Evidence that the carboxy groups of Asp158 in papain and caricain have abnormally low $pK_a$ values and thus do not contribute the key ionisations with $pK_a > 4$ that generate catalytic competence

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Studies on a number of cysteine proteinases of the papain (EC 3.4.22.2) family using pyridyl- and pyrimidyl- disulphide reactivity probes have demonstrated that the catalytic site (Cys)-S-(His)-Im- is the ion-pair state of these enzymes at low $pH$ where the enzymes are inactive and that catalytic competence is generated by a protonic dissociation across a $pK_a > 4$ [1-4]. The assignment of the $pK_a > 4$ to the most obvious candidate, Asp158 (papain numbering), is not supported by studies on Asp158Asn mutants of papain [5] and papaya proteinase $\Omega$ (caricain, EC 3.4.22.30) [6]. In a present work studies on the reactions of papain and caricain with 4-chloro-7-nitrobenzofurazan ($\text{Nbf-Cl}$) using $pH$-dependent kinetics, spectral analysis of the resulting enzyme derivatives and computer modelling suggest $pK_a$ values for the carboxy groups in papain and caricain to be 2.8 and < 2 (possibly ca. 1.5) respectively.

Earlier studies on papain using $\text{Nbf-Cl}$ as a thiol-specific reactivity probe demonstrated abnormally high $pH$ activity at low $pH$ [7-9]. In the present work, improved stopped-flow methodology, together with the availability of SKETCHER [10] for incisive evaluation of multiprotonic state $pH$-$k$ data and the $pK_a$ values for (Cys)-S-(His)-Im- ion-pair formation now known to be 3.4 for papain [1] and 2.9 for caricain have allowed the characteristics of the $pH$-$k$ profiles for the reactions of papain and caricain with $\text{Nbf-Cl}$ to be properly defined.

The second-order rate constants ($k$) for the reactions of both enzymes in the $pH$ range ca. 2.5-7 at 25°C in aqueous buffer (pH 0.1, containing 6.7% v/v ethanol) were determined by stopped-flow kinetic analysis at 412nm in the case of papain (the isobestic point of the reaction of the product $\text{S-Nbf-papain}$ and at 417nm in the case of caricain (the $pH$-independent value of $\lambda_{max}$ of $\text{S-Nbf-caricain}$). The reactions, which were shown to obey overall second-order kinetics at pH 3 and pH 4.5, were studied using [electrolyte]$_0$=100mM and [Nbf-Cl]$_0$=800mM. Initial rates (v) were determined from the linear increase in $A_{412}$ or $A_{417}$ recorded over times ranging from 10s to 4 min and values of $k$ in M$^{-1}$ s$^{-1}$ were calculated by using the values of [enzyme]$_0$, [Nbf-Cl]$_0$, $e_{412}$=11360 M$^{-1}$ cm$^{-1}$ for the papain reactions and $e_{417}$=8400 M$^{-1}$ cm$^{-1}$ for the caricain reactions.

Inspection of the molecular environments of Asp158 in the crystal structures of the two enzymes and modelling of the S-Nbf-enzyme products were carried out using the computational techniques described in [11] using principally CHARMM and QUANTA.

The $pK_a$ profile for the reaction of $\text{Nbf-Cl}$ with papain in acidic media consists of a skewed bell-shaped curve ($pK_a$=2.8, $pK_a$=3.4, $pK_a$=4.0; $\tilde{\lambda}_{412}=97$ M$^{-1}$ s$^{-1}$, $\tilde{\lambda}_{417}=2.5$ M$^{-1}$ s$^{-1}$, $\tilde{\lambda}_{417}=1.9$ M$^{-1}$ s$^{-1}$). The major bell-shaped component bounded by $pK_1$ and $pK_2$ is interpreted as reaction of the thiolate anion in the (Cys255)-S-(His159)-Im- ion-pair produced across $pK_a$=3.4 with Nbf-Cl in a minor ionization state (i.e. from crossed-over ionisations) assisted by the undissociated carboxy group of Asp158 and inhibited by the carboxylate anion of this residue. The accumulation of electron density on the nitro group in the transition-state for the formation of the Meisenheimer-type intermediate would be stabilised by hydrogen-bond donation by (Asp158)-CO$_2$H and destabilised by (Asp158)-CO$_2$-. Computer modelling of the product, S-Nbf-papain, supports the possibility of these interactions. If the activation of Nbf-Cl in its reaction with papain is by direct interaction, the only hydrogen bond donor able to donate a proton to the nitro group of Nbf-Cl is (Asp158)-CO$_2$- and its $pK_a$ is thus determined as 2.8.

In marked contrast to the reaction of Nbf-Cl with papain, the reaction with caricain is not characterised by a bell-shaped $pH$-$k$ profile. Rather, $k$ increases with increase in $pH$ along a succession of sigmoidal components ($pK_a$=2 and probably ca. 1.5, $pK_a$=2.9, the $pK_a$= of (Cys)-S-(His)-Im- ion-pair formation, $pK_a$=3.9 and $pK_a$=5.8, $\tilde{\lambda}_{412}=0.1$ M$^{-1}$ s$^{-1}$, $\tilde{\lambda}_{417}=0.08$ M$^{-1}$ s$^{-1}$, $\tilde{\lambda}_{417}=0.9$ M$^{-1}$ s$^{-1}$, $\tilde{\lambda}_{417}=13$ M$^{-1}$ s$^{-1}$). The difference between the forms of the $pH$-$k$ profiles for the reactions of papain and caricain is suggested to be due to the different microenvironments of Asp158. Thus in papain one oxygen atom of the carboxy side-chain is exposed and available for interaction with the Nbf-Cl ligand, the other being hydrogen bonded to its own backbone -NH and that of Ala136. In caricain, however, both oxygen atoms of the Asp158 side-chain accept two hydrogen bonds from both the backbone -NH and the side-chain -NH$_3^+$ of Lys137 in one case and from both the backbone NH and the side-chain -OH of Ser136 in the other. The additional hydrogen bonding of the carboxylate in caricain could account for the lower $pK_a$ value (ca. 1.5) than that of the analogous group in papain ($pK_a=2.8$).

Support for the hypothesis that both ionisation states of the side-chain of Asp158 in papain interact with Nbf-Cl whereas neither ionisation state of the analogous side-chain in caricain has a major effect is provided by the electronic absorption spectra of the respective S-Nbf-derivatives. Thus $\lambda_{max}$=417nm for the caricain derivative and does not vary with $pH$ in the range 2.1-4.9. For the papain derivative, however, $\lambda_{max}$=424nm when the Nbf- label is in the presence of (Asp158)-CO$_2$H and $\lambda_{max}$=408nm when in the presence of (Asp158)-CO$_2$-.