Evidence for an Acyl–Pepsin Intermediate: a Re-appraisal

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Summary A reported trapping of a putative acyl–pepsin intermediate in the pepsin-catalysed hydrolysis of dipeptide substrates using radioactive methanol cannot be confirmed, and the intermediacy of an acyl–enzyme therefore remains in doubt.

IT has recently been reported by Akhtar and Al-Janabi[1] that, using radioactive methanol as a water analogue, evidence for an acyl–enzyme in pepsin-catalysed reactions has been obtained. Thus pepsin was incubated in the presence and absence of a pseudo-substrate (Z–L-Tyr) and of a substrate (Z–L-Tyr–L-Tyr) at pH 4·6 and 37° for 24 hr. in solutions containing 2·5% (v/v) CT_3OH. It was reported that after incubation in the absence of substrate or pseudo-substrate the protein had not incorporated any radioactivity after precipitation in trichloroacetic acid, whereas in the presence of such materials a significant fraction of the pepsin had been specifically esterified. Since during the long incubation time, enzyme autolysis gives rise to significant concentrations of substrate-like material (this phenomenon is readily observed by the pepsin "blank" rate when assayed by the appearance of ninhydrin-positive material[3]), it seemed to us surprising that no radioactivity was associated with the protein sample in the absence of explicitly added substrate or pseudo-substrate. A closer examination of these reactions reveals that the claim of specific esterification of pepsin is in error, and that the indications of an acyl–enzyme in pepsin-catalysed reactions continue to rest on indirect arguments of analogy to the neutral proteases.[2]

Under the conditions used earlier[1] it has now been found that the same level of radioactivity is associated with precipitated enzyme either in the presence of (a) Z–L-Tyr or Z–L-Tyr–L-Tyr, or (b) in the absence of any explicitly added substrate or pseudo-substrate. From the arguments above, it was important to check whether the radioactivity associated with the protein sample in (b) arises from the presence of autolytic substrate-like protein fragments. Accordingly, the following further controls were performed: (c) pepsin, incubated in the absence of CT_3OH, was precipitated into a solution of trichloroacetic acid containing CT_3OH; (d) pepsin, purified from small peptide impurities (which commercial pepsin usually contains) by gel filtration, was incubated as in (a) and (b); (e) experiments as in (a) and (b) were performed, and the incubation stopped after 10 min., 2 hr., and 24 hr.; and (f) pepsin was incubated with CT_3OH in the presence of the competitive inhibitor Z–D-Tyr, which is not a pseudo-substrate (in the sense that it is not a substrate for pepsin-catalysed ^{18}O-exchange with $H_2{}^{18}O$). In *all* of the above experiments, between 800 and 1000 c.p.m. were associated with washed protein precipitates from 0·9 ml. of reaction mixture. In the time-course study (e), the radioactivity associated with protein appeared to depend on the incubation time, and rose from close to 800 c.p.m. to approximately 1000 c.p.m. All experiments were performed in duplicate, the experiments agreeing to $\pm 3\%$.

The above experiments indicate that, at least by the method reported,[1] the acyl–enzyme postulate cannot be validated. Other methods of rapid precipitation of enzyme (*e.g.* by acetone) have yielded similar results. The radioactivity associated with precipitated protein appears [from experiments (c) and (d)] to be largely due to occluded CT_3OH, though a small contribution from direct esterification of the 39 free carboxyl groups[4] in native pepsin is also possible [experiment (e)].

As has been pointed out elsewhere[5] such experiments cannot rule out the intermediacy of an acyl–enzyme in reactions catalysed by pepsin, but until the existence of unequivocal data on this point, mechanisms avoiding the unnecessary postulate of acyl–enzyme formation can reasonably be preferred.[5]

The question of direct proof for a covalent *amino*–enzyme intermediate continues to concern us.[6] Exactly analogous problems of small amounts of radioactive material adventitiously bound to precipitated protein have

to be faced in this case too, and direct proof of the amino–enzyme intermediate must await a demonstration that pepsin has been *specifically* labelled by substrate to an extent and in a time consistent with the achievement of the steady state.

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[1] M. Akhtar and J. M. Al-Janabi, *Chem. Comm.*, 1969, 1002.
[2] See A. J. Cornish-Bowden, P. Greenwell, and J. R. Knowles, *Biochem. J.*, 1969, **113**, 369.
[3] A. J. Cornish-Bowden and J. R. Knowles, *Biochem. J.*, 1969, **113**, 353.
[4] T. J. Rajagopalan, S. Moore, and W. H. Stein, *J. Biol. Chem.*, 1966, **241**, 4940.
[5] J. R. Knowles, *Phil. Trans.*, Ser. *B*, in the press.
[6] M. Akhtar and J. M. Al-Janabi, *Chem. Comm.*, 1969, 859; J. R. Knowles, R. S. Bayliss, A. J. Cornish-Bowden, P. Greenwell, T. M. Kitson, H. C. Sharp, and G. B. Wybrandt, "International Symposium on Structure, Function Relationships of Proteolytic Enzymes," Copenhagen, June 1969, in the press; C. Godin and C. Y. Yuan, *Chem. Comm.*, 1970, 84.