THE CASE FOR ASSIGNING A VALUE OF APPROXIMATELY 4 TO pKa_I OF THE ESSENTIAL HISTIDINE–CYSTEINE INTERACTIVE SYSTEMS OF PAPAIN, BROMELAIN AND FICIN

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Received 2 December 1974

1. Introduction

One of the p*K*a values that characterizes the pH-dependence of the kinetic parameters of reactions catalysed by the thiol proteases papain (EC 3.4.22.2.) bromelain (EC 3.4.22.4.) and ficin (EC 3.4.22.3.) is near to 4 [1–5]. All three enzymes each possess a histidine imidazole group within 5Å of their essential thiol group [6].

Of these enzymes, only papain is well characterized structurally: its essential (and only) thiol group (cysteine-25) is 7.5Å from the carboxyl side chain of aspartic acid-158 and 3.4Å from the N-1 of the imidazole group of histidine-159 [7].

It is commonly assumed that a particular state of ionization of one or other of these two groups is crucial to the catalytic process and that it is the ionization of this group (p*K*a ~ 4) that is reflected in the pH-dependence of the kinetic parameters. There is continuing discussion of whether this essential group of p*K*a ~ 4 should be assigned to aspartic acid-158 or to histidine-159. A recent paper by Murachi and Okumura [8] claims to show that the imidazolium ion of histidine-159 of papain and the essential imidazolium ion of bromelain are characterized by 'normal' p*K*a values near to 7 and thus are unlikely candidates for the essential groups of p*K*a 4 in these enzymes. Another recent paper [9] claims to show that the carboxyl group of aspartic acid-158 is part of the catalytic site of papain and quotes the work of Murachi and Okumura [8] in support of its dismissal of histidine-159 as the essential group of p*K*a 4.

In our view the conclusions drawn by Murachi and Okumura [8] and by Löffler and Schneider [9] are inappropriate (see Results and discussion section). These authors appear to have overlooked a number of papers [10–17] from three different laboratories. In these papers, evidence is presented strongly suggestive of a p*K*a value near to 4 for the essential imidazolium ions (or more generally the histidine-cysteine systems – see later) of these enzymes.

The present paper reports for the first time kinetic evidence that the active centre of papain is characterized not merely by one p*K*a value near to 4, but by two such values in addition to the p*K*a ~ 9 commonly assigned to the thiol group of cysteine-25 (but see later!). Similar sets of values appear to characterize the active centres of bromelain and ficin.

2. Experimental

Fully active papain, bromelain and ficin were prepared by covalent chromatography using the method of Brocklehurst et al. [18]. All enzyme preparations contained 1.0 mol of thiol with high reactivity towards 2,2′dipyridyl disulphide (2PDS) at pH 4 (see [14]) per mol of protein. Kinetic studies on the reactions of the three enzymes with excess of

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2PDS were carried out under pseudo first-order conditions at 25.0°C, I = 0.1 using an Aminco Morrow Dual Wavelength Stopped-Flow Spectrophotometer. The formation of 2-thiopyridone was monitored at 343 nm (see [14]) and the reference wavelength was either 385 nm or 450 nm.

A molecular model of papain based on the co-ordinate determinations of Drenth et al. [7] was purchased from Labquip, Reading, UK.

3. Results and discussion

We have reported pH-apparent second order rate (k_2) profiles for the reactions of 2PDS with papain [11,13] and with bromelain [12]. The most striking features of these profiles are the large rate maxima at pH values ~ 4 where the reactions are much faster than the reactions of 2PDS with the thiolate ions of these enzymes in the plateau regions at high pH. We here report that the reaction of ficin with 2 PDS is characterized by a pH-k_2 profile of similar at least in the pH region above 3.8.

These rate maxima provided the first compelling evidence that the active centre cysteine thiol groups of these enzymes each interact with another group to provide a nucleophilic state additional to that obtained when both side chains are deprotonated. The rate maxima were considered to represent reactions of these interactive nucleophilic states with 2PDS protonated or partially protonated on one of its nitrogen atoms.

These results were obtained using equimolar second order conditions necessitated, using conventional spectrophotometry, by the rates of the reactions ($k_2 \sim 10^4$ M^{-1} s^{-1}). The features of the profiles that prompted further study of these reactions were the apparent identity of the molecular acid dissociation constants (see [19]) that characterize the rate maxima (pKa_I and pKa_{II}) and in particular the fact that the value of pKa_I (approx. 3.8) is substantially greater than the value of pKa_{II} of $2PDSH_2^{2+}$ (2.45, see [14]).

It seems probable that adsorptive complex formation could precede the reactions of the thiol groups of these enzymes with 2 PDS. The obvious alternative explanations of the non-identity of pKa_I of the profiles with pKa_{II} of $2PDSH_2^{2+}$ are (i) perturbation of pKa_{II} of $2PDSH_2^{2+}$ by adsorptive complex formation of $2PDSH^+$ with the enzyme and (ii) both pKa_I and pKa_{II} of the profiles characterize free enzyme ionizations.

If the latter is the case, these results could provide an important contribution to the description of the active centres of these enzymes.

It was possible to decide between the above alter-

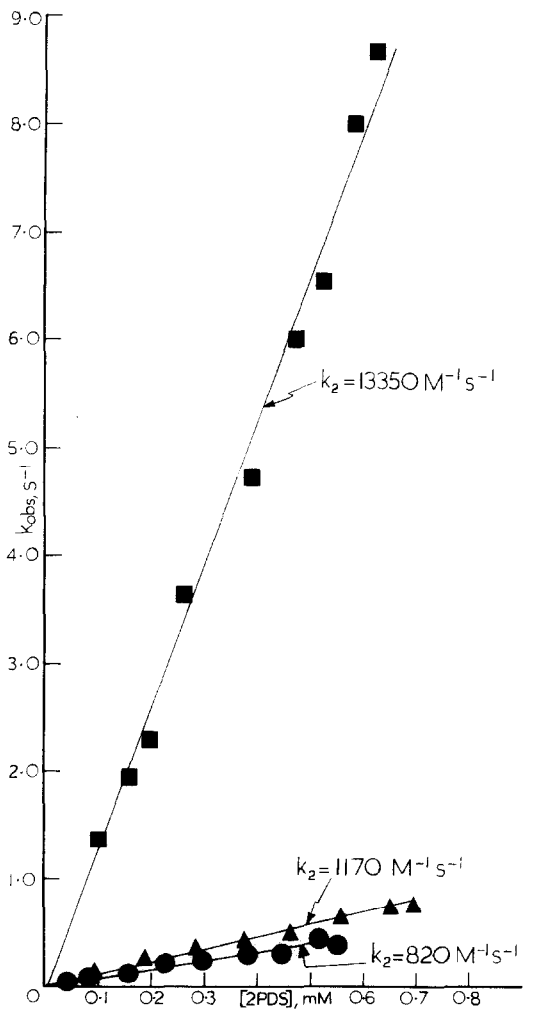

Fig.1. Dependence on [2PDS] of k_{obs}, the pseudo first order rate constant for the reaction of papain with 2PDS at 25°C, I = 0.1: ■–■–■, sodium formate buffer, pH 3.75; (●–●–●), phosphate (KH_2PO_4 + NaOH) buffer, pH 6.55; ▲–▲–▲, Tris–HCl buffer, pH 8.55. All solutions contained 1 mM EDTA. The highest [2PDS] is approx. 50% of its solubility in aqueous media.

natives by studying the reactions under pseudo first order conditions using an excess of 2PDS. For all three enzymes the reactions were studied at pH values 3.75, 6.55 and 8.55, where in each case one of the major reactive protonic states of the systems predominates [11,12]. In all cases the observed pseudo first order rate constants (k_{obs}) were linear in [2PDS]. Typical k_{obs} vs. [2PDS] plots are shown in fig.1. Because no degree of saturation of the enzyme by 2PDS is apparent, the p*K*a values of the profiles characterize free enzyme ionizations and not perturbed 2PDS ionizations. The acid limbs of the profiles are not due to denaturation. Sampling experiments established that the rates of loss of catalytic activity and of the high reactivity of the thiol groups towards 2PDS at pH 4 (see [10,14]) are very much slower (approx. 10^3 times) than the rates of the 2PDS reactions.

Further discussion is given in terms of papain, the only enzyme of the three for which detailed structural information is available. The rate of the reaction of the thiol group of cysteine-25 with 2PDS depends upon three ionizing groups in the papain molecule, characterized by p*K*a values approximately 4, 4 and 9. A molecular model of papain shows that the active centre region contains only three ionizing groups (cysteine-25, aspartic acid-158 and histidine-159) and it seems reasonable to associate the three molecular p*K*a values with these groups. The geometry of the active centre suggests that the high rate of reaction of papain with 2PDS at pH values around 4 may be attributed to the reaction of the cysteine-25 thiol group, rendered nucleophilic by its interaction with the imidazole group of histidine-159, with 2PDS hydrogen bonded to the carboxyl group of aspartic acid-158.

If one of the p*K*a values of 4 approximates to the group p*K*a of aspartic acid-158 this leaves molecular p*K*a values of 4 and 9 to describe successive proton losses from the imidazolium–thiol system of papain. A general description of this interactive system may be given in the elegant terms used by Dixon and Tipton [20] to describe the ionization of dibasic acids. Thus in a proportion of papain molecules, histidine-159 may be considered to ionize with p*K*a approximately 4 and cysteine-25 to ionize with p*K*a approximately 9. In the rest of the papain molecules, cysteine-25 ionizes with p*K*a 4 and histidine-159 with p*K*a 9. The proportion of each remains uncertain despite the interesting attempt by Polgar [21] to titrate both types of thiol group in papain. The ambiguity in Polgar's analysis arises from not knowing the molar extinction coefficient of the papain thiol group in any protonic state other than that at high pH where both cysteine-25 and histidine-159 are deprotonated.

A possible explanation of the identity of pKa_I and pKa_{II} of the profile (i.e. $pKa_{II} - pKa_I < 0.6$) is that the protonations of aspartate-158 and the conjugate base of the cysteine-25 thiol-histidine-159 imidazolium pair are positively cooperative (see [19]).

The above description of the papain active centre, provided by the 2PDS reactivity probe, is incompatible with the recent descriptions by Murachi and Okumura [8] and Löffler and Schneider [9]. Murachi and Okumura showed that the rates of both histidine loss and of activity loss by papain and by bromelain consequent on photooxidation using methylene blue are dependent formally on the base form of a group of p*K*a approximately 6.5. The rate of activity loss, however, is significantly greater than the rate of histidine loss. These results, therefore, provide no evidence that the essential histidine residues are characterized by p*K*a 6.5. Activity loss by papain consequent on photooxidation using proflavin results from the conversion of the indole ring of tryptophan-177 to a formylkynurenine residue and photooxidation of this residue seems to be preceded by photooxidation of tryptophan-69 [22]. Since tryptophan-177 shields histidine-159 from solvent [7,17] it may also shield it from the photosensitizing dye. Murachi and Okumura's results could be explained by a rate-limiting photooxidation of tryptophan-69 controlled by a p*K*a of 6.5. This may permit photooxidation of tryptophan-177 and subsequently of histidine-159. A possible candidate for this controlling p*K*a may be the interactive phenolic hydroxyl groups of tyrosine-67 and tyrosine-61. The expected similarity of the intrinsic p*K*a values of these groups in isolation predicts a large perturbation of the system p*K*a values, see [25].

Löffler and Schneider [9] showed that papain loses activity when one carboxyl group (presumed to be aspartic acid-158) is converted to an amide in which glycine ethyl ester is the amino component. If it is aspartic acid-158 that is modified by this large

group it is not surprising that papain loses activity because the backbone carbonyl group of this residue is probably part of the substrate binding site [26]. This result, however, in no way constitutes evidence that aspartic acid-158 rather than histidine-159 provides the additional nucleophilic state of cysteine-25.

Perturbation of the active centre p*K*a values by acylation of cysteine-25 in papain during catalysis or by alkylating this residue with a reporter group [27] will be discussed elsewhere. The dramatic difference in the pH-k_2 profiles for the reactions of 2PDS with purified bromelain (pH optimum 4, k_2 max 5×10^4 M^{-1} s^{-1}) and with partially purified bromelain (pH optimum 6, k_2 max 10^3 M^{-1} s^{-1}) (see [12]) is due to the tight binding by bromelain of a naturally occurring polypeptide which has been isolated by affinity chromatography using Sepharose-insolubilised bromelain [28].

Acknowledgements

We thank the Science Research Council for providing Research Studentships for M.S., M.P.J.K. and J.P.G.M. and a Postdoctoral Fellowship for T.S., the Medical Research Council for providing an Aminco Morrow Stopped Flow Dual Wavelength Spectrophotometer and Miss Angela Duffy for skilled technical assistance.

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