decrease in emission intensity accompanies the blue shift of the maxima. The effects of guanidine hydrochloride on the fluorescence properties of the 2-oxoacyl derivatives are more complicated, depending on the nature and length of the peptides involved. In general, the emission intensity increases with increasing guanidine hydrochloride concentrations, with very little change to the maxima. On the other hand, guanidine hydrochloride decreases the intensity of the emission of quinoxaline derivatives (results not shown).

## DISCUSSION

The fact that the 2-oxoacyl and quinoxaline derivatives of peptides are both fluorescent is interesting because of their possible use as probes for detecting *N*-terminal regions of proteins and peptides. In this connection, both the 2-oxacyl and the quinoxalinyl derivatives are formed under mild conditions and are relatively stable at neutral pH. Even though the quantum yields of the 2-oxoacyl derivatives are low, the 2-oxoacyl group has the advantage of being a very small fluorophore and consequently is less likely to perturb the fine structures of the *N*-terminal regions of proteins than most commonly employed fluorescent probes. The energy transfer with an internal aromatic residue of the peptide chain has been shown to occur in the present study and the sensitivity of the fluorophore to the solvent polarity, salts, denaturants and chemical quenchers are all useful propertes for the use of these groups as probes.

As shown by Dixon & Fields (1972), the quinoxalinyl residue formed at the *N*-terminal residue can be easily cleaved at acid pH. This reaction removes the first residue and makes the second residue of the original chain the new *N*-terminus. The finding that the quinoxaline derivatives of amino acids are fluorescent and the substituent at the 3 position of the 2-hydroxyquinoxaline is the side chain of the amino acid from which it is derived, makes this a possible alternative method for peptide sequence determination to the commonly employed Edman degradation (Edman & Henschen, 1975; Walsh *et al.*, 1981). The low rates of formation of the quinoxalinyl derivative and the cleavage reaction are definite disadvantages. However, the reaction can be accelerated by temperature or other factors which are to be further explored.

It is remarkable that the fluorescence properties as well as the effects of quenchers, denaturants and organic solvents on both the 2-oxoacyl and the quinoxalinyl fluorophores are profoundly affected by the length and the composition of the oligopeptides from which they are derived. It is now known that short peptides have their own conformations (Hruby *et al.*, 1985; Wright *et al.*, 1988) even though the conformation of a peptide is flexible and very much dependent on the environment. The facts that the fluorescence derivatives of oligopeptides with different chain lengths and compositions behave differently towards ethylene glycol, NaCl and guanidine hydrochloride suggest that these peptides are somewhat different from one another not only in conformation but also in their stability. Therefore The 2-oxoacyl and quinoxalinyl fluorophores could be useful probes for the study of the conformations and stabilities of peptides.

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