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FERROCENOPAPAIN, AN ORGANOMETALLIC PROTEIN FORMED BY SITE-SPECIFIC INACTIVATION OF PAPAIN USING CHLOROACETYLFERROCENE

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Papain is rapidly, specifically and irreversibly inactivated by chloroacetylferrocene in a time-dependent, two-stage process which involves initial, physical complexation between the enzyme and the organometallic reagent followed by chemical reaction. The kinetics of the process show saturation kinetics with respect to inactivator concentration. The modification occurs with a 1:1 stoichiometry and the degree of loss of enzymatic activity is directly reflected in the loss of thiol groups in the active-site that are accessible to Ellman's reagent. The modification is faster at higher pH. The ferrocenium ion of the modified papain can be generated at low pH by anaerobic treatment with ferric nitrate and shows a 180 percent increased lifetime at pH 3.3 compared to the acetylferrocenium ion under identical conditions. The spectral properties of the ferrrocenopapain indicate that the ferrocene is in an unusual environment at the protein active-site and the implications of this are discussed.

KEY WORDS: Papain, chloroacetyl-ferrocene, chemical modification, enzyme inhibition, protease, thiol-directed reagent.

INTRODUCTION

The metallocenes, sandwich compounds of various metals between a pair of parallel cyclopentadienyl rings, have been used in biochemistry for a variety of purposes. The commonest of them, ferrocene (**1**) has been incorporated into various drugs and enzyme inhibitors as a hydrophobic moiety, sometimes to resemble the phenyl ring of phenylalanine.[1–4] For example, β-ferrocenylalanine (a putative analogue of phenylalanine) has been tested as an inhibitor of phenylalanine hydroxalase.[1] They have also been used, as they are almost spherical, as electron-transfer agents in tests of the Marcus theory for proteins.[5] More recently, ferrocenium ions, formed from (**1**) by removal of one electron, have been shown to inhibit the proteinase that cleaves the aminoterminal propeptides from procollagen.[6] Metallocenes have been used as general markers of proteins, e.g. the use of (**2**) in electron microscopic immunohistochemistry for visualization of antigen-antibody complexes.[7–10] Metallocene-like molecules such as the half-sandwich compounds, tetramethylcyclobutadiene nickel (**5**) and maleic anhydride irontetracarbonyl (**6**), have been used to covalently modify alcohol dehydrogenase[11] and ribonuclease A,[12,13] respectively with the general

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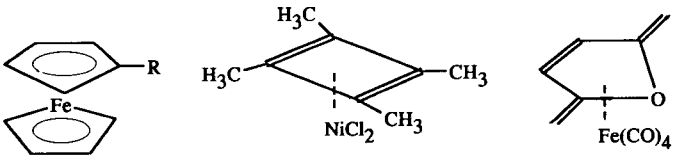
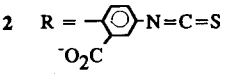


1 R = H

2 R = 

3 R = $-COCH_2Cl$

4 R = $-COCH_3$

5

6

purpose of exploiting the spectral properties of metallocenes as probes. On a different tack, dichlorometallocene complexes (η^5-$(C_5H_5)_2MCl_2$ where M = Ti, V or Nb) have been shown to have antitumour activities *in vivo*[14,15] and, as attack on DNA was proposed to explain this[16,17], studies of their complexes with purines have been reported.[18] Yet a further interesting application of metals in biochemistry has been the incorporation of organometallics and inorganic complexes into proteins to modify the reactivity of the organometallic or metal site. Thus, avidin has been incorporated into a diphosphinerhodium (I) asymmetric hydrogenation catalyst.[19] Incorporation of a water-soluble Fe–S cluster in aqueous solution into insulin and bovine serum albumin led to changes in redox potential and in stabilisation of the cluster towards oxygen.[20] As a step towards obtaining a combination of organometallic reactivity with a carefully-controlled and stereochemically-defined enzymatic binding-site we have studied the modification of papain, a protein of known three-dimensional structure and specificity[21] by a suitably-activated metallocene model system, chloroacetylferrocene (**3**), and now report on this study.

MATERIALS AND METHODS

Reagents

Titanocene dichloride, acetylferrocene, acetonitrile and dithiothreitol were obtained from the Aldrich Chemical Co. (Poole, Dorset, UK). The following materials were obtained from Sigma Chemical Co. (Poole, Dorset): papain (Type III, twice crystallised), N-benzyloxycarbonylglycine p-nitrophenyl ester, L-cysteine, β-mercaptoethanol, aminoacetonitrile, bovine serum albumin. Agarose-gly-gly-tyr(OBzl)-arg affinity chromatographic medium was from Miles-Yeda Ltd (Illinois). All commercial chemicals were of the highest purity available. All water was glass-distilled and deionised before use.

Enzyme Assays and Kinetics

UV-visible spectra and rate measurements were performed using a Pye-Unicam SP8-100 spectrophotometer with the cuvette compartment thermostatted at 25°C by

water circulating from a Churchill Thermostat. Other UV/visible spectra were obtained at 25°C using a Carlo-Erba Spectracomp 601 instrument. Proton NMR spectra were obtained at 60 MHz using a Varian EM360 spectrometer. All pH measurements were made using a Philips PW 9409 digital pH meter. Phosphate buffer for the activation and assay of papain contained 0.02 M phosphate with 10 mM EDTA and 6.8% (v/v) acetonitrile at the required pH (pH 6.80 was used for the assay of papain).

Enzyme

Commercial papain was further purified by affinity chromatography on agarose-gly-gly-tyr(OBzl)-arg as described.[22] When commercial papain was used it was activated[23] with L-cysteine or dithiothreitol, isolated by Sephadex® G-25 gel filtration and stored in pH 7.0 phosphate buffer under nitrogen at 4°C. To determine approximate papain concentrations a value[24] of $E^{1\%}_{1\,cm}$ of 23,000 daltons was used. For accurate concentration determination of papain the free sulphydryl titre was measured using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) and an extinction coefficient of 14,150 $M^{-1}\,cm^{-1}$ for the 5-thio-2-nitrobenzoate ion.[26] Papain activity was assayed spectrophotometrically at 400 nm at pH 6.80 using N-benzyloxycarbonylglycine p-nitrophenyl ester,[27] the concentration of which was determined by alkaline hydrolysis to the 4-nitrophenolate ion.

Inhibition Studies

Time-dependent inhibition studies were carried out by incubating together enzyme and inhibitor in the appropriate medium on an ice-bath at 4°C. Aliquots (10–50 μl) were removed and assayed for remaining enzyme activity. The incubation medium was degassed with nitrogen before addition of enzyme. Inhibition was detected by plots of activity remaining versus time. The inhibitors were dissolved in the minimum volume of dimethyl sulphoxide. Control incubation mixtures contained all components except for inhibitor and the amount of dimethyl sulphoxide was held constant. Solutions containing (**3**) were protected from light.

Synthesis

Chloroacetylferrocene (**3**), synthesized by Friedel-Crafts acylation of ferrocene after the method of Schlogel and Egger[28], had mp 92–94°C (lit.[28] 93–95°C), showed a single spot on TLC and had the correct ^{1}H NMR spectrum.

Data Analysis

Enzyme inhibition data were analysed by means of the Enzfitter[c] program by R.J. Leatherbarrow, distributed by Elsevier-BIOSOFT.

RESULTS

The inhibition of papain by (**3**) was found to be time-dependent at pH 6.80 and pH 7.50. By the end of 3–4 hours incubation at either pH there was >97% inhibition

of the papain activity. At this point for the pH 6.80 run, both inhibitor-containing and control incubation mixtures were run separately down a Sephadex® G-25 column to separate protein from small molecules (e.g. (**3**) and its hydrolysis products). Relative to similarly-treated control, papain incubated with (**3**) had lost >94% of its activity even after this desalting step. Dialysis of modified papain against assay buffer confirmed the irreversibility of the modification.

The time-course of the inhibition followed first-order kinetics. The rate of inhibition of papain by chloroacetylferrocene at pH 6.80, 4°C was dependent on the concentration of (**3**), rate data being collected in Table 1 and displayed in Figure 1. The

TABLE I
Rate constants (k_i) for inactivation of papain by chloroacetylferrocene at 4°C in pH 6.80 phosphate buffer; [papain] = 5.9×10^{-5} M as free –SH

[Chloroacetyl-ferrocene], mM	$10^2 \times k_i$ (min^{-1})
1.65	5.50
0.850	4.90
0.413	4.20
0.248	3.34
0.207	2.84

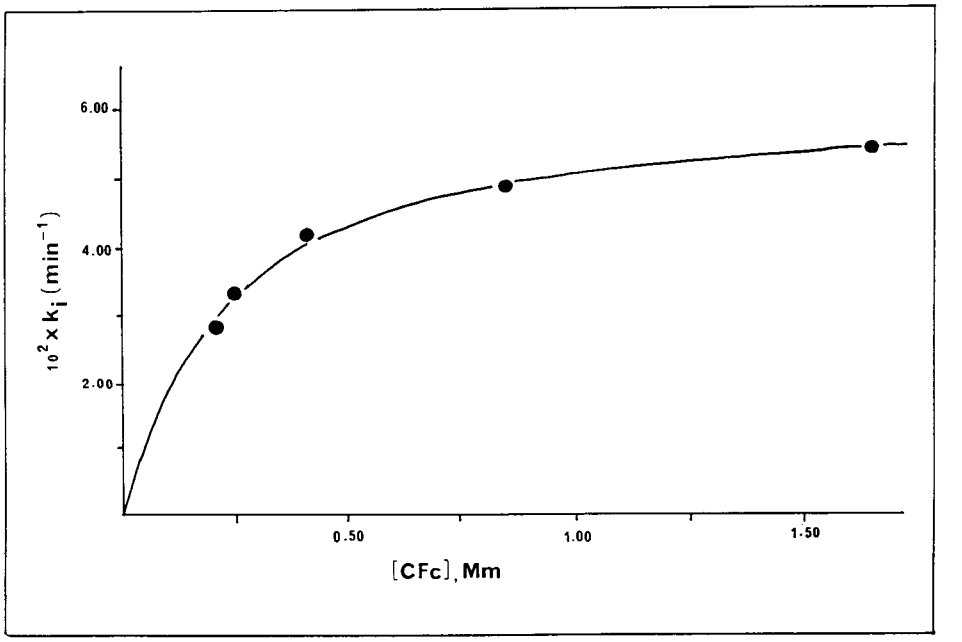


FIGURE 1 Concentration dependence of the pseudo first-order rate constants for inhibition (k_i) of papain (5.90×10^{-5} M, free SH) by various concentrations of chloroacetylferrocene in pH 6.80 phosphate buffer at 4°C. Points are experimental: line is theoretical using equation (1) and values of $k_i^{max} = 0.0627\ min^{-1}$ and $K = 2.26 \times 10^{-4}$ M.

data were fit by equation (1) using direct nonlinear regression analysis, where k_i^{max} is the maximal rate constant for inactivation of papain by chloroacetylferrocene (CFc)

$$k_i = \frac{k_i^{max}[CFc]}{K + [CFc]} \quad (1)$$

when the concentration of CFc is much greater than the constant, K, in equation (1). Values obtained were $k_i^{max} = 0.0627 \pm 0.0018\ min^{-1}$ and $K = 2.26 \pm 0.222 \times 10^{-4}$ M.

The stoichiometry of this inactivation was studied as follows. A series of solutions of fixed papain concentration (3.95×10^{-5} M) was incubated with a range of CFc concentrations (from $0.42–8.40 \times 10^{-5}$ M) to give a range of [CFc]/[papain] ratios from 0.11 to 2.13. Incubation was carried out in a cold room (4°C) for 24 hours, after which time the mixtures were assayed for remaining papain activity, relative to a control sample of papain not treated with CFc. The results are shown in Figure 2 which plots the percentage enzyme activity remaining against the molar ratio of CFc

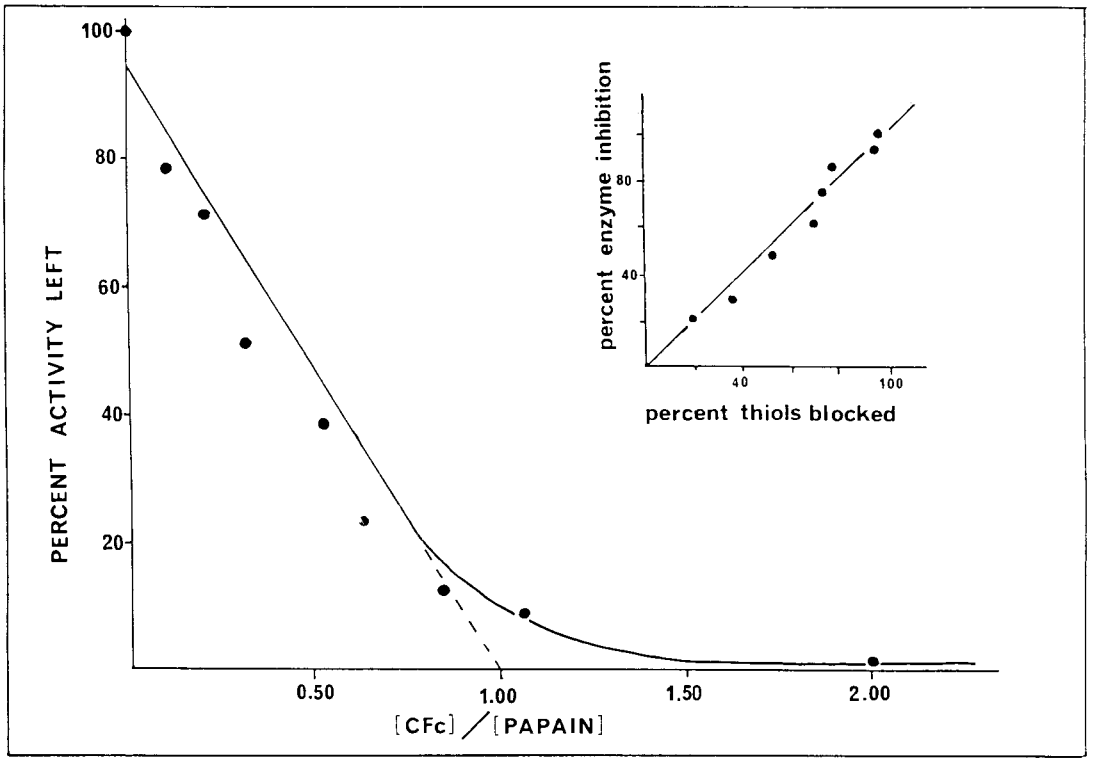


FIGURE 2 Plot of percentage of enzymatic activity remaining, relative to control papain, after incubation of papain with chloroacetylferrocene at various molar ratios of chloroacetylferrocene/papain (papain concentration based on active-site thiol titre). The inset shows a plot of percentage inhibition of papain activity on chloroacetylferrocene treatment as a function of papain active-site thiol titre expressed as percentage of available thiol blocked (points are experimental; the line is derived by linear least square regression analysis and has a slope of 1.04, intercept −0.09).

to papain. The papain concentration was based on the Ellman free –SH titre. From extrapolation of the initial part of the curve (at [CFc]/[papain] < 1) it can be seen that inactivation occurs with a 1:1 stoichiometry of CFc:papain. In the inset to Figure 2 is shown a plot of the percentage inhibition measured for these solutions *vs* the percentage thiol groups which were inaccessible to Ellman's reagent (% RSH blocked) for the same solutions. The slope of the line drawn through the points is 1.00 indicating a 1:1 relationship between remaining activity and the accessible thiol titre (see Table II).

The papain which had been treated with CFc and subjected to gel filtration (or dialysis) was named ferrocenopapain and relative to papain, showed an additional light absorption at 320 nm (see Figure 3) indicating a change in the nature of the reisolated protein. Preliminary studies of the magnetic circular dichroism spectrum of ferrocenopapain indicated a bound ferrocene species (A.F. Drake, O.S. Ejim and K.T. Douglas, unpublished observations).

As one of the chemical characteristics of ferrocene is its oxidation to ferrocenium ion (redox potential[29] of 0.771 v), we tried a number of oxidising agents to generate the ferrocenium ion of the enzyme-bound ferrocene and found that the most convenient was ferric nitrate at pH 3.30. As a model system acetylferrocene (**4**) was studied under similar conditions, the acetylferrocenium ion having a weak absorption band at 630 nm. Although this absorption is weak, there is no possibility of spectral complications from contributions by absorbing protein natural chromophores (λ_{max} 280 nm) or the ferric nitrate oxidant. On addition of ferric nitrate solution to ferrocenopapain in a trial run, new peaks of absorption from the ferrocenium form appeared at 325 nm (E_M 4400 M^{-1} cm^{-1}) and 626 nm (E_M 100 M^{-1} cm^{-1}) but because of ready aerial oxidation these decayed quickly. Studies of the ferrocenium ions of ferrocenopapain and acetylferrocene were made by degassing a solution of the appropriate ferrocene at pH 3.30 in a quartz cuvette by bubbling nitrogen gas through them for 20 min *via* syringe needles in a rubber septum cap. Care was taken not to

TABLE II

Results of determination of remaining enzymatic activity and free –SH titre for a series of papain solutions containing various amounts of chloroacetylferrocene in pH 7.0 phosphate buffer after 24 hours at 4°C. The enzyme concentration (based on –SH titration) was 3.95×10^{-5} M throughout and the reactions were run in the dark under nitrogen

10^5 [CFc], M	$\frac{[CFc]}{[papain]}$	Percentage inhibition relative to control	Percentage SH groups blocked
8.40	2.13	99.5	96.0
4.20	1.10	94.3	95.0
3.78	1.06	90.7	75.0
3.36	0.85	87.1	78.0
2.56	0.64	76.4	74.0
2.10	0.53	61.4	70.0
1.26	0.32	48.6	52.0
0.84	0.21	28.6	36.0
0.42	0.11	21.4	20.0
0	0.00	0.00	0.0

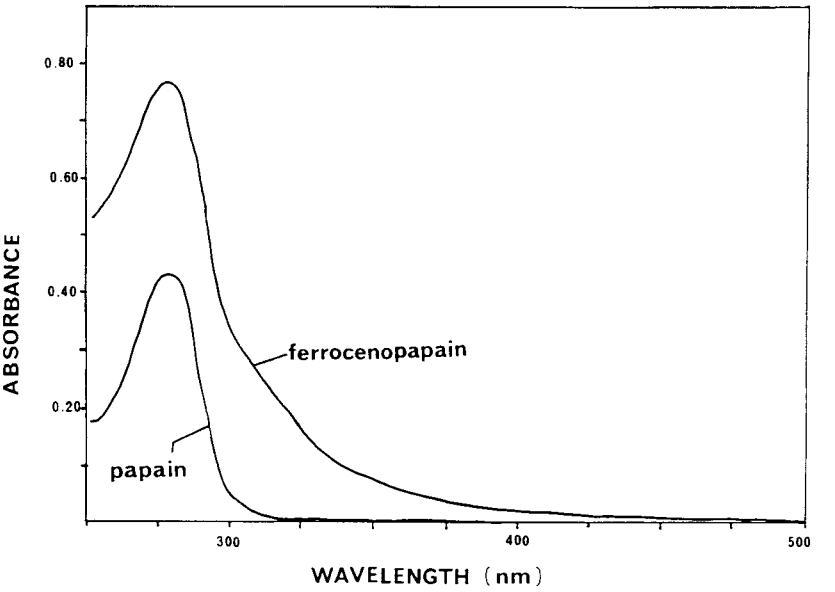


FIGURE 3 Ultraviolet spectra for papain and ferrocenopapain at 25°C in pH 6.80 phosphate buffer. Each protein solution has been desalted by G-25 gel filtration prior to spectral measurement of the tubes containing the fractions most concentrated in each protein as appropriate. Elution conditions were identical as were initial protein concentrations so that papain and ferrocenopapain concentrations are closely comparable.

denature the protein in this process. After degassing, a mole equivalent of Fe^{3+} as its nitrate was injected, the solution mixed thoroughly and the absorbance at 630 nm recorded with time for both systems, carefully noting the moment of mixing. The data, normalised to unit relative absorbance at $t = 0$ for direct comparison, are plotted in Figure 4. The ratio of half-lives of the acetylferrocenium ion and the protein-bound ferrocenium ion was calculated to be 1.8 with the protein-bound ion being the more stable. After loss of the band at 625 nm with time, further injections of mole equivalents of Fe^{3+} ions regenerated it (3 cycles tested).

DISCUSSION

The reaction of CFc with papain is likely to be a covalent modification in view of its irreversibility by the criteria of gel filtration and dialysis. The 1:1 stoichiometry and 1:1 correspondence of remaining activity with free –SH titre indicate a specified reaction either at or very close to cys_{25}, the catalytic thiol residue. A number of chloroacetyl- or bromoacetyl species have been found to alkylate cys_{25} including iodoacetamide and related species,[30,31], N-tosyl-L-phenylalanine chloromethyl ketone and related molecules[32,33] as well as several others.[34–36] The saturation dependence of the rate of alkylation of papain by CFc argues for a specific interaction when taken together with the 1:1 stoichiometry and the parallel losses of –SH titre and enzyme activity. CFc probably forms a physical (Michaelis-type) complex (E.CFc)

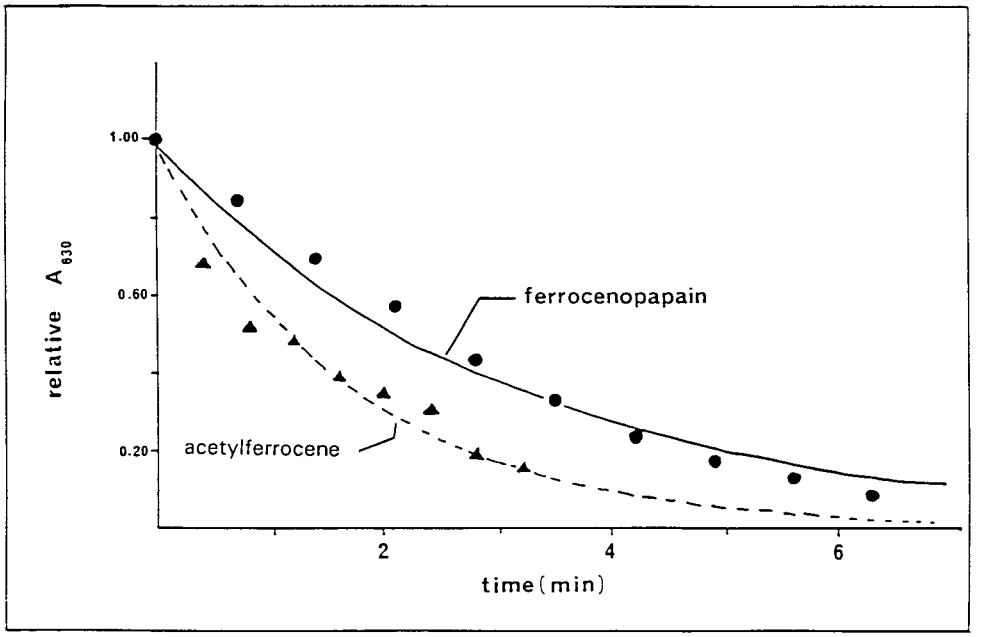
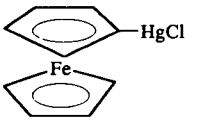


FIGURE 4 Plot of relative absorbance at 630 nm *versus* time for the ferrocenium ions derived from acetylferrocene (—▲—▲—) and ferrocenopapain (—●—●—) at pH 3.30 after Fe^{3+} oxidation as described in the text. Points are experimental; lines are for single exponential decays in each case with rate constants of 0.0323 min^{-1} and 0.0594 min^{-1} for the ferrocenopapain and acetylferrocene systems, respectively.

prior to chemically alkylating the enzyme, according to equation (2), where E is papain and FcPAP is ferrocenopapain.

$$E + CFc \underset{K}{\rightleftharpoons} E.CFc \xrightarrow{k_i^{max}} FcPAP \qquad (2)$$

It has been suggested that the carbonyl group of α-halocarbonyl reagents may hydrogen-bond to either the backbone NH of cys_{25} or the amide NH_2 of gln_{19}[36] and in the ferrocene case such an interaction might allow the stability of E.CFc to be such that it can be kinetically-detected. However, the ferrocenyl portion must be exerting some specific binding contribution as saturation kinetics have not been reported widely for simple alkylating agents with papain. Finally, the mercurial derivative of ferrocene (7) did not inhibit papain even at high concentrations under our conditions, again arguing for a specific modification mechanism.



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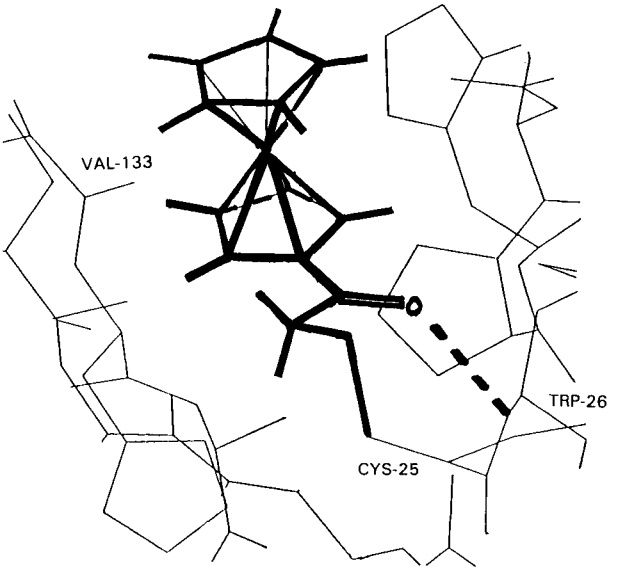

FIGURE 5 Molecular graphics view of the active-site of papain modified by alkylation of cys_{25} by chloroacetylferrocene (**3**) using the coordinates for papain from reference 33 and for acetylferrocene from reference 37. The hydrogen-bond between the acetylferroceno group and the backbone NH of trp_{26} is shown as a dotted line.

There is evidence that the environment of the ferrocene bound at the papain active-site is distinct from ferrocene in bulk solution (i.e. that it does not freely protrude from the enzyme surface into the solvent). The UV-visible spectrum is significantly changed in the 345/460 nm regions relative to free acetylferrocene. Thus, the band at 464 nm ($E_M = 676\ M^{-1}\ cm^{-1}$) found for acetylferrocene in pH 6.03 water was not detected for ferrocenopapain and the peak at 345 nm of acetylferrocene in pH 6.03 water had $E_M = 1409\ M^{-1}\ cm^{-1}$ whilst the corresponding band for ferrocenopapain was at <325 nm appearing really only as a weak shoulder on the intense 280 nm band. Moreover, the lifetime of the ferrocenium ion formed from ferrocenopapain was slightly increased ($t_{1/2}$ being raised $\sim$2-fold) relative to the acetylferrocenium ion under identical conditions. This small change cannot be thoroughly discussed yet as acetylferrocene may not be the best model for ferrocenopapain. It is likely that the environment around the enzyme-bound ferrocene is significantly changed relatively to any easily envisaged and readily-accessible 'classical' ferrocene from organometallic chemistry. For example, it is held at a water:protein interface, one side being hydrophilic, the other most likely hydrophobic. Only one cyclopentadienyl ring on the iron will be free to rotate (see Figure 5) giving potential novel NMR properties. Also, the site in which the ferrocene has been bound is formed by the 'surface' side-chains of the local L-amino-acids around the confines of the active-site. The ferrocene is now effectively chiral because of its relationship to the fixed chirality of the active-site, close in its proximity. If reactions were carried out at such an immobilised organometallic in general, they would be expected to be enantioselective as a result of this chiral environment.

Molecular graphics analysis of papain modified at cys_{25} by reaction with (**2**) using the coordinates of papain with cys_{25} alkylated by Z-phe-ala-CH_2Cl[33] allows the C=O group of the ferrocenoketomethylene-group ($FcCOCH_2$–) to make a hydrogen-bond with trp_{26} backbone NH in the manner observed in the crystal structure of papain which had reacted with Zphe-ala-CH_2Cl.[33] This locates the ferroceno group into a hydrophobic pocket adjacent to val_{133}.

The changed reactivity of the ferrocenium ion of ferrocenopapain makes it reasonable to expect that carefully-modified organometallic compounds (selectively bound, with appropriate enzymes as one of their ligands!) would have unusual and novel chemical reactivities. In addition, CFc (**3**) has potential as a heavy metal label for use in protein crystallography/electron microscopy.

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