THE BINDING OF A SPIN-LABELLED DIPEPTIDE TO PAPAIN

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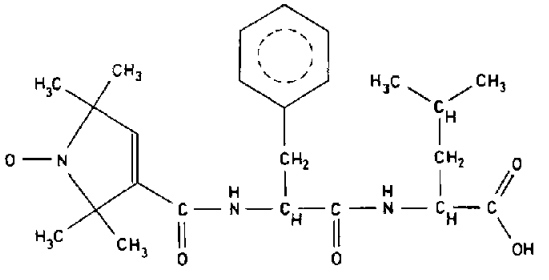
1. Introduction

Stable nitroxide radicals have found extensive use as spin-labels in the study of biopolymers [1] and their interactions with small molecules [2,3]. Using e.s.r. spectroscopy, information may be obtained about the mobility of the nitroxide group and hence the binding of a spin label to a protein may be monitored. In addition, if a spin label is attached to a protein, the magnetic relaxation of the protein nuclear spins is strongly enhanced by dipolar interactions with the electron spin ($\alpha\ r^{-6}$) [3]. In principle therefore, the relative positions of the spin label and protein nuclei may be determined from the enhancement of the relaxation rates of nuclear spins in the protein.

We report a study of the interaction of the proteolytic enzyme papain with *N*-(2,2,5,5-tetramethylpyrroline-1-oxyl-3-carbonyl)-L-phenylalanyl-L-leucine (SL-Phe-Leu),



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This spin-labelled dipeptide is of a type found by Berger and Schechter [4] to be a strong (product) inhibitor of papain at about pH 4.

2. Experimental

Details of the preparation of SL-Phe-Leu are published elsewhere [5]. Stock solutions of SL-Phe-Leu were prepared by dissolving 3–4 mg in dimethyl sulphoxide (1 ml) and dispersing the solution in double-distilled water (9 ml) through a fine glass capillary.

Papain was isolated from dried papaya latex [6] and fractionated by the method of Sluyterman and Wijdenes [7], to give pure mercuripapain (PapSHg) and irreversibly-oxidised papain ($PapSO_2^-$) after extensive dialysis against double-distilled water. Active papain (PapSH) was generated in situ by the addition of cysteine (10 mM) and EDTA (2 mM).

The activity of the papain was determined from the rate of hydrolysis of benzoylarginine ethyl ester (BAEE) (Merck) at pH 4.2 and 25°C with a pH-stat (Radiometer, Copenhagen). The inhibition of papain by the dipeptide was determined under the same conditions using 3.2×10^{-5} M SL-Phe-Leu with different concentrations of BAEE (4–33 mM). The reaction mixture contained 10 mM cysteine, 2 mM EDTA, 0.2 μM papain and 0.05 or 0.3 M KCl.

X-band ESR measurements were carried out using a Varian E-4 spectrometer at room temperature using

an aqueous sample cell. The microwave power 20 mW, was non-saturating. It was ascertained that in SL-Phe-Leu solutions without enzyme, the peak height was proportional to the dipeptide concentration. Spectra of the SL-Phe-Leu bound to papain were obtained with the aid of a Varian C-1024 time averaging computer. Four scans of the ESR spectrum of SL-Phe-Leu in the presence of papain were accumulated, after which the spectrum of a SL-Phe-Leu solution withou papain was subtracted until the free (narrow) signal had almost disappeared.

3. Results and discussion

The Lineweaver-Burk plots of the hydrolysis of BAEE by papain with and without SL-Phe-Leu are shown in fig.1. The inhibition constant K_i was determined from the ratio of the gradients of the two plots, which is $1 + [I]/K_i$ where [I] is the inhibitor concentration. The corresponding binding constant (K_i^{-1}) for SL-Phe-Leu was found to be 2.5×10^4 M^{-1} in both 0.05 and 0.3 M KCl. This compares well with the binding constants of similar phenylalanine-leucine derivatives determined by Berger and Schechter [4] (1.5×10^4 M^{-1} for *N*-acetyl- and *N*-methoxycarbonyl- and 4×10^4 for *N*-butoxycarbonyl-).

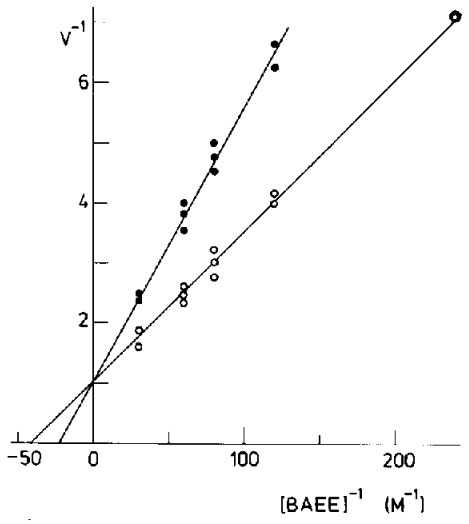

Fig.1. Lineweaver–Burk plots for the hydrolysis of BAEE by papain with (o) and without (o) 3.2×10^{-5} M SL-Phe-Leu, at pH 4.2 with 0.05 M KCl. The reciprocal rate (V^{-1}) is expressed in arbitrary units.

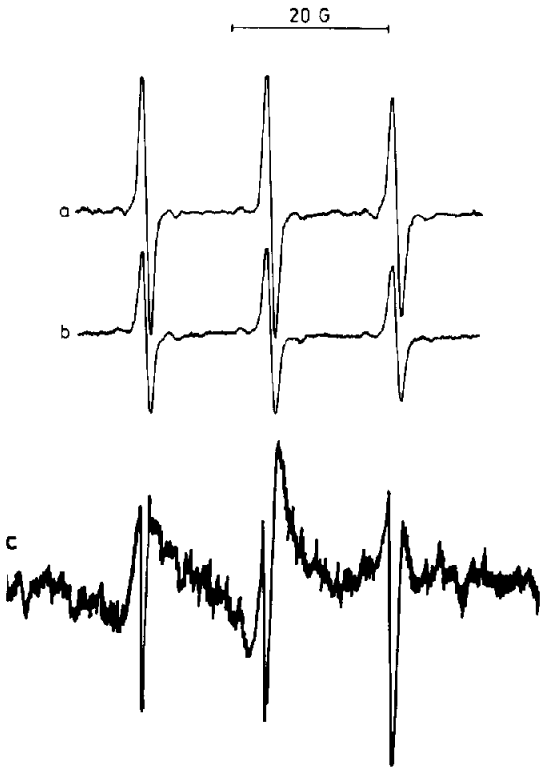

Fig.2. E.s.r. spectra of SL-Phe-Leu in 0.03 M sodium acetate buffer pH 4.2, containing 0.05 M KCl. (a) 0.25×10^{-4} M SL-Phe-Leu. (b) as (a) with 1.65×10^{-4} M PapSH. (c) A difference spectrum showing the spectrum of SL-Phe-Leu bound to papain. The sharp peaks arise from 'unbound' signal and are caused by spectrometer drift (SL-Phe-Leu, 10^{-4} M; PapSH, 0.44×10^{-4} M).

The addition of active papain to an aqueous solution of SL-Phe-Leu at pH 4 was found to reduce the intensity of the e.s.r. spectrum of the free nitroxide (fig.2 (a) and (b)). The addition of the cysteine or EDTA used to generate PapSH from PapSHg had no effect on the e.s.r. signal. The reduction of the free nitroxide signal intensity was accompanied by the appearance of a broader signal. This broad signal arising from the bound dipeptide (obtained as described in the experimental section and shown in fig.2 (c)) is that of a moderately-immobilised spin label, typical of a nitroxide radical with a correlation time, τ_c, of between 3 and 7×10^{-9} s [8,9]. This compares favourably with the τ_c of 8×10^{-9} s in water at 20°C estimated, from the X-ray crystallo-

graphic dimensions of papain [10], using the Stokes–Einstein equation.

From the three-dimensional model of a papain derivative in which the enzyme has been alkylated with a peptide-like chloromethylketone [11], it appears that SL-Phe-Leu will fit comfortably into the active site groove. Some mobility of the nitroxide group is possible, which would give rise to a slightly shorter correlation time for the nitroxide group than for the protein itself.

The integrated intensity of the central line of the spin label bound to papain was compared with that of the free label present in solution. It was assumed that all the lines were Lorentzian in shape; the relationship of integrated intensity being proportional to $h\Delta^2$, where h is the peak height and Δ the peak-to-peak linewidth in the first derivative spectrum, was used. The integrated intensity of the bound label was found to be in good agreement with the 'loss' of free label intensity on addition of the protein. The contribution of the bound label signal to the free label peak height was found to be neglibible for levels of binding below 60%. Observing the latter conditions, free and bound labels proportions were determined directly from peak height measurements.

The pH dependence of the binding of SL-Phe-Leu to PapSH at two different protein concentrations is shown in fig.3. Both plots show maximum binding around pH 4 whereas at pH values above 4.4 the binding decreases rapidly. This behaviour agrees well with that found for other product inhibitors of papain [12,13].

The binding constant, K_b, is defined as $K_b = [EL]/[E][L]$ where [EL] is the concentration of the enzyme–SL-Phe-Leu complex, [E] is that of the free enzyme and [L] that of the unbound label. K_b (at pH 4.2) was determined from the total concentrations of SL-Phe-Leu and the enzyme and the relative reduction of the free nitroxide e.s.r. signal arising from the addition of PapSH, assuming one binding site per enzyme molecule. This K_b appeared to be independent of the total spin label concentration (between 6×10^{-6} and 10^{-4} M) but was dependent on papain concentration. This is illustrated in fig.4. At the lowest papain concentrations, K_b (2 to 3×10^4 M^{-1}) agrees with the association constant, K_i^{-1} (2.5×10^4 M^{-1}) from the inhibition studies, which were also carried out at low papain concentrations (0.2 μM).

In order to check whether the lower K_b values at higher papain concentrations might be due to incomplete activation as the PapSHg/cysteine ratio increased, the PapSHg was dialysed against a large volume of buffer solution containing cysteine (10 mM) and EDTA (2 mM) under nitrogen. The resulting K_b values (open circles in fig.4) showed the same concentration dependence as those obtained without prior dialysis.

The binding of SL-Phe-Leu to papain was also independent of the incubation time, ruling out the

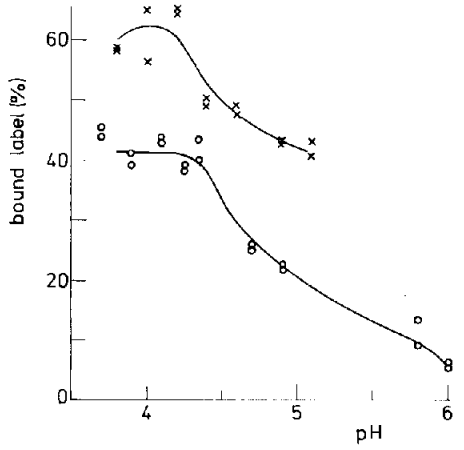

Fig.3. The percentage of SL-Phe-Leu (0.25×10^{-4} M) bound to papain as a function of pH. Papain concentrations 8.5×10^{-4} M (x) and 1.65×10^{-4} M (o) in 0.03 M sodium acetate, 0.05 M KCl.

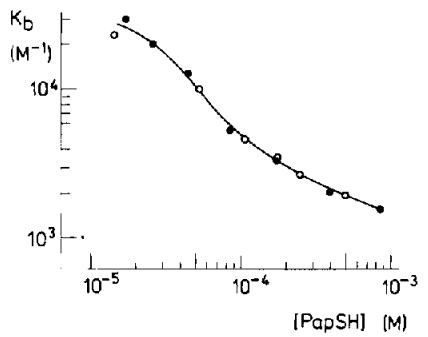

Fig.4. K_b as a function of active papain concentration in 0.03 M sodium acetate, 0.05 M KCl, 10 mM cysteine, 2 mM EDTA, pH 4.2.

possibility that the observed low K_b values might be attributed to hydrolysis of the spin label.

SL-Phe-Leu was found to bind to the inactive papain derivatives $PapSO_3^-$ and PapSHg although to a much lesser extent than to active papain. The pH dependence of the binding was similar to that of the active enzyme. K_b for these derivatives at pH 4.2 was about $800 \pm 100\ M^{-1}$ and was independent of protein concentration. A similar K_b value at pH 4.2 was found for a papain alkylated at its thiol group by α-*N*-tosyllysylchloromethylketone (Pap TLCK) (generously donated by Mr H. M. Swen). The binding of SL-Phe-Leu to these inactive papains is not necessarily substrate-like, as in the case of Pap TLCK, a large part of the active site is occupied by the TLCK moiety. It is also possible that a similar non-specific binding may occur with active papain. At a PapSH concentration of 0.8 mM the binding constant is only twice that for the inactive derivatives.

At papain concentrations below 0.2 mM most of the bound spin label will be bound substrate-like, in the acyl-enzyme form. At these protein concentrations it is possible with modern techniques to obtain good PMR spectra in a reasonable time. The position of the unpaired electron spin in the protein could be determined from the differential line broadening of protons located at various distances from the label. The most appropriate resonances are perhaps tryptophan N H resonating between 10 and 11.5 ppm (δ) in H_2O [5]. From the molecular model of papain, it appears that the five Trp residues are at distances ranging from 10 to 30 Å from the expected position of the unpaired electron spin. Calculations according to Wein et al. [3] indicate that the line broadening will be from 150 to 0.2 Hz in that distance range for a protein with $\tau_c \approx 10^{-8}$ s. This should at least make feasible the assignment of the Trp N H resonances of papain.

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