# THE EFFECT OF ARGININE MODIFICATION ON THE pH DEPENDENCE OF PEPSIN ACTIVITY

T.M. KITSON and J.R. KNOWLES
*The Dyson Perrins Laboratory, University of Oxford, Oxford, OX1 3QY, England*

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The pH dependence of $k_{cat}$ or $k_{cat}/K_m$ for the pepsin-catalysed hydrolysis of small synthetic neutral peptides shows apparent $pK_a$-values of about 1.0 and 4.7 [1]. Knowles et al. [2] have discussed the possible ionising groups to which these $pK_a$-values might be assigned and have concluded that each of the two ionisations observed kinetically is probably due to an enzyme carboxyl group. If this is so, then the question arises as to why the first $pK_a$ of the pepsin system is so low (the 'normal' value for protein carboxyl groups being in the range 2.2–5.1 [3]). If two ionising groups of closely similar $pK_a$ form a hydrogen-bonded pair, then we may expect that the first $pK_a$ of the system will be much lower than the $pK_a$ of either group in isolation (the two $pK_a$-values of maleic acid are 1.83 and 6.07, and of fumaric acid, for which such an interaction is impossible, 3.03 and 3.15). Another possibility for the stabilisation of a carboxylate group relative to the protonated form would be provided by the formation of an electrostatic interaction between the carboxylate anion and an adjacent positive charge. In pepsin there is paucity of positive charges which might fulfil this requirement; the single lysine and the terminal α-amino group of the single polypeptide chain have been shown by acylation to have no effect on the proteolytic activity [4], and photooxidation of the single histidine shows this residue also to be non-essential [5]. There remain the two previously uninvestigated arginine residues, and this report is concerned with the action of an arginine-specific reagent on the activity of pepsin.

Of the reagents which have been used specifically to modify arginine residues in proteins, there are two which react in the pH range of stability of pepsin. These reagents are a crystalline trimer of butan-2,3-dione (biacetyl) developed by Yankeelov [6, 7], and phenylglyoxal used by Takahashi [8, 9]. The latter is readily available, can be easily prepared labelled with [$^{14}$C], and has the advantage, for pepsin modification, of having an aromatic group and being a rough substrate analogue of pepsin. Phenylglyoxal has been used in the pH range 5.5 to 8.0; the reaction is faster in the slightly alkaline region, but the side-reactions with lysine and histidine which are observed at pH 8.0 are virtually absent at pH 5.5 [8]. In the present work pH 5.5 was used since pepsin denatures at pH values above about 6.0.

Pepsin (5 mg/ml) was allowed to react at room temperature with 100 mM phenylglyoxal hydrate solution in pH 5.5 acetate buffer for 24 hr. Haemoglobin assay [10] showed that the remaining proteolytic activity was approximately 45% of that of a control solution containing no phenylglyoxal. A time-course study showed that this extent of inactivation was reached after about 4 hr and then remained constant, and was not increased upon the dissolution of further phenylglyoxal in the reaction mixture.

The haemoglobin assay is limited to a narrow pH range around 1.3. In order to investigate the properties of modified pepsin over a wider pH range, the continuous automatic ninhydrin technique of Cornish-Bowden and Knowles [1] was used. The modified enzyme was isolated by passage through a column of Sephadex G25 and assayed using *N*-acetyl-L-phenylalanyl-L-phenylalanylglycine as substrate. Again, at pH 1.3 a residual activity of the modified enzyme of about 45% was found; this increases linearly with pH until at about pH 4.7 the activity of the modified and unmodified enzyme are the same. Below pH 1.3 the activity of the modified enzyme increases rapidly suggesting that the inactivation is reversed in solutions of this acidity.

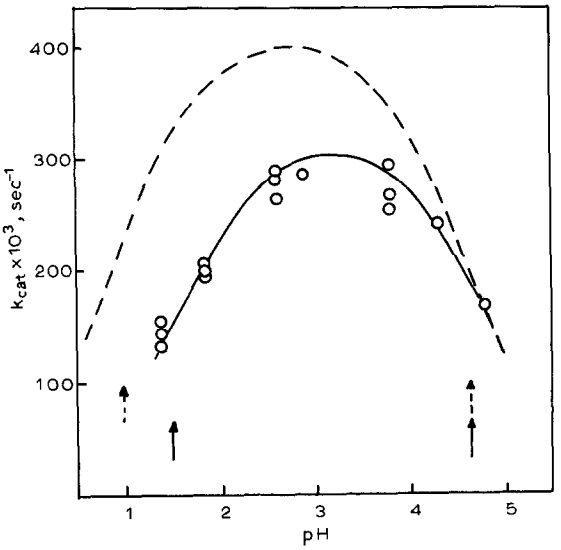


Fig. 1. The dashed curve is the pH dependence of $k_{cat}$ for native pepsin [11]. The lower curve is the corresponding plot for modified enzyme (see text). The arrows indicate the apparent $pK_a$-values for each curve.

Lineweaver-Burk plots show that there is little or no difference in the magnitude of $K_m$ for the modified and unmodified enzyme, whereas there is a significant difference in $k_{cat}$. This means that the inactivation takes place not by blocking of the enzyme's active site, but by an effect on the catalytic process subsequent to enzyme–substrate binding. We can also equate the percentage residual activity of treated pepsin at a particular pH directly with the ratio of the $k_{cat}$-values for the modified and unmodified enzyme. Thus from the plot of pH dependence of $k_{cat}$ given by Cornish-Bowden [1, 11] for native pepsin we have constructed a similar plot for the modified enzyme (fig. 1). This shows quite clearly that the $pK_a$ of 4.7 exhibit by the native enzyme is unchanged after modification by phenylglyoxal, whereas the $pK_a$ of 1.0 is raised to approximately 1.5.

In order to find the number of residues modified by phenylglyoxal in, for example, ribonuclease A, Takahashi [8] has used amino acid analysis after acid hydrolysis of the protein. This technique was applied in the present work but no difference in the arginine content of the modified and unmodified enzyme was observed. The result is expected in the view that the modification appears to be reversible below about pH 1.3. Takahashi performs the modification reaction at pH 7.0 or 8.0, in contrast to the pH of 5.5 used here; the greater stability of the modified residues when the reaction is performed at the higher pH is paralleled in the case of the other reagent mentioned above, butan-2,3-dione [6]. Thus as an alternative to amino acid analysis we are at present determining the number of groups modified, using $^{14}C$-labelled phenylglyoxal. If only one arginine residue reacts then it should be simple to elucidate which, since both occur in a region of known sequence near the carboxyl end of the polypeptide chain [12].

In summary, it has been shown that an arginine-specific reagent rapidly inactivates pepsin, causing an increase in the lower $pK_a$ exhibited by pepsin of approximately 0.5 of a pH unit. This may be due to the disruption of an interaction between one (or perhaps both) of the arginine residues in pepsin and the catalytically essential carboxylate anion. Interestingly, however, this can only be a partial answer to the question asked initially of why the $pK_a$ of the carboxyl group is so low, since even in the modified enzyme its value is only 1.5. Presumably some other factor (possibly hydrogen-bonded interaction, *vide supra*) is also effective.

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