The pH-Dependence of the Rate of Acylation of Papain by *N-trans*-Cinnamoylimidazole

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BENDER and BRUBACHER[1] have reported the preparation from papain (EC 3.4.4.10) and *N-trans*-cinnamoylimidazole (NTCI) of *trans*-cinnamoylpapain (TCP) in which the cinnamoyl group is covalently attached to the active site of the enzyme, probably as a thiol ester. These authors have shown that the deacylation of TCP to provide cinnamic acid and papain is dependent upon a single ionizing group of pK_a 4·69 which is required in the base form.

By making the simplifying assumption that the catalysis of the hydrolysis of NTCI by papain may be represented by (1) it can be shown that a knowledge of the pH-dependence of the maximum concentration of ES′ which occurs during catalysis[2,3] and k'_3[1] severally permits the computation of the pH-dependence of the pseudo first-order rate constant for acylation, $k_2/(1 + K_s/\mathrm{E}_0)$.

$$\mathrm{E} + \mathrm{S} \xrightarrow{K_s} \mathrm{ES} \xrightarrow{k'_2} \underset{+\mathrm{P}_1}{\mathrm{ES}'} \xrightarrow{k'_3} \mathrm{E} + \mathrm{P}_2 \quad (1)$$

It is here reported (see the figure) that acylation of papain by NTCI is dependent upon two ionizing groups of pK_a 4·48 and 5·25, one of which is required in the acid form and the other in the base form. A curve which is closely similar to that shown in the figure is obtained for the pH-dependence of the observed first-order rate constant for the hydrolysis of NTCI by papain, after correction for the aqueous hydrolysis.[3] The values of pK_a obtained from this curve are 4·39 and 5·31.

It is of interest to compare the papain-catalysed hydrolysis of NTCI with that of α-*N*-benzoyl-L-arginine ethyl ester (BAEE) a substrate which is used more commonly to assay this enzyme. Smith and Parker[4] have evaluated the kinetics of the hydrolysis of BAEE by means of the usual Michaelis–Menten formulation (2). The pH-dependence of the rate of deacylation of TCP is

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_0} \mathrm{E} + \mathrm{P} \qquad (2)$$

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[1] M. L. Bender and L. J. Brubacher, *J. Amer Chem. Soc.*, 1964, **86**, 5333.

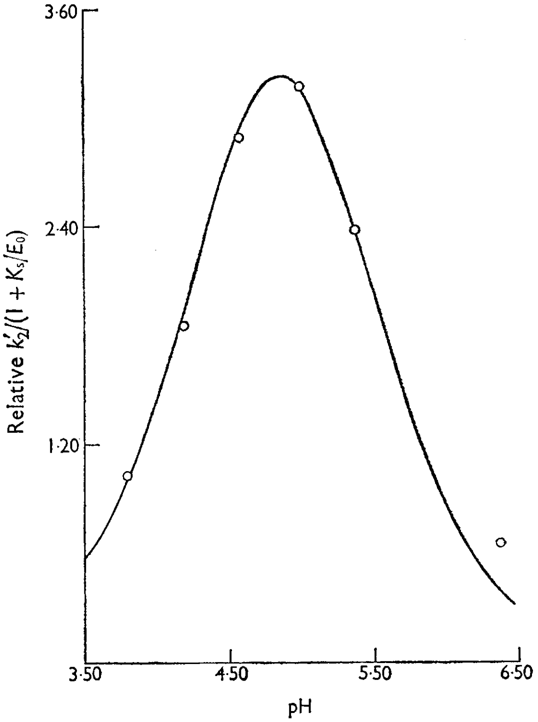
[2] K. Brocklehurst and A. P. Mathias, unpublished results.

[3] K. Brocklehurst and A. R. Njogu, unpublished results.

[4] E. L. Smith and M. J. Parker, *J. Biol. Chem.*, 1958, **233**, 1387.

similar to the pH-dependence of k_0 for the hydrolysis of BAEE. The pH-dependence of k_1 for this substrate shows that two ionizing groups of pK_a *ca.* 4 and 8 are required in the enzyme for activity, one is the acid form and the other in the base form. This curve, which probably represents the pH-dependence of the acylation of papain by BAEE, is ambiguous in that it does not specify which group is required in the acid form and which in the base form. By contrast, the pH-profile for the acylation of papain by NTCI, which contains an ionizing group of pK_a *ca.* 5 conjugated to its electrophilic centre, is not ambiguous; the group which is required in the acid form is probably the imidazole group of NTCI. Thus the group of pK_a *ca.* 4, which must be a group in the enzyme, must be required in the base form. By making the assumption that the same groups in papain are required for the acylation of the enzyme both by NTCI and by BAEE, the ambiguity of the pH-dependence of k_1 for the hydrolysis of BAEE may be eliminated. The group of pK_a *ca.* 4 is required in the base form and that of pK_a *ca.* 8 is required in the acid form.

It has been suggested that the group of pK_a *ca.* 8 is a thiol group.[4] That a thiol group is essential for the activity of papain is well established[5] and recently it was shown that a thiol group in this enzyme is acylated during its catalysis of the hydrolysis of methyl thionohippurate.[6] The results obtained with NTCI suggest that if the group of pK_a *ca.* 8 is a thiol group, it is required for activity as an unionized thiol group and not as a thiolate anion.



Dependence on pH of the rate of acylation of papain by NTCI at 25·0°.
The points are calculated from experimental values of $k_3'^1$ *and* $[ES']_{max}$.[3] *and the curve is theoretical for two ionizing groups of* pK_a 4·48 *and* 5·25.

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[5] E. L. Smith and J. R. Kimmel, in P. D. Boyer, H. Lardy, and K. Myrback, "The Enzymes", Academic Press, New York, 1960, Vol. 4, p. 133.

[6] G. Lowe and A. Williams, *Proc. Chem. Soc.*, 1964, 140.