Mechanism of the Reaction of Papain with Substrate-Derived Diazomethyl Ketones

IMPLICATIONS FOR THE DIFFERENCE IN SITE SPECIFICITY OF HALOMETHYL KETONES FOR SERINE PROTEINASES AND CYSTEINE PROTEINASES AND FOR STEREOELECTRONIC REQUIREMENTS IN THE PAPAIN CATALYTIC MECHANISM

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The reactions of papain (EC 3.4.22.2) with substrate-derived diazomethyl ketones reported by Leary, Larsen, Watanabe & Shaw [*Biochemistry* (1977) **16**, 5857–5861] are unusual in that (i) these reagents fail to react with low-molecular-weight thiols and (ii) the rate of reaction with the papain thiol group does not decrease to near-zero values across a pK_a of 4 as the pH is decreased. Existing data are shown to suggest an interpretation involving neighbouring-group participation via transient thiohemiketal formation, rate-determining protonation by imidazolium ion and alkylation on sulphur via a three-membered cyclic transition state. Implications for (a) the difference in site-specificity exhibited by halomethyl ketones in their reactions with serine proteinases and cysteine proteinases and (b) stereoelectronic requirements in the mechanism of papain-catalysed hydrolysis are discussed. The possibility of two tetrahedral intermediates between adsorptive complex and acyl-enzyme is indicated.

The thiol group of papain is in the side chain of cysteine-25 and is part of the catalytic site of the enzyme, being transiently acylated during catalysis (see Lowe, 1976). The reactions of this thiol group with a wide variety of electrophilic reagents have been studied under conditions of reagent concentration that provide for the order of reaction with respect to concentration to be 2, first-order in enzyme and first-order in reagent. Since for these reactions the apparent second-order rate constant, k , is less than $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at all pH values, the pH-dependence of k probably provides macroscopic pK_a values characteristic of the free reactant molecules (Brocklehurst & Dixon, 1977).

Despite the variety of shapes of pH- k profile for different reactions of the papain thiol group (see, e.g., Wallenfels & Eisele, 1968; Chaiken & Smith, 1969; Polgar, 1973; Furlanetto & Kaiser, 1973; Shipton & Brocklehurst, 1977, 1978) all possess one common feature, a decrease in k to essentially zero at low pH dependent on a pK_a value approx. 4. This decrease in k would be predicted if the thiol group does not possess nucleophilic character when it is unionized and in the environment of the imidazolium ion of histidine-159, i.e. the XH_3^+ state:

Stepwise ionization of this diad at higher pH values provides two protonic states of the active centre in

which the sulphur atom possesses nucleophilic character (see Shipton & Brocklehurst, 1978). The first ionization (pK_a approx. 4) produces the XH state:

and the second (pK_a approx. 8–9) produces the X state:

Reactions are known in which the reactivity of the XH state of the enzyme is greater than that of the X state (see, e.g., Wallenfels & Eisele, 1968; Chaiken & Smith, 1969; Furlanetto & Kaiser, 1973; Shipton & Brocklehurst, 1977) as it is in the acylation step of the catalytic act (see Lowe, 1976). There are other reactions, however, in which the reactivity of the X state of the enzyme is greater than that of the XH state (see, e.g., Polgar, 1973; Shipton & Brocklehurst, 1978). It is important to emphasize that, whatever the relationship of the reactivities of the X and XH states of the enzyme in all these various reactions, it was invariably found that k decreases in acidic media across a pK_a of approx. 4 as the unreactive XH_2 state is approached.

It is the purpose of the present paper to point to an exception to this generalization and to suggest an interpretation of the anomalous pH- k profile, which (1) could provide an explanation for the difference in site-specificity exhibited by halomethyl ketones

towards serine proteinases and cysteine proteinases and (2) may have implications for the catalytic mechanism of papain.

Discussion

I. Mechanism of the reactions of substrate-derived diazomethyl ketones and halomethyl ketones with papain

Leary *et al.* (1977) reported that the value of k for the reactions of papain with benzyloxycarbonyl-phenylalanyl diazomethyl ketone and benzyloxycarbonylphenylalanylphenylalanyl diazomethyl ketone in which the papain thiol group becomes alkylated decreases with increasing pH above pH 6.5 according to a pK_a approx. 8.5 and remains constant in the pH range 4.0–5.5 at a value approx. 78% of that at pH 6.5. The pH-dependence of these reactions was contrasted with that of the reactions of papain with substrate-derived chloromethyl ketones (Bender & Brubacher, 1966; Whitaker & Perez-Villasenor, 1968) and with chloroacetamide (Chaiken & Smith, 1969). In these reactions the reactivity of the X state of the enzyme is greater than that of the XH state, i.e. k increases with pH above 6.5.

Leary *et al.* (1977) suggested that the pH-dependence of k for the reactions of papain with the diazomethyl ketones might be accounted for by the mechanism shown in Scheme 1(a), which is analogous to that proposed by French *et al.* (1963) for the reaction of glutamine amidotransferase with azaserine (see also Buchanan, 1973). Another possible mechanism is shown in Scheme 1(b). For both mechanisms it is necessary to propose that the protonation on carbon of the bound [Scheme 1a, reaction (i)] or covalently bonded (Scheme 1b, reactions 4 and 5) reagent is rate-determining. For Scheme 1(b) it is proposed that low quasi-equilibrium concentrations of the various ionic forms of the thiohemiketal intermediate are formed rapidly at all pH values in the range studied. The pH-dependence of k in this range should then reflect the ionization of the active-centre imidazolium ion of the free enzyme (see Brocklehurst & Dixon, 1977). Only at pH values where the concentration of the nucleophilic XH state of papain has decreased to a value that makes thiohemiketal formation (Scheme 1b, reaction 2) rate-determining would the value of k be expected to decrease markedly with decreasing pH. There is no reason to suppose that this decrease in k should depend upon pK_1 (approx. 4) of the papain active centre (i.e. the pK_a for $XH_2 \rightleftharpoons XH$). The 'mirage pK_a ' (see Dixon, 1973) that would result from such a change in rate-determining step could well be much less than 4. In the analogous pH- k profiles for the formation of acetoxime and furfuraldoxime (Jencks, 1959) the mirage pK_a values (approx. 3 and 1.5 respectively) are (a) significantly different from each

other and (b) well below the pK_a of the hydroxylammonium ion (approx. 6).

Some arguments in favour of Scheme 1(b) as against Scheme 1(a) are given below.

(1) Whatever the exact structure of the XH state of papain (see Polgar, 1974, 1977; Shipton *et al.*, 1975; Dixon, 1976; Lowe, 1976; Malthouse & Brocklehurst, 1976; Klinman, 1978) any unionized thiol that is present is probably hydrogen-bonded to the neutral histidine imidazole group and it is difficult to envisage that this shared proton would be readily available for protonation of the diazoketone.

(2) A more probable protonating site is the active-centre imidazolium ion, which is present in both the XH_2 and XH states of the enzyme. The difficulty with Scheme 1(a) that the proton of the active-centre cysteine-histidine system of the XH state of papain may be constrained to lie between the sulphur and nitrogen atoms and thus may not be available for reagent protonation is removed if the sulphur atom is engaged in thiohemiketal formation as it is in Scheme 1(b).

(3) The nucleophilic character of the XH state of papain (which will exist in finite amount even at pH 4) and the ability of keto groups to undergo rapid and reversible addition of nucleophiles (see Lewis & Wolfenden, 1977) including thiols (see, e.g., Lienhard & Jencks, 1965) compel the view that the most probable covalency change that would follow adsorptive binding in a manner analogous to substrate binding would be thiohemiketal formation. Poulos *et al.* (1976) (see also Weiner *et al.*, 1966; Kézdy *et al.*, 1967) have presented cogent argument that the alkylation of the active-centre imidazole groups of serine proteinases by halomethyl ketones proceeds through the intermediacy of hemiketals involving the active-centre serine hydroxy groups and the reagent keto group.

It thus seems possible or even probable that adduct formation involving a reagent keto group and an active-centre hydroxy or thiol group may be a common feature of the reactions of halomethyl ketones and diazomethyl ketones with both serine proteinases and cysteine proteinases. If this is the case it is necessary to seek an explanation for the difference in the alkylation site observed for the two types of enzyme [i.e. imidazole in the serine proteinases (see Poulos *et al.*, 1976) and thiol in the cysteine proteinases (see Bender & Brubacher, 1966; Whitaker & Perez-Villasenor, 1968)]. The difference might have been explained in terms of hard-soft acid-base considerations (Pearson, 1963; Pearson & Songstad, 1967) and the general insensitivity of thiolate nucleophilicity to the thiol pK_a , for mechanisms like that in Scheme 1(a). Intermediate adduct mechanisms, however, at first sight seem to demand alkylation on imidazole for both serine proteinases and cysteine proteinases. One property of bivalent

Scheme 1. Two mechanisms for the alkylation of the thiol group of papain by substrate-derived diazomethyl ketones (a) Mechanism due to Leary *et al.* (1977); (b) mechanism involving the formation of intermediate thiohemiketals. The forms shown that do not involve covalent bonding of enzyme and reagent represent adsorptive complexes. Free protons are omitted for clarity. For further details see the text.

sulphur as against bivalent oxygen that could account for the difference in site-specificity by intermediate adduct mechanisms is the property of sulphur to participate in three-membered cyclic transition states (see Capon & McManus, 1976), which contrasts markedly with the lack of ability in this respect of bivalent oxygen (see, e.g., Peters & Walker, 1923; Bartlett & Swain, 1949). Thus in Scheme 1(b), after the rate-determining proton transfer from nitrogen to carbon, the sulphur atom could readily be alkylated either before or during collapse of the tetrahedral thiohemiketal.

II. Implications for the papain catalytic mechanism

The above interpretation of the mechanism of the reaction of papain with diazomethyl ketones and with halomethyl ketones may have implications for the mechanism of papain-catalysed hydrolysis. The reaction of diazomethyl ketones is unusual among the many reactions that have been used to study the reactivity of the papain thiol group in that the atom in the reagent that becomes protonated by the papain imidazolium ion is the same atom that finally becomes bonded to the papain sulphur atom. It seems

reasonable to suppose that S-C bond formation in the three-membered cyclic transition state as against N-C bond formation will be facilitated by or may even require movement away of the histidine imidazole group. The probable steric requirements of this unusual reaction of papain support the idea that movement of the histidine side chain may need to occur to permit the catalytic act. The X-ray crystallographic data indicate the possibility of rotation of this side chain by 30° around the C_(β)-C_(γ) bond (Drenth *et al.*, 1976), and it has been proposed that this type of movement is necessary to permit protonation of the leaving group in acylation (Lowe, 1976).

If the movement of the histidine side chain is restricted to that suggested by the X-ray crystallographic study, the existence of two tetrahedral intermediates between adsorptive complex and acyl enzyme would be predicted from the stereo-electronic theory proposed by Deslongchamps *et al.* (1972, 1973, 1975) as applied to acylation of α-chymotrypsin by Bizzozero & Zweifel (1975). Thus for papain the developing lone-pair orbital on the nitrogen atom of the scissile peptide bond of the substrate should be antiperiplanar to the new C-S bond of the initial tetrahedral intermediate. In this intermediate, protonation of the nitrogen atom by the imidazolium ion of histidine-159 could not occur because the lone-pair orbital of the nitrogen atom of the leaving group points in the wrong direction. Inversion at this nitrogen atom would be required to permit protonation. Inversion would produce a second tetrahedral intermediate in which the orientations of the lone-pair orbital and of the N-H bond are interchanged. Structural changes far more extensive than those indicated by X-ray crystallographic study, e.g. rotation around the C_(α)-C_(β) bond of the side chain of histidine-159, would be necessary to permit attack by the cysteine-25 sulphur atom and protonation by the imidazolium ion of histidine-159 without inversion at the nitrogen atom of the tetrahedral intermediate.

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