Pyrylium-mediated Transformations of Natural Products. Part 5.[1] Reactions of Gelatin and Chymotrypsin with 4-(4-Methoxy-3-sulphophenyl)-2,6-bis-(4-sulphophenyl)pyrylium Perchlorate

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The title pyrylium salt reacts with lysine residues in gelatin and chymotrypsin in aqueous buffer to form rapidly the corresponding divinylogous amide and pseudobase in a ratio depending on the protein concentration. First-order ring closure of the divinylogous amide proceeds at rates comparable to those observed with the analogous intermediate from free lysine.

Numerous methods have been used for the selective modification of amino groups in proteins.[2a] Pyrylium salts, which are selective reagents [3] for primary amino groups, were first applied in 1971, when O'Leary and Samberg [2b] described the reaction of 2,4,6-trimethylpyrylium perchlorate with six (±1) of the ε-amino groups of lysine residues in α-chymotrypsin. This application remained an isolated example until our own work on the amino groups of proteins.

We have synthesised a series of water-soluble pyrylium salts [4] for the selective transformation of primary amino groups in natural products into other functionalities *via* pyridinium salts. We have described [5] preparative conditions and kinetic rate constants for the reactions of 4-(4-methoxy-3-sulphophenyl)-2,6-bis-(4-sulphophenyl)pyrylium perchlorate (1) with simple primary amines and with lysine. We now present a study of the kinetics of the reaction of (1) with gelatin and with α-chymotrypsin, proteins of known amino acid composition, containing 30 and 14 residues of lysine, respectively.[6-9] Pseudo-first-order conditions were used in which the effective lysine concentration was at least 30 times that of (1).

Reaction of Gelatin with the Pyrylium Salt (1).—As we showed previously [5] the addition of a solution of the pyrylium salt (1) in 0.01N-HCl to a buffer solution (pH 10.1) containing an amine results in the immediate formation of a new, strong absorbance band at *ca.* 480 nm whose magnitude (at constant pH) depends on the amine concentration. The new absorption band is due to the divinylogous amide (4) (Scheme); the pseudobase (2) and its anion (3) absorb at 339 and 487 nm, respectively. The progress of the reaction can be followed by monitoring the disappearance of characteristic absorbance at *ca.* 480 nm.

Gelatin was treated with the pyrylium salt (1) in carbonate buffer (pH 10.1; $I = 0.85$) at 35.0 ± 0.1 °C, to reduce the viscosity of the gelatin solutions. Spectral changes during the reaction of gelatin with the pyrylium salt (1) (Figure 1) are interpreted, as before, in terms of a fast conversion of (1) into (2) and (4) followed by slow decay of (2) and (4). The divinylogous amide absorbance at 483 nm appeared immediately after the addition of (1) and then decreased to give the characteristic pyridinium salt (5) band at 320 nm. In equation

$$A_t = B_0 e^{-k_C t} + C_0 e^{-k_D t} \quad \text{(i)}$$

(i), k_C is the rate constant for formation of pyridinium salt from divinylogous amide, k_D is the rate constant for decomposition of pseudobase, A_t is the total absorbance at 483 nm, B_0 is the absorbance of divinylogous amide at $t = 0$ and at 483 nm, and C_0 is the absorbance of the anion of the pseudobase (3) at 483 nm at $t = 0$.

The disappearance of the divinylogous amide (4) was, as previously observed,[5] a typical biphasic process (Figure 2), and the individual pseudo-first-order rate constants were calculated by the subtraction method [5,10] according to equation (i).

Extrapolation of the slow portion of the curve (Figure 2) gives a value of absorbance of the anion (3) at $t = 0$. Knowing this and also the original concentration of the pyrylium ion (1), we calculated the initial concentrations of pseudobase (2) and divinylogous amide (4) by the method described in our previous paper.[5] Results of these calculations and values of k_C and k_D are shown in Table 1.

Increasing the gelatin concentration increases the initial concentration of divinylogous amide (4) and decreases that of the pseudobase (2) (Table 1). Species (2) and (4) are formed by nucleophilic attack of hydroxy or amino groups, respectively, at position 2 of the pyrylium ring.[4,11-13] Individual second-order rate constants k_{OH} and k_{VA} (Scheme) are too fast to be measured, but k_{VA}/k_{OH} was determined as 0.23 by using equations (ii)—(iv) (Table 3), where K_a' is the acidity constant of lysine corrected for the ionic strength of the buffer used.[14]

$$[\text{lysine residues}] = [\text{Lys res.}]_{\text{stoich.}} K_a'/(K_a' + H^+) \quad \text{(ii)}$$

$$[VA]/[PB] = k_{VA}[\text{Lys res.}]/k_{OH}[OH^-] \quad \text{(iii)}$$

$$k_{VA}/k_{OH} = [VA][OH^-]/[PB][\text{Lys res.}] \quad \text{(iv)}$$

The observed rate constant $k_C = 3.0 \times 10^{-3}$ s^{-1} for cyclization of the divinylogous amide (4) to the pyridinium salt (5) is independent of the concentration of gelatin (Table 3). Rate constant k_D calculated from equation (i) is also independent of the concentration of gelatin. The value of k_D (1.0×10^{-4} s^{-1}) is within experimental error (Table 3) of the rate constant for decomposition of the pseudobase (2) at 35 °C in buffer solution pH 10.1 ($k_D = 0.8 \times 10^{-4}$ s^{-1}). Even reaction of the pseudobase (2) with gelatin at $c = 3 \times 10^{-4}$M carried out according to method B (see Experimental section) gives $k_D = 1.0 \times 10^{-4}$ s^{-1}.

These results indicate that the rate constants k_1 and k_2 are small in relation to the rate constants k_C and k_D (Scheme), and neither the reaction of the pseudobase (2) with gelatin nor the equilibrium between pseudobase and divinylogous amide (4) is significant.

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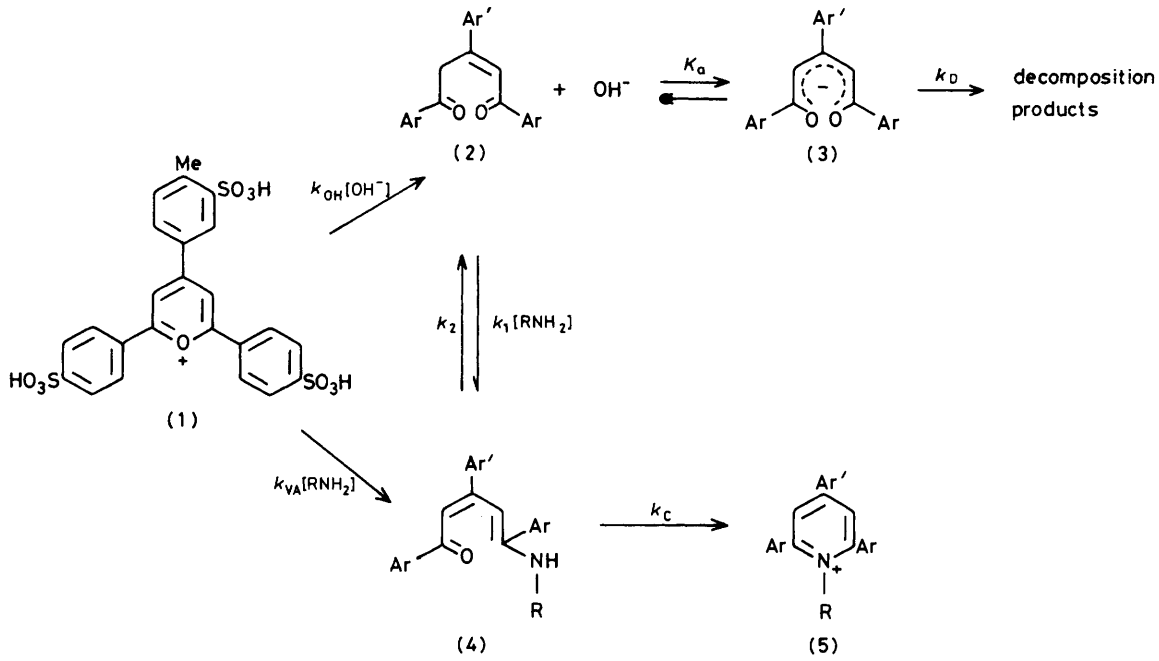

Scheme. R = lysine residue from gelatin or α-chymotrypsin

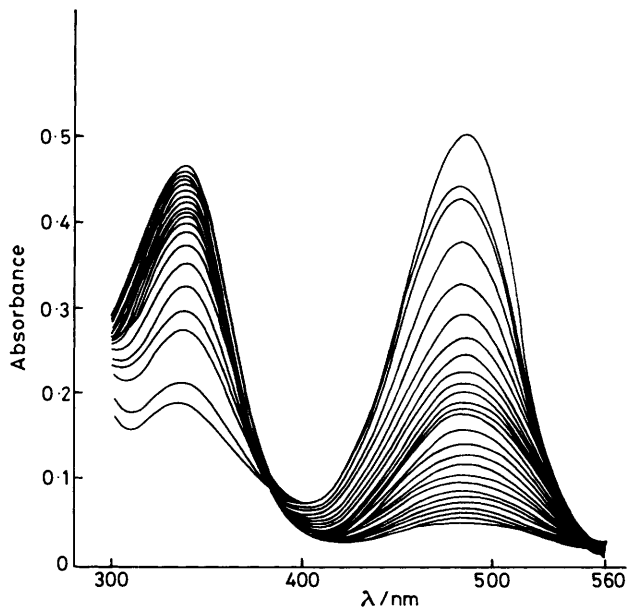

Figure 1. Scanning u.v.–visible study of the reaction of the pyrylium salt (1) (2×10^{-5} mol l^{-1}) with gelatin (2×10^{-4} mol l^{-1}) at pH 10.1 and 35 °C in water. Traces 1—13 taken at 10 min intervals, traces 14—26 at 30 min intervals

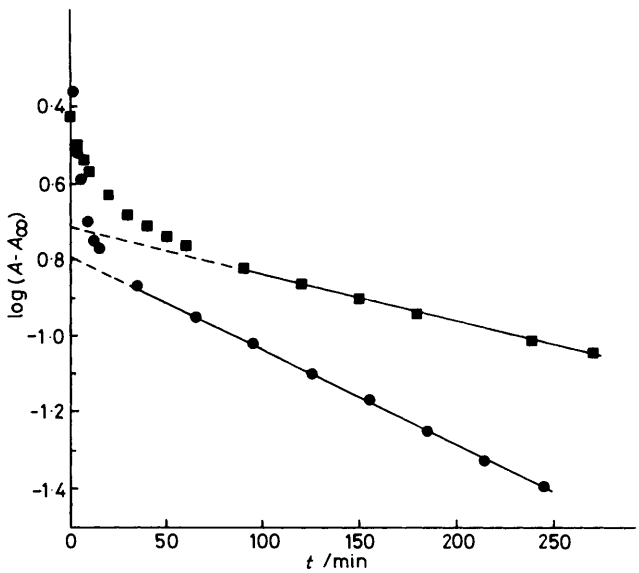

Figure 2. Biphasic plot of log $(A - A_\infty)$ *vs.* time for reaction of the pyrylium salt (1) (2×10^{-5} mol l^{-1}) with: gelatin (2×10^{-4} mol l^{-1}) at 35 ° (filled circles); α-chymotrypsin (5×10^{-4} mol l^{-1}) at 25 °C (filled squares) at pH 10.1 in water

Reaction of α-Chymotrypsin with the Pyrylium Salt (1).—The reaction of α-chymotrypsin with the pyrylium salt (1) was carried out in carbonate buffer solution (pH 10.1, I = 0.85) at 25.0 ± 0.1 °C. The spectral changes observed are essentially the same as in the reaction with gelatin. Therefore, the same kinetic model of reaction was applied (Scheme) and the relevant rate constants were calculated as before.

Data are tabulated in Tables 2 and 3. Again no reaction of pseudobase (2) with lysine residues was observed. The average value for the rate constant for cyclization of (4) to the pyridinium salt (5), $k_C = 4.7 \times 10^{-4}$ s^{-1}, is in good agreement with the same rate constant determined earlier [5] at 25 °C for reaction of lysine with the pyrylium salt (1) (Table 3).

Conclusions.—Comparisons of the data in Table 3 indicate that the general kinetic models for the reaction of the pyrylium salt (1) with proteins such as gelatin and α-chymotrypsin, containing 30 and 14 lysine residues, respectively, are identical.

However, there appear to be significant differences in the reactivity of lysine itself, as reported previously,[5] and the

Table 1. Rate constants [a] for reaction of the pyrylium salt (1) [c] with gelatin at pH 10.1 and 35.0 ± 0.1 °C

[gelatin]/mol l^{-1}	[Lys res.]/ mol l^{-1}	$10^4 k_D/s^{-1}$	$10^3 k_C/s^{-1}$	A_0^{PB}	10^5[pseudobase]/mol l^{-1}	10^5[divinylogous amide]/ mol l^{-1}
5×10^{-5}	1.5×10^{-3}	1.7		0.360	2.0	0
1×10^{-4}	3×10^{-3}	0.6	3.1	0.207	1.14	0.86
1.5×10^{-4}	4.5×10^{-3}	0.6	3.1	0.176	0.97	1.03
2×10^{-4}	6×10^{-3}	1.0	3.2	0.165	0.91	1.09
2.5×10^{-4}	7.5×10^{-3}	*b*	2.7	0.104 [b]	0.57	1.43
3×10^{-4}	9×10^{-3}	*b*	2.9	0.093 [b]	0.51	1.49

[a] $r > 0.999$. [b] [pseudobase] was determined from A_∞ values for faster part of kinetic curve. Rate constant (k_D) was not determined for these concentrations of gelatin. [c] Concn. 2×10^{-5} mol l^{-1}.

Table 2. Rate constants [a] for reaction of the pyrylium salt (1) [b] with α-chymotrypsin at pH 10.1 and 25.0 ± 0.1 °C

[chymotrypsin]/ mol l^{-1}	[Lys res.] mol l^{-1}	$10^4 k_D/s^{-1}$	$10^4 k_C/s^{-1}$	A_0^{PB}	10^5[pseudobase]/mol l^{-1}	10^5[divinylogous amide]/ mol l^{-1}
1.248×10^{-4}	1.747×10^{-3}	0.4	2.8	0.247	1.32	0.68
2.312×10^{-4}	3.237×10^{-3}	0.5	6.4	0.223	1.19	0.81
4.856×10^{-4}	6.798×10^{-3}	0.4	4.5	0.185	0.99	1.01
5×10^{-4}	7×10^{-3}	0.5	4.9	0.193	1.03	0.97
1×10^{-3}	1.4×10^{-2}	0.5	4.9	0.134	0.72	1.28

[a] $r > 0.995$. [b] Concn. 2×10^{-5} mol l^{-1}.

Table 3. Comparison of kinetic data for reaction of the pyrylium salt (1) and/or pseudobase (2) with lysine, gelatin, and α-chymotrypsin at pH 10.1.

Compound	*T*/°C	k_{VA}/k_{OH}	$10^4 k_C/s^{-1}$	$10^4 k_D/s^{-1}$	$10^4 k_1$/l mol^{-1} s^{-1}
Gelatin	35	0.23 ± 0.05	30 ± 2	1.0 ± 0.5	
α-Chymotrypsin	25	0.16 ± 0.06	4.7 ± 1.3	0.5 ± 0.1	
Lysine [a]	25	0.13	6.4 ± 0.5	0.5 ± 0.1	3.3

[a] Data for lysine from ref. 5.

lysine residues in protein molecules. In particular, the pseudobase (2) reacts with lysine ($k_1 = 3.34 \times 10^{-4}$ l mol^{-1} s^{-1}),[5] but this reaction is not observed for the proteins investigated. The formation of pyridinium salts from pseudobases involves as rate-determining step the formation of divinylogous amide. This is always slower than divinylogous amide formation from pyrylium salts.[5,15-17] Availability of the non-protonated ε-amino groups in lysine residues in proteins, because of intramolecular interaction, is lower than in free lysine. Hence pseudo-first-order rate constants ($k_1[RNH_2]$) for formation of divinylogous amide from pseudobase are much lower than those for decomposition (k_D) of the pseudobase.

The same strong intramolecular protein interactions could stabilize the divinylogous amide (4), decreasing the decomposition rate constant (k_2).

Because of the ratio of pyrylium salt to protein chosen, on average only one lysine residue per protein reacts. It is not known whether there are significant differences in reactivity of the various lysine amino groups in the protein molecule.

From the kinetic results presented in this paper, we conclude that pyrylium salts could provide a convenient means of transforming amino groups in complex and sensitive natural products into other functionalities.

O'Leary and Samberg have reported [2b] the second-order rate constant for the reaction of α-chymotrypsin with the 2,4,6-trimethylpyrylium ion as 2×10^{-3} l mol^{-1} s^{-1} at pH 9.0 and 20 °C. However, under the conditions described, the measured rate constant applies to the decreasing concentration of pseudobase. The value of 2×10^{-3} l mol^{-1} s^{-1} is *ca.* 8 times that for reaction of the pseudobase (2) with lysine [5] (*cf.* Table 3). This comparison, and the conditions of the reactions, indicate that the rate constant determined by O'Leary and Samberg is composite, describing two parallel processes, *i.e.* formation of pyridinium salt and cleavage of the pseudobase of the 2,4,6-trimethylpyrylium salt (*cf.* ref. 5).

Experimental

U.v.–visible spectra were recorded with a Perkin-Elmer 330 apparatus. 4-(4-Methoxy-3-sulphophenyl)-2,6-bis-(4-sulphophenyl)pyrylium perchlorate was obtained according to the literature method.[4] Photographic gelatin and α-chymotrypsin from bovine pancreas, three times crystallized and lyophilized (Sigma), was used. Other reagents were of analytical grade.

Kinetic Procedure.—Method A. A stock 0.005M-solution of the pyrylium salt (1) was prepared in 0.01N-HCl. Reactions were initiated by injection of 10 μl of a stock solution of (1) with a Hamilton syringe into a thermostatted (25.0 ± 0.1 °C for α-chymotrypsin; 35.0 ± 0.1 °C for gelatin) u.v. cell containing 2.5 ml of a buffered protein solution. Reactions were followed by monitoring the disappearance of the characteristic absorbance of the divinylogous amide at 483 nm.

Method B. The pyrylium salt (1) (8.5 mg) was dissolved in 5 ml of a buffer solution and after 5 min mixed with a buffered solution of the appropriate protein and diluted with buffer to 25 ml. The reaction mixture was thermostatted to 35.0 ± 0.1 °C. At appropriate intervals 1 ml samples were withdrawn and diluted to 25 ml with buffer pH 11.0. Reaction was followed by monitoring the disappearance of the characteristic absorbance at 487 nm of the anionic form of the pseudobase.

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