Differences between the electric fields of the catalytic sites of papain and actinidin detected by using the thiol-located nitrobenzofurazan label as a spectroscopic reporter group

Keith BROCKLEHURST, Erdjan SALIH, and T. Stephen LODWIG

Department of Biochemistry, Medical College of St. Bartholomew's Hospital, University of London,
Charterhouse Square, London EC1M 6BQ, U.K.

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1. The catalytic-site thiol groups of papain (EC 3.4.22.2) and actinidin (EC 3.4.22.14) were each labelled with the nitrobenzofurazan (Nbf) chromophore by reaction with 4-chloro-7-nitrobenzofurazan at pH 4.4. 2. The electronic-absorption spectra of both labelled enzymes were determined in aqueous solution, in the pH ranges approx. 2–5 for *S*-Nbf-papain and approx. 3.3–8 for *S*-Nbf-actinidin, and for the latter also in 6M-guanidinium chloride. 3. The spectrum of *S*-Nbf-papain is characterized by $\lambda_{\text{max.}} = 402\text{ nm}$ at pH 5 and by $\lambda_{\text{max.}} = 422\text{ nm}$ at pH 2.18. The pH-dependent shift in $\lambda_{\text{max.}}$ accompanies a pH-dependent change in A_{430} , the nature of which is consistent with its dependence on a single ionizing group with $\text{p}K_a$ 3.7. 4. The spectrum of *S*-Nbf-actinidin is pH-independent in the pH range approx. 3.3–8 and is characterized by $\lambda_{\text{max.}} = 413\text{ nm}$. This absorption maximum shifts to 425 nm in 6M-guanidinium chloride. 5. These results are discussed and related to those reported previously from studies on papain and actinidin with various reactivity probes. 6. Despite the close similarity in the catalytic sites of papain and actinidin deduced from X-ray-diffraction studies, the considerable differences in their reactivity characteristics are mirrored by differences in their electric fields detected by the Nbf spectroscopic label. The microenvironment in the catalytic site of actinidin appears to favour the existence of ions significantly more than in the corresponding region in papain.

Continued study of the cysteine proteinases papain (EC 3.4.22.2) and actinidin (3.4.22.14) is demanded particularly by the seemingly contradictory observations that their catalytic sites display considerably different reactivity characteristics whereas the X-ray-diffraction studies appear to reveal closely similar structures. According to the X-ray-diffraction data reported for papain by Drenth *et al.* (1971, 1976) and for actinidin by Baker (1977, 1980), the catalytic sites of the two enzymes appear to be virtually identical.

A particularly sensitive method of detecting structural differences makes use of chemical reactivity and of its perturbation by the proton as the sterically least demanding ligand. Kinetic study of the modification of the catalytic-site thiol group of each of these enzymes may be particularly relevant for the enzyme's catalytic mechanism because it plays a nucleophilic purpose in the

catalytic act itself (for reviews see Lowe, 1976; Brocklehurst *et al.*, 1981a; Polgár & Halász, 1982). Some of the factors that control the expression of its nucleophilic character towards electrophilic reactivity probes, therefore, may control also its nucleophilic capability in the catalytic act. The catalytic sites of papain and actinidin have been investigated previously by using four types of reactivity probe each targeted on the thiol group of cysteine-25 in each enzyme, and all point to striking differences between the two enzymes (see Allen & Lowe, 1973; Shipton *et al.*, 1976; Brocklehurst & Malthouse, 1980; Brocklehurst *et al.*, 1981b, 1983; Salih & Brocklehurst, 1983).

The most obvious explanations for at least some of the differences between the catalytic-site reactivities of papain and actinidin involve differences in the relative dispositions of the ionic side chains (S^- , ImH^+ and CO_2^-) of the catalytic-site residues. The problem is that the crystallographic data provide no evidence for such conformational

Abbreviation used: Nbf, 7-nitrobenzofurazan.

differences. In the present work the nitrobenzofurazan group was covalently bonded to the catalytic-site thiol groups of papain and actinidin to serve as a spectroscopic reporter group. The electronic-absorption spectrum of this chromophore is sensitive to the electric field created by the catalytic site of each enzyme, and differences between the two enzymes detected by it are discussed.

Materials and methods

Papain was prepared as described by Baines & Brocklehurst (1979), and actinidin as described by Brocklehurst *et al.* (1981b). Samples of *S*-Nbf-papain and *S*-Nbf-actinidin were prepared for spectral analysis by reaction of the enzyme (20–35 μ M) with Nbf-Cl (1 mM) in 0.1 M-sodium acetate buffer, pH 4.4, followed by gel filtration on Sephadex G-25, essentially as described for *S*-Nbf-papain by Allen & Lowe (1973). Buffers were prepared as described by Dawson *et al.* (1969) and by Long (1971). Spectral analysis was carried out in a Cary 118C spectrophotometer on solutions of the *S*-Nbf-enzymes prepared from the stock solutions by dilution into various buffers. Concentrations of the order of 1 μ M were used to conserve material, and this necessitated the use of the 0–0.02 *A* range of the spectrophotometer.

Results and discussion

4-Chloro-7-nitrobenzofurazan (Nbf-Cl, I) has been used previously in studies on papain and actinidin as a reactivity probe, and in the case of papain also to provide a fluorescent reporter group (Allen & Lowe, 1973; Shipton *et al.*, 1976; Brocklehurst *et al.*, 1981b). The electronic-absorption spectrum of the Nbf label is sensitive to the nature of the substituent on C-4 (Birkett *et al.*, 1970) and is pH-independent in acidic media. The pH-dependent change in the spectrum of Nbf-Cl with apparent pK_a approx. 10 is due to Meisenheimer adduct formation by attack of OH^- at C-6 (Baines *et al.*, 1977).

Both Allen & Lowe (1973) and Shipton *et al.* (1976) considered the carboxy group of aspartic

acid-158 to be the most likely candidate responsible for the increase in the rate of the reaction of the papain thiol group with Nbf-Cl with decrease in pH (pK_a approx. 3.5). Inspection of a molecular model of papain suggests that, in the attack of the thiolate anion of cysteine-25 at C-4 of Nbf-Cl, the carboxy group of aspartic acid-158 could be close to the nitro group on C-7 or to N-1 of the ring system, where electron density would be expected to accumulate in the transition state. This transition state might be destabilized, therefore, by the carboxylate anion of aspartic acid-158 and/or stabilized (by hydrogen-binding) by the undissociated carboxy group. No analogous increase in rate as the pH is decreased is observed for the reaction of Nbf-Cl with the catalytic-site thiol group of actinidin (Brocklehurst *et al.*, 1981b).

In the present work the catalytic-site thiol groups of papain and actinidin were labelled by reaction with Nbf-Cl at pH 4.4. Allen & Lowe (1973) reported the long-wavelength band of the spectrum of *S*-Nbf-papain to have λ_{max} 402 nm and ϵ_{402} 12100 $\text{M}^{-1} \cdot \text{cm}^{-1}$, and that on 'denaturation' either with 6 M-guanidinium chloride or with an unstated concentration of HCl λ_{max} shifted to 422 nm. This value of λ_{max} is close to those (420–425 nm) reported by Birkett *et al.* (1970) for low- M_r thiols labelled by reaction of Nbf-Cl. In the present work the spectrum of *S*-Nbf-papain was shown to be characterized by $\lambda_{\text{max}} = 402$ nm at pH 5.0 and by $\lambda_{\text{max}} = 422$ nm at pH 2.18. The pH-dependent (and fully reversible) shift in λ_{max} provides for a relatively large pH-dependent change in A_{430} , and this change is shown in Fig. 1 to be consistent with its dependence on a single ionizing group with pK_a 3.7. The perturbation of the spectrum to shorter wavelengths from that typical of a labelled low- M_r thiol appears to occur therefore as a result of protonic dissociation with pK_a 3.7, and there is no evidence that this involves deprotonation of more than one group per molecule. Spectroscopic study of *S*-Nbf-actinidin showed λ_{max} (a) to be 413 nm and invariant in the pH range 3.3–8.0 and (b) to shift to 425 nm in 6 M-guanidinium chloride. Thus the spectrum of the Nbf label located on the catalytic-site thiol group of actinidin is perturbed by its microenvironment to a somewhat smaller extent than the spectrum of the Nbf label in papain. Furthermore, this perturbing influence is not removed by protonation either of a group with pK_a approx. 4 as it is in the case of papain or of a group with pK_a 5.5. A group associated with a pK_a of 5.5 appears to control the reactivity of the S^-/ImH^+ ion-pair in free actinidin towards the benzofuroxan reactivity probe (Salih & Brocklehurst, 1983). Thus the group in actinidin of pK_a 5.5 detected by benzofuroxan would appear not to be analogous to the group in *S*-Nbf-papain with pK_a

Fig. 1. Dependence on pH of ϵ_{430} of *S*-Nbf-papain in aqueous solution at 25°C

The points are values of ϵ_{430} calculated from values of A_{430} taken from absorption spectra of *S*-Nbf-papain (1.03 μM) in aqueous buffers (sodium formate and sodium acetate, 10.1). The continuous line is theoretical for:

$$\epsilon_{430} = \frac{\tilde{\epsilon}_{430} - \tilde{\epsilon}_{430}}{1 + K_a/[H^+]}$$

where $\tilde{\epsilon}_{430} = 6738 \text{ M}^{-1} \cdot \text{cm}^{-1}$, $\tilde{\epsilon}_{430} - 11417 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $pK_a = 3.7$. The concentration of *S*-Nbf-papain was calculated by using the recorded value of A_{402} at pH 5.0 and the value of $\epsilon_{402} = 12100 \text{ M}^{-1} \cdot \text{cm}^{-1}$ reported by Allen & Lowe (1973).

house, 1980). The inability of the Nbf label to detect the removal of the perturbing influence in actinidin by protonation in the pH range 3.3–8.0 suggests (a) that the group in actinidin with pK_a 5.5, detected by benzofuroxan, is not analogous to aspartic acid-158 in papain, i.e. aspartic acid-161, and (b) that, if aspartic acid-161 is responsible for the perturbation of the spectrum of the Nbf label, its pK_a is less than 3.3. If the group in actinidin with pK_a 5.5 that modulates the reactivity of S^-/ImH^+ is not the carboxy group of aspartic acid-161, it must be remote from the immediate catalytic-site region because SH (cysteine-25), ImH^+ (histidine-162) and CO_2H (aspartic acid-161) are the only ionic or ionizable groups in this region of the enzyme molecule.

Inspection of a molecular model suggests that if the S, ImH^+ and CO_2H groups of the papain catalytic site remain reasonably close to their crystallographically determined positions in *S*-Nbf-papain, N-3 of the Nbf group could be close to ImH^+ but ImH^+ would always be remote from N-1 and the NO_2 group. It suggests also that N-1 and the NO_2 group could be close to the carboxy group of aspartic acid-158, as was proposed for the transition state in the formation of *S*-Nbf-papain. Protonation of Im (which could be characterized by pK_a 3.7, a value close to pK_a values that control deacylation; see, e.g., Lowe, 1976) might be expected to shift λ_{max} from 402 nm to even shorter wavelengths. A more reasonable candidate for the perturbing group of pK_a 3.7 would seem to be the carboxy group of aspartic acid-158. The carboxylate anion was invoked to destabilize the transition state for *S*-Nbf-papain formation, and this anion might be expected to destabilize the excited state of the $\pi-\pi^*$ transition of the Nbf chromophore. The fact that the value of λ_{max} , when the group of pK_a 3.7 is proton-rich is close to its value for *S*-Nbf-papain in 6M-guanidinium chloride suggests that the undissociated form of the carboxy group affords no more stabilization to the excited state than solvent water.

The simplest interpretation of all of the data discussed above is that the state of ionization of the carboxy group of aspartic acid-158 in papain affects the reactivity of the S^-/ImH^+ ion-pair and the electronic-absorption spectrum of *S*-Nbf-papain. The perturbation of the absorption spectrum of *S*-Nbf-actinidin could be due to the carboxylate anion of aspartic acid-161. The fact that this perturbation is smaller than in *S*-Nbf-papain, and the fact that the perturbation is not removed by change of pH in range 3.3–8.0, suggests that the microenvironment of this carboxy group is not identical with that of aspartic acid-158 in papain. The pK_a of this group would seem to be significantly lower (<3.3) than that of the papain catalytic-

3.7 detected by the Nbf label. The simplest interpretation of the identity of this group with pK_a 3.7 is that it is the carboxy group of aspartic acid-158 and may be the same group that affects the kinetics of the reactions of the S^-/ImH^+ ion-pair with both 2,2'-dipyridyl disulphide and Nbf-Cl (see Shipton *et al.*, 1976; Brocklehurst & Malt-

site carboxy group (suggested to be 3.7). The lower pK_a deduced for this group may be indicative of a general effect that contributes to the properties of the whole of the catalytic site. Reactivity-probe studies (Brocklehurst *et al.*, 1981b) suggested that the production of the S^-/ImH^+ ion-pair is associated with a pK_a value approx. 3, a value lower by almost 1 unit than the pK_a that controls the production of the ion-pair in papain. The existence of a microenvironment in the catalytic-site region of actinidin that favours the existence of ions significantly more than the corresponding region in papain would account for the lower pK_a values controlling the formation of S^- and CO_2^- , the higher pK_a value controlling the deprotonation of ImH^+ and the marked preference (see Brocklehurst *et al.*, 1983) for an anionic reactivity probe expressed by actinidin, which contrasts with the relative rejection of the same probe by papain. The low isoelectric point of actinidin (3.1; McDowell, 1970) and the high isoelectric point of papain (approx. 9; see Brocklehurst *et al.*, 1981a) might have been expected to result in a lower pK_a for the carboxy group of aspartic acid-158 in papain than for the carboxy group of aspartic acid-161 in actinidin, which is the reverse of the situation that appears to obtain. Evidence that the catalytic site of papain might be affected by this general field effect was reported by Bendall & Lowe (1976). The results reported in the present paper suggest that the catalytic site of actinidin is markedly affected by a field effect that is more localized and specific than that deriving from overall surface charge.

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