THE LABELLING OF PEPTIDES
AND PROTEINS WITH A NEW FLUOROPHORE:
N-ACETYLDL-(p-\(N',N'\)-DIMETHYLAMINO)PHENYLALANINE

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Received 17 July, 1986

It was found that N-acetyl-dL-(p-\(N',N'\)-dimethylamino)phenylalanine,
in the form of azlactone, can be introduced into a peptide or protein
molecule as a new convenient fluorescent marker. A fluorophore of similar
properties: N-acetyl-(p-amino)phenylalanine can be introduced into a peptide
chain by the reaction with the azlactone of N-acetyl-(p-nitro)phenylalanine
followed by reduction of nitro group to amino group. This method, however,
cannot be applied to proteins.

The spectrofluorimetric technique based on Föster's theory of fluorescence
energy transfer [1] permits direct measurement of intramolecular distance
between two fluorophores in solution. One of the advantages of this method
is the possibility of carrying out measurements at very low concentration of
the solutions (10\(^{-5}\) mole/dm\(^3\)). Recently this method found wide application
in studies on the conformation of peptides. In 1981 Jankowski et al. [2]
proposed a new fluorescent marker: \(\text{p-aminophenylalanine}\)\(^1\) which together
with tyrosine was used for determining intramolecular distances in peptides.

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\(^*\) This work was supported by the Polish Academy of Sciences, grant CPBR 3.13.4, 3.3.
\(^1\) Abbreviations used: APhe, dL-(p-amino)phenylalanine; DMAPhe, dL-(p-dimethylamino)
phenylalanine; (NO\(_2\))Phe, dL-(p-nitro)phenylalanine; Ac-DMAPhe, N-acetyl-dL-(p-\(N',N'\)-dimethyl
amino)phenylalanine; Ac(NO\(_2\))Phe, N-acetyl-(p-nitro)phenylalanine; Ac-APhe-Gly-Gly-OEt, N-acetyl-dL-(p
amino)phenylalaninylglycylglycine ethyl ester; Ac-DMAPhe-Gly-Gly-OEt, N-acetyl-dL-(N',N'-
dimethylamino)phenylalaninylglycylglycine ethyl ester; Ac(NO\(_2\))Phe-Gly-Gly-OEt, N-acetyl-dL-(p-nitro)
phenylalaninylglycylglycine ethyl ester; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea.
As regards its fluorescence quantum yield this marker is similar to tryptophan, however the possibility of quenching of its fluorescence by acidification of the sample is of advantage. This simplifies separation of emission bands and permits determination of the fluorophore fluorescence quantum yield in the absence of energy transfer. p-Aminophenylalanine was successfully used in the conformational studies of an enkephalin analogue [2].

The aim of the present work was to develop a method of introducing APhe or a marker of similar properties into a protein molecule. The required properties of the marker were: (i) the possibility of quenching the fluorescence by acidification of the solution; (ii) large critical Förster's distance, and (iii) ready binding of the marker to a protein molecule. In addition to APhe, p-(N',N'-dimethylamino)phenylalanine was also examined. The latter compound has not been used as a fluorescent marker before.

The method developed to introduce the APhe group consisted of the reaction of a peptide with azlactone of N-acetyl-DL-(p-nitro)phenylalanine followed by catalytic reduction of the nitro group with hydrogen.

The DMAPhe group can be introduced into a protein or peptide by direct action of the azlactone of N-acetyl-(p-N',N'-dimethylamino)phenylalanine. To determine the conditions of the reaction between azlactones and amino groups of peptides, model reactions between the azlactone and diglycine ethyl ester or lysozyme were studied.

The applicability of azlactones as protein-modifying reagents has been earlier demonstrated [3, 4, 5] and the spectroscopic properties of APhe were thoroughly investigated [2, 6].

In the present work, the spectral characteristics of DMAPhe have been determined to evaluate the applicability of this group as a fluorescent marker.

**MATERIALS AND METHODS**

**Reagents.** Lysozyme (a product of Carl Both, Karlsruhe, F.R.G.) was used without further purification. In the fluorescence quantum yield measurement APhe (Sigma, Switzerland) was used as a standard. DL-Phenylalanine was purchased from Reanal (Hungary), diglycine ethyl ester hydrochloride was prepared according to Schot et al. [7]; DL-(p-nitro)phenylalanine was obtained according to Bergel & Stock [8].

**N-Acetyl-DL-(p-nitro)phenylalanine.** DL-(p-Nitro)phenylalanine (8.8 g, 34.9 mmol) was dissolved in 50 cm³ of 10% NaOH solution and then 19 cm³ of acetic anhydride (threefold molar excess) was added under vigorous stirring. After 30 min the solution was brought to pH 1 using concentrated HCl. The precipitate was recrystallized from water. Yield 6.65 g (63%); m.p. 192 - 194°C. Elemental analysis (C₁₁H₁₂N₂O₃): 11.2% N found, 11.1% N calc.; IR (KBr, cm⁻¹): 3320, 2930, 1720, 1605, 1515, 1340, 1190, 850.
N-Acetyl-DL-(p-N',N'-dimethylamino)phenylalanine. Ac(NO₂)Phe (7.68 g, 30.4 mmol) was dissolved in 300 cm³ of methanol and then 30 cm³ of 40% formaldehyde was added. After introducing the catalyst (5% Pd/C) the reduction was carried out with hydrogen at atmospheric pressure until no substrate could be detected chromatographically (about 7 h). After filtering off the catalyst the solution was evaporated to dryness under reduced pressure. The residue was recrystallized from methanol. Yield 5.0 g (66%); m.p. 200 - 202°C. Elemental analysis (C₁₃H₁₈N₂O₃): 11.2% N found, 11.2% N calc.; IR (KBr, cm⁻¹): 3340, 2825, 1710, 1620, 1560, 1240, 812.

Azlactone of N-acetyl-DL-(p-nitro)phenylalanine. To 0.54 g (2.14 mmol) of Ac(NO₂)Phe suspended in 15 cm³ of ethyl acetate 0.442 g (2.14 mmol) of dicyclohexylcarbodiimide was added. The mixture was stirred for 2 h. The precipitate of dicyclohexylurea was filtered off and the filtrate was evaporated at reduced pressure. The single product formed was extracted with boiling isooctane. Azlactone crystallized on cooling. Yield 0.15 g (33.3%); m.p. 69.5 - 70°C. Elemental analysis (C₁₁H₁₀N₂O₄): 11.7% N found, 12.0% N calc.; IR (film of uncryrstallized product, cm⁻¹): 2930, 1820, 1680, 1600, 1515, 1230, 900, 855, 700. ¹H NMR (CDCl₃, int. TMS) 2.13 (doublet 3H), 3.30 (octet 2H), 4.13 (quartet 1H), 7.40 - 8.25 (quartet 4H). In the spectrum a homoallylic coupling (⁻J = 2 Hz) between H² and CH₃ group, analogous to that found in the spectra of other azlactones of aromatic N-acetyl amino acids [9], is clearly visible.

Azlactone of N-acetyl-DL-(p-N',N'-dimethylamino)phenylalanine. To 1.06 g (4.23 mmol) of Ac-DMAPhe suspended in 15 cm³ of ethyl acetate, 0.874 g (4.23 mmol) of DCC was added. The mixture was stirred for 2 h at room temperature. The DCU precipitate was filtered off and the filtrate was evaporated under reduced pressure. The resulting oil was dissolved in 3 cm³ of ethyl acetate, the remaining DCU filtered off and the solvent evaporated again; 0.82 g (83.6%) of azlactone in the form of very unstable yellow oil was obtained. The product transformed back to Ac-DMAPhe when left in air. IR (film, cm⁻¹): 1820, 1680, 1620, 1525, 1230, 820; ¹H NMR (CDCl₃, int. TMS): 1.94 (doublet 3H), 2.79 (singlet 6H), 2.99 (octet 2H), 4.29 (multiplet 1H), 6.50 - 7.07 (quartet 4H). As before, the homoallylic coupling (⁻J = 2.0 Hz) was observed in the spectrum.

Ethyl ester of N-acetyl-DL-(p-nitro)phenylalanylglycylglycine. Hydrochloride of glycylglycine ethyl ester (0.4 g, 2.03 mmol) was dissolved in 25 cm³ of Clark-Lubs phosphate buffer which was a mixture of 50 cm³ of 0.1 M monopotassium phosphate and 39 cm³ of 0.1 M NaOH. During the reaction pH of the solution was adjusted to 7.4 with 0.1 M NaOH. Then 0.5 g (2.13 mmol) of azlactone of Ac(NO₂)Phe in 2 cm³ of dioxane was introduced dropwise under vigorous stirring. At the beginning, some opalescence appeared which vanished after 1 h. The solution was left overnight in a refrigerator; 0.7 g of crystalline product was obtained. Yield 82.4%; m.p.
187°C. Elemental analysis (C_{17}H_{22}N_{4}O_{7}): 14.4% N found, 14.2% N calc.; IR (KBr, cm\(^{-1}\)): 3270, 3070, 2980, 1750, 1660, 1570, 1550, 1515, 1345, 1215, 1110, 1025, 885, 855; UV (methanol), \(\varepsilon (\lambda_{\text{nm}})\): 0.997 \times 10^{4} (271.7), 1.90 \times 10^{4} (202.4).

Ethyl ester of N-acetyl-DL-(p-amino)phenylalanl glycylglycine. Ethyl ester of N-acetyl-DL-(p-nitro)phenylalanl glycylglycine (0.369 g, 0.94 mmol) suspended in 30 ml of methanol to which the catalyst (5% Pd/C) was added, was reduced in a stream of hydrogen at room temperature for 5 h. The substrate dissolved as the reaction proceeded. After filtering off the catalyst, the solution was evaporated under reduced pressure. The non-crystalline product obtained was purified by recrystallization from the ethyl acetate - isooctane mixture. Yield 0.110 g (35%), m.p. 155 - 157°C. Elemental analysis (C_{17}H_{24}N_{4}O_{7}): 15.6% N found, 15.4% N calc.; IR (KBr, cm\(^{-1}\)): 3425, 3360, 3070, 2980, 2850, 1750, 1650, 1550, 1250, 1200, 1035, 815; UV (methanol), \(\varepsilon (\lambda_{\text{nm}})\): 1.21 \times 10^{3} (292.4), 0.879 \times 10^{4} (242.1), 3.19 \times 10^{4} (202.4).

Ethyl ester of N-acetyl-DL-(p-N',N'-dimethylamino)phenylalanl glycylglycine. A solution of 0.1 g (0.43 mmol) of azlactone of Ac-DMA Phe in 0.5 cm\(^3\) of dioxane was combined with a solution of 0.10 g (0.51 mmol) of hydrochloride of diglycine ethyl ester in 5 cm\(^3\) of Clark-Lubs buffer (pH 8), stirred for 40 min and left overnight. The solution was then extracted five times with 3 ml portions of ethyl acetate and the combined extracts were washed with Na\(_2\)CO\(_3\) solution and with water, then dried with anhydrous MgSO\(_4\). After evaporating the solvent an oil was obtained which was crystallized from 1 ml of ethyl acetate to yield 0.051 g (29.9%). M.p. 142 - 144°C. Elemental analysis (C_{19}H_{28}N_{4}O_{3}): 13.8% N found, 14.2% N calc.; IR (KBr, cm\(^{-1}\)): 3290, 3070, 2980, 2800, 1750, 1630, 1520, 1350, 1200, 1025; UV (methanol), \(\varepsilon (\lambda_{\text{nm}})\): 1.53 \times 10^{3} (303.0), 1.39 \times 10^{4} (259.7), 1.93 \times 10^{4} (205.1).

Modification of lysozyme with azlactones. The modification of lysozyme was carried out in Clark-Lubs phosphate buffer. For azlactone of Ac-DMA Phe pH of the solution was 7.4, 6.5 or 6.4, whereas for azlactone of Ac(NO\(_2\)) Phe it was 7.4. Lysozyme (80 mg) was dissolved in 10 cm\(^3\) of the buffer; 13 mg of an azlactone dissolved in 1.0 cm\(^3\) of dioxane was added to the lysozyme solution and the mixture left for 18 h. Then 2 cm\(^3\) of the reaction mixture was introduced to the column filled with Sephadex G-25 (length 50 cm, volume 120 cm\(^3\)) and eluted with 0.001 M HCl. The protein content in the eluate was monitored by measuring the absorbance at 280 nm. The fractions rich in lysozyme were collected and rechromatographed under the same conditions to obtain a single elution peak. After rechromatography the spectrum of the modified lysozyme remained unchanged.

Number of marker molecules per lysozyme molecule. The number of marker molecules bound to one lysozyme molecule was determined spectrophotometrically by the method of Jaffé & Orchin [10]. The measurements were made in aqueous solutions at pH 8. In the case of proteins labelled with
Ac-DMAPhe the measurements were performed at 247 and 291 nm. In the calculations the following values of the molar absorption coefficients were used \([\varepsilon(\lambda_{nm})] = 2.08 \times 10^4 \text{ (247)}, 3.44 \times 10^4 \text{ (291)}\) for lysozyme and 0.99 \times 10^4 \text{ (247), 1.60 \times 10^3 \text{ (291)}}\) for Ac-DMAPhe. For the proteins labelled with the Ac(NO\(_2\))Phe group the wavelengths used were 220 and 282 nm. The molar absorption coefficients were \([\varepsilon(\lambda_{nm})] = 2.70 \times 10^4 \text{ (220), 6.01 \times 10^3 \text{ (182)}}\) for lysozyme and 3.50 \times 10^3 \text{ (220), 8.86 \times 10^3 \text{ (282)}}\) for Ac(NO\(_2\))Phe. The absorption coefficients were determined in aqueous solutions at pH 8.

Attempts at reducing nitro groups of the lysozyme modified with azlactone of N-acetyl-dl-(p-nitro)phenylalanine. Lysozyme modified with azlactone of Ac(NO\(_2\))Phe was dissolved in 0.001 M HCl and exposed to hydrogen at atmospheric pressure in the presence of Pd/C as catalyst. The concentration of lysozyme was 10\(^{-4}\) mol/dm\(^3\). The reaction was followed spectrophotometrically but no changes in the UV spectra of the reaction mixture were observed. Another sample was treated with NaHSO\(_3\) in Clark-Lubs phosphate buffer, pH 8. A 24-fold excess of NaHSO\(_3\) was used with respect to the number of nitro groups. The concentration of lysozyme was 10\(^{-3}\) M. The reaction products were analysed spectrophotometrically after removing NaHSO\(_3\) on a Sephadex G-25 column. No changes in the UV spectrum of the modified lysozyme were found after the reaction.

Spectroscopic measurements. The fluorescence spectra were recorded with a Perkin-Elmer 204 spectrometer equipped with a Perkin-Elmer 56 recorder; UV and IR spectra were recorded with Specord UV-VIS and Specord 75 IR spectrometers, respectively. \(^1\)H NMR spectra were recorded with a Tesla 100 MHz apparatus. The quantum yield of fluorescence was determined using p-aminophenylalanine as a standard (\(\Phi = 0.086\)). The relationship \(\Phi = F_xE_x/N_x/F_sE_s\) was used, where \(E_x\) and \(E_s\) are absorbances at 280 nm of a substance and the standard, respectively, and \(F_x\) and \(F_s\) are the fluorescence intensities, at the maximum of the emission band, of a substance and the standard, respectively. The critical Förster's distance was determined according to Jankowski et al. [2].

Determination of \(pK_a\). \(pK_a\) values for amino group in APhe and for \(N',N'-\text{dimethylamino} \) group in DMAPhe were determined from the plots of absorbance at the maximum of \(^1\)L\(_a\) band (236 and 247 nm) as a function of pH. The measurements were made using Ac-DMAPhe and Ac-APhe-Gly-Gly-OEt at a concentration of 10\(^{-4}\) mol/dm\(^3\).

RESULTS AND DISCUSSION

For modification of lysozyme and model reaction with diglycine ethyl ester, azlactones of N-acetyl-dl-(p-nitro)phenylalanine and N-acetyl-dl-(p-N', N'-dimethylamino)phenylalanine (for chemical formulae see Scheme 1) were
used. The former azlactone reacts with diglycine ethyl ester with good yield, forming \( N\)-acetyl-DL-(p-nitro)phenylalanylglucylglycine ethyl ester, the hydrogenation of which in the presence of Pd proceeds quantitatively to yield \( N\)-acetyl-DL-(p-amino)phenylalanylglucylglycine ethyl ester. The reaction of diglycine ethyl ester with azlactone of \( N\)-acetyl-DL-(N',N'-dimethylamino)phenylalanine yields \( N\)-acetyl-DL-(N',N'-dimethylamino)phenylalanylglucylglycine ethyl ester. The structure of \( Ac(NO_2)\)Phe-Gly-Gly-OEt, Ac-APhe-Gly-Gly-OEt and Ac-DMAPhe-Gly-Gly-OEt was confirmed by IR and UV spectra analyses and by elemental analysis.

The modification of lysozyme with azlactones of Ac(\( NO_2 \))Phe and of Ac-DMAPhe was carried out at pH 7.4. Under these conditions in both cases 2.3 mole of (\( NO_3 \))Phe per mole of lysozyme was found. Attempts at reduction with hydrogen of nitro groups built into lysozyme, in the presence of Pd or with sodium hydrosulphite gave a negative result, as evidenced by the lack of any changes in the UV spectra during the experiment.

The modification of lysozyme using azlactone of Ac-DMAPhe was carried out at pH 6.0, 6.5 and 7.4. A clear dependence of the degree of modification on pH was observed (Table 1). At lower pH values (6.0 - 6.5) the modification concerned mainly N-terminal \( \alpha \)-amino groups of lysozyme. With increasing pH the fraction of modified \( \varepsilon \)-amino groups of lysozyme increased. The character of the relationship between the amount of the marker bound to lysozyme and pH does not prove that the reaction is selective, and

<table>
<thead>
<tr>
<th>pH</th>
<th>Ac-DMA-Phe/lysozyme</th>
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<tr>
<td>6.0</td>
<td>0.98</td>
</tr>
<tr>
<td>6.5</td>
<td>1.25</td>
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<tr>
<td>7.4</td>
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Table 1

The number of Ac-DMAPhe groups introduced into a lysozyme molecule as a function of pH

even at pH 6, in addition to \( \alpha \)-amino groups, \( \varepsilon \)-amino groups of lysine become modified. Since one lysozyme molecule contains 6 lysine residues, it can bind up to 7 Ac-DMAPhe groups. Within the range of pH studied the highest content of Ac-DMAPhe (2.1 groups) was found in the lysozyme sample modified at pH 7.4. Changes in the lysozyme UV spectrum after modification are shown in Fig. 1. For the lysozyme sample containing covalently bound marker dissolved in solution of pH 3, when the marker itself does not fluoresce, the maximum of the fluorescence spectrum was at 335 nm. This value is consistent with that reported by Jankowski &
Siemion [11] for unmodified lysozyme, which indicates that the spatial structure of lysozyme is not significantly changed by modification.

In order to assess the applicability of Ac-DMAPhe moiety as a fluorescent marker, \( pK_a \), fluorescence quantum yield and location of the UV absorption and emission bands were determined. The measurements were made for Ac-DMAPhe aqueous solutions at pH 7.4 (Table 2). In Ac-DMAPhe spectra three bands occur (Fig. 2). Bands \( 1L_b \) and \( 1L_a \) overlap, thus preventing an accurate localization of the position of the \( 1L_b \) band without numerical

| Table 2 |
| The comparison of APhe and DMAPhe spectral properties in aqueous solutions of pH 7.5 at 20°C |
|---|---|---|
|  | APhe* | DMAPhe |
| Position of \( 1L_b \) absorption band (nm) | 284.3 | 295.0 |
| Position of \( 1L_a \) absorption band (nm) | 236.0 | 247.0 |
| Fluorescence band maximum (nm) | 345.0 | 363.0 |
| Fluorescence quantum yield | 0.086 | 0.220 |
| \( pK_a \) of aromatic amino group | 4.25 | 5.40 |
| Föster's critical distance (nm) | 1.06 | 1.08 |

* For the free amino acid (data after [2] and [6]).
Fig. 1. Comparison of UV spectra of lysozyme modified with azlactone of Ac-DMAPhe (2) and unmodified lysozyme (1). pH 8, concentration $3 \times 10^{-4}$ M

Fig. 2. UV spectra of Ac-DMAPhe in aqueous solution. 1, pH 8.0, concentration $9.6 \times 10^{-5}$ mol/dm$^3$; 2, pH 1.0, concentration $1.0 \times 10^{-3}$ mol/dm$^3$
analysis of the spectrum. All absorption bands of DMAPhe are red shifted as compared to those of APhe. After acidification of the Ac-DMAPhe solution below its pK_a, the $^1L_b$ band moves toward shorter waves; the molar absorption coefficient decreases and there appears a vibrational fine structure of the absorption band, accompanied by a drastic reduction in fluorescence intensity. The emission band of DMAPhe is also shifted toward higher wavelength values as compared with that of APhe (Fig. 3).

![Fluorescence spectrum of Ac-DMAPhe in aqueous solution. Excitation wavelength 285 nm, pH 7.0, concentration $2.61 \times 10^{-3}$ mol/dm$^3$](image)

Thus DL-(p-dimethylamino)phenylalanine, because of the greater value of the Förster's critical distance and the higher fluorescence quantum yield seems to be a better fluorescence marker than DL-(p-amino)phenylalanine.

**Concluding remarks.** The azlactone of N-acetyl-DL-(p-nitro)phenylalanine can be used for introducing APhe groups into small peptides which do not contain disulphide bridges. However, the application of this compound in
the case of proteins is limited by the lack of a selective and quantitative method of reducing nitro groups to amino groups in the presence of disulphide bridges. On the other hand, there is no need for reduction when Ac-DMApHe groups are used. These groups can be directly introduced into a polypeptide chain by reacting it with azlactone of N-acetyl-DL-(N',N'-dimethylamino)phenylalanine. This permits the Ac-DMApHe group to be used, together with endogenous tyrosine, for spectrofluorimetric measurements of intramolecular distances in peptides and proteins.

The authors are grateful to the Alexander v. Humboldt Stiftung Bonn for making accessible to them the spectrofluorimeter.

REFERENCES