feasible and that these electrons are available for addition to organic compounds.

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Pepsin as an Esterase

Sir:

Current interest in the mechanism of pepsin action prompts us to report the rapid cleavage, by crystalline swine pepsin, of the depsipeptide benzyloxycarbonyl-L-histidyl-*p*-nitro-L-phenylalanyl-β-phenyl-L-lactic acid methyl ester (Z-His-Phe(NO_2)-Pla-OMe) at the ester bond linking the Phe(NO_2) and Pla residues. At pH 4, the rate of this hydrolysis exceeds the rate of cleavage of the peptide bond linking the Phe(NO_2) and Phe residues in the corresponding peptide benzyloxycarbonyl-L-histidyl-(*p*-nitro)L-phenylalanyl-L-phenylalanine methyl ester (Z-His-Phe(NO_2)-Phe-OMe). Both the depsipeptide and the peptide are cleaved by pepsin more rapidly at pH 4 than at pH 2, thus exhibiting a pH dependence similar to that observed previously for the action of pepsin on synthetic substrates of the type Z-His-Phe-Phe-OEt.[1]

Z-His-Phe(NO_2)-Pla-OMe (mp 134–136°) was prepared in 64% yield by the azide method, with Z-Phe-(NO_2)-Pla-OMe (mp 135–136°) as an intermediate, the latter having been made (65% yield) by the benzenesulfonyl chloride method.[2] The peptide Z-His-Phe-(NO_2)-Phe-OMe (mp 217–218° dec) was obtained in 85% yield by the azide method, with Z-Phe(NO_2)-Phe-OMe (mp 167–168°) as an intermediate, the latter having been made (93% yield) by the N,N′-dicyclohexylcarbodiimide method. The final products and all intermediates gave satisfactory elemental analyses and were homogeneous by thin layer chromatography.

Because these substrates, like those recently developed in this laboratory,[1] have a site of protonation at the imidazolyl group, they are moderately soluble in aqueous buffered media in the pH range 2–5, and the addition of an organic solvent is not required; such solvents have been shown to inhibit the action of pepsin on synthetic substrates.[3] In contrast to widely used pepsin substrates (*e.g.*, Ac-Phe-Tyr), the compounds used in the present work do not contain a free carboxylate group; the presence of such a group adjacent to the sensitive bond has been shown to inhibit pepsin action.[1] The replacement of the central L-phenylalanyl residue of Z-His-Phe-Phe-OMe by a *p*-nitro-L-phenylalanyl residue permits spectrophotometric measurement at favorable wavelengths of the kinetics of enzymic cleavage, but does not affect markedly the rate of pepsin action at the sensitive peptide bond. The results obtained by the spectrophotometric method and the ninhydrin method[1] for the cleavage of Z-His-Phe(NO_2)-Phe-OMe were identical. For measurement of the rate of cleavage of the ester linkage in Z-His-Phe(NO_2)-Pla-OMe the ninhydrin procedure is not applicable, and spectrophotometry is the method of choice.

The enzymic cleavage of the two substrates is restricted to the scission of the ester or amide bond between the Phe(NO_2) residue and the Pla or Phe residue. This was demonstrated for the depsipeptide by the isolation, from the peptic hydrolysate, of Z-His-Phe(NO_2) (mp 236° dec) in 78% yield, and thin layer chromatography showed β-phenyllactic acid methyl ester to be the only other product of hydrolysis. In the cleavage of Z-His-Phe(NO_2)-Phe-OMe, the increase in ninhydrin color stopped after 100% hydrolysis of one peptide bond, Z-His-Phe(NO_2) was isolated in 86% yield, and thin layer chromatography showed phenylalanine methyl ester to be the only ninhydrin-reactive component of the hydrolysate.

The initial rates of hydrolysis, as determined spectrophotometrically, were linear, and satisfactory Michaelis–Menten kinetics were observed; the values of K_M (app) and k_{cat} are shown in Table I. Comparison of

Table I. Kinetics of Pepsin Action on Synthetic Substrates[a]

Substrate	$K_M \times 10^5$, *M* pH 2.0	pH 4.0	$k_{cat} \times 10^2$, sec^{-1} pH 2.0	pH 4.0
Z-His-Phe(NO_2)-Pla-OMe[b]	40 ± 8	40 ± 3	13 ± 3	77 ± 4
Z-His-Phe(NO_2)-Phe-OMe[c]	52 ± 8	46 ± 3	7 ± 2	29 ± 3

[a] Enzyme preparation, twice-crystallized swine pepsin (Worthington lot PM 708); substrate concentration, 0.05–0.25 m*M* (5 points in plots of S/v against S); pH controlled by sodium citrate buffers (0.04 *M*); 37 ± 0.1°; initial rates followed with a Cary Model 15 recording spectrophotometer equipped with automatic sample changer. At pH 4, $\Delta\epsilon_{310} = 1060$ for the cleavage of the Phe(NO_2)-Pla bond, and 800 for the cleavage of the Phe(NO_2)-Phe bond; at pH 2, $\Delta\epsilon_{265} = -420$ for both substrates. Control experiments, in which either the enzyme or the substrate was omitted, showed no significant change in absorbance during the time period of the kinetic measurements. [b] Enzyme concentration 0.02 mg/ml (5.72×10^{-7} *M*) at pH 2; 0.005 mg/ml (1.43×10^{-7} *M*) at pH 4. [c] Enzyme concentration 0.04 mg/ml (1.14×10^{-6} *M*) at pH 2; 0.02 mg/ml (5.72×10^{-7} *M*) at pH 4.

the kinetic constants at pH 4 for Z-His-Phe(NO_2)-Phe-OMe with those for Z-His-Phe-Phe-OEt reported previously[1] ($K_M = 1.8 \times 10^{-4}$ *M*; $k_{cat} = 0.31$ sec^{-1}) indicates that the principal effect of the *p*-nitro group is to increase K_M slightly, without marked effect on k_{cat}. Of special importance is the finding that the values of K_M for Z-His-Phe(NO_2)-Phe-OMe and for Z-His-Phe(NO_2)-Pla-OMe are nearly the same, but that the value of k_{cat} at pH 4 for the ester is nearly three times that for the amide. The slower rate of hydrolysis of both substrates at pH 2 is a reflection of lower k_{cat} values, the K_M values being nearly the same at pH 2 and pH 4.

Benzyloxycarbonyl-L-histidyl-L-phenylalanyl-D-phenylalanine ethyl ester (mp 187–188°) and the corresponding LDL compound (mp 137–139°) are completely resistant to cleavage by pepsin and were found to be competitive inhibitors of the enzymic hydrolysis of both Z-His-Phe(NO_2)-Phe-OMe and Z-His-Phe(NO_2)-Pla-OMe. Lineweaver–Burk plots gave the same value of K_I (2.8×10^{-4} *M*) at pH 4.0 and 37° for the LDL compound with the two substrates, and

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similar values ($1.9 \pm 0.1 \times 10^{-4}$ *M*) for the LDL compound.

Lokshina, *et al.*,[4] have reported that, in the presence of 0.027 m*M* pepsin, acetyl-L-phenylalanyl-(β-phenyl)-L-lactic acid (2.7 m*M*) is cleaved to 30% (as measured by the disappearance of material reacting with NH_2OH to form a hydroxamate) in 48 hr at 37° and pH 2 (10–15% ethanol was present); no hydrolysis was observed at pH 4. When expressed as moles of substrate cleaved per minute per mole of enzyme, the rate measured by Lokshina, *et al.*, at pH 2 is approximately 0.01 min^{-1}; this may be compared to the hydrolysis of Z-His-Phe-(NO_2)-Pla-OMe (0.25 m*M*) by 0.00014 m*M* pepsin at an initial rate of 20 min^{-1} at 37° and pH 4. The present work, in confirming the conclusion reached by Lokshina, *et al.*, thus places the esterase activity of pepsin on a sounder experimental basis and provides suitable substrates for the study of the mechanism of pepsin action at ester bonds.

In view of the current interest in the development of specific chemical reagents for pepsin,[5–8] it may be noted that the results reported here make it desirable to determine the effect of chemical modification of this enzyme not only with proteins and synthetic peptides as substrates but also with suitable synthetic esters. In the present work it was found that the specific reagent L-1-diazo-4-phenyl-3-tosylamidobutanone[8] inhibits both the esterase activity (substrate, Z-His-Phe(NO_2)-Pla-OMe) and the peptidase activity (substrate, Z-His-Phe(NO_2)-Phe-OMe) of pepsin.

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Pre-annihilation Electrochemiluminescence of Rubrene

Sir:

Several reports of electrochemically generated luminescence resulting from electrolysis of solutions of fluorescent aromatic molecules have recently been published.[1–6] In most cases, the luminescence has been attributed to a radical anion–radical cation annihilation reaction which leaves one of the resulting neutral molecules in an excited state.

Alternative explanations for light-emitting reactions seen under some conditions have involved luminescent oxidation of the anion by either cation decomposition products[2] or solvent oxidation products.[3] Although we have found that the luminescence is brightest and most generally seen under conditions where the annihilation process is most likely, we have also observed light under conditions such that none of the above explanations is pertinent.[2,6]

Specifically, with certain aromatic hydrocarbons light can be detected while oxidizing the anion or reducing the cation at potentials insufficiently anodic or cathodic, respectively, to generate the oppositely charged radical ion and at potentials where there is no appreciable background electrolysis. Rubrene is a highly electrochemiluminescent molecule which typifies this kind of behavior, and therefore we report a more detailed study of the pre-annihilation electrochemiluminescence of this molecule.

The light out putfrom a 1×10^{-3} *M* solution of rubene in DMF with 0.1 *M* tetrabutylammonium perchlorate (TBAP) as supporting electrolyte was observed with a photomultiplier in two series of double-potential-step experiments. In the first series, designed to study the light output obtained while oxidizing the anion (formed at −1.37 v *vs.* sce and moderately stable), the potential of the first step was held at −1.6 v for 5 sec, and the potential of the second step was increased incrementally in the positive direction. The light output was measured 0.2 sec after initiation of the second step. A similar series of experiments was used to study the light produced while reducing radical cation (formed at +0.95 v and considerably less stable). In this case, the potential of the first step was held at +1.0 v, and the potential of the second step was shifted incrementally in the negative direction.

Light was detected while oxidizing at the anion voltages positive of −0.2 v and while reducing the cation at voltages negative of −0.95 v. The intensity of this pre-annihilation light, observed while oxidizing the anion and reducing the cation, was about one and two orders of magnitude, respectively, less than that observed when the potential excursion was great enough to produce the maximum intensity of anion–cation annihilation light.

Both of these threshold voltages are sufficiently far removed from the potentials at which the oppositely charged ion is produced to rule out a simple annihilation reaction as the source of light. Also it is apparent that light is produced while oxidizing the anion and reducing the cation at overvoltages well below the energy of the rubrene 0–0 singlet transition (2.3 ev). However, only singlet emission is seen. This requires a mechanism more involved than direct excitation to the singlet. One possibility is that this pre-annihilation light-emitting process initially generates triplets by a direct heterogeneous electron transfer which is followed by a triplet–triplet annihilation reaction to produce singlets.[7] This has been held

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