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Transpeptidation by Porcine Pepsin Catalyzed by a Noncovalent Intermediate Unique to Its Iso-mechanism*

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Porcine pepsin proteolysis of the hexapeptide Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe (where OMe = methoxy and Nle = norleucine) in the presence of dipeptide Leu-Leu synthesizes a new hexapeptide Leu-Ser-*p*-nitro-Phe-Leu-Leu. Contrary to transpeptidation kinetics of other proteases, which depend upon an acyl-enzyme intermediate, the time course for pepsin-catalyzed transpeptidation displays a distinct lag before reaching a steady-state reaction velocity. Moreover, this lag is coupled to burst kinetics for the formation of proteolytic products, Leu-Ser-*p*-nitro-Phe and Nle-Ala-Leu-OMe. The lag requires that free Leu-Ser-*p*-nitro-Phe accumulate in the reaction medium during the lag phase and subsequently rebind for transpeptidation. Consistent with this dissociative kinetic mechanism are normal solvent isotope effects on formation of the proteolytic products Leu-Ser-*p*-nitro-Phe (v_H/v_D = 2.2 ± 0.2) and Nle-Ala-Leu-OMe (v_H/v_D = 1.8 ± 0.1) as opposed to an inverse effect on the formation of the transpeptidation product Leu-Ser-*p*-nitro-Phe-Leu-Leu (v_H/v_D = 0.40 ± 0.09). Because proteolysis is slower in D_2O but transpeptidation is faster, the isotopically sensitive step must occur *after* release of both products of proteolysis, which precludes putative acyl-enzyme covalent intermediates. Isotopically enhanced transpeptidation is a new type of isotope effect but one that is consistent with the Uni Bi iso-mechanism previously postulated on the basis of solvent isotope effects on V_{max} but not on V_{max}/K_m (Rebholz, K. L., and Northrop, D. B. (1991) *Biochem. Biophys Res. Commun.* 179, 65–69) and confirmed by solvent isotope effects on the onset of inhibition by pepstatin (Cho, Y.-K., Rebholz, K. L., and Northrop, D. B. (1994) *Biochemistry* 33, 9637–9642). As a new biochemical mechanism for peptide bond synthesis that has a potential for applications in biotechnology, it is here proposed that the energy necessary to drive peptide synthesis from free peptides comes from the sizable free energy drop associated with rehydration of the active site of pepsin in 55 M water.

Porcine pepsin has long been known to catalyze transpeptidation as well as proteolysis (1). By analogy with serine proteases, the following mechanism was envisioned,

$$E + P_2\text{-}P_1 \rightleftharpoons E \cdot P_2\text{-}P_1 \xrightarrow{-P_1} P_2\text{-}E \xrightarrow{+H_2O} P_2 + E$$
$$P_2\text{-}E \xrightarrow{+N} E \cdot P_2\text{-}N \rightarrow P_2\text{-}N + E$$

SCHEME I

where E is the proteolytic enzyme, P_2-P_1 is the polypeptide substrate with the scissile bond indicated by the hyphen, P_1 is the first peptide product released by proteolysis, P_2 is the second peptide product, N is an accepting peptide, and P_2-N is the product of transpeptidation. P_2-E is a covalent acyl-enzyme intermediate that is the key to the transpeptidation mechanism in serine proteases; the covalent bond can be transferred to a hydroxyl of water to form a free carboxyl group, or it can be transferred to the amino terminus of an accepting peptide to form a new peptide bond. This has long been thought to be the only possible mechanism for transpeptidation. For example, Kasche (2) has written emphatically: "Only proteases that form covalent acyl-enzyme intermediates can be used as catalysts in kinetically controlled[1] peptide synthesis." However, amid considerable controversy on how such an acyl-enzyme might form with aspartic proteases, various reports of transpeptidation yielding a product akin to N-P_1 appeared, which led to an alternative proposal of an amino-enzyme intermediate, E-P_1 (3), although no direct evidence was ever reported (4, 5).

In opposition, James and Sielecki (6) argued from x-ray crystallographic data that the formation of a covalent intermediate is not likely to occur. More convincingly, Antonov *et al.* (7) reported the incorporation of ^{18}O from water into the scissle carbonyl of P_2-N transpeptidation products, which requires that P_2 attain a free carboxyl at some intermediate stage. More recently, Hyland *et al.* (8) invoked an enzyme-bound amide hydrate adduct to explain similar ^{18}O exchange data with the human immunodeficiency virus aspartic protease. In addition, the relatively long incubation times[2] and low yields raised possibilities that the transpeptidation products of aspartic proteases may not be initial products but rather may arise from some secondary reaction or perhaps from a contaminating protease (6).

Nevertheless, no alternative to a covalent mechanism was forthcoming, at least not until Rebholz and Northrop (9) found that pepsin obeys an iso-mechanism in which the enzyme finishes the chemistry of proteolysis in a different form than it began, and the isomerization back to the starting form is kinetically significant. This finding offered new possibilities for the chemical mechanism of transpeptidation by aspartic proteases. In an attempt to link the two, efforts were made to induce transpeptidation by manipulation of reaction conditions known to perturb the iso-mechanism. Solvent isotope effects were successful and are described below.

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[1] Kasche (2) makes a distinction between *kinetic-controlled* and *equilibrium-controlled* transpeptidation. In the former, the transpeptidation product is a transient intermediate arising from a sacrificial peptide amid an unfavorable equilibrium; in the latter, the equilibrium favors peptide bond synthesis, because the concentration of water is lowered by the addition of organic solvents.

[2] Long incubation times result, in part, from the necessity of using very dilute pepsin to avoid autolysis.

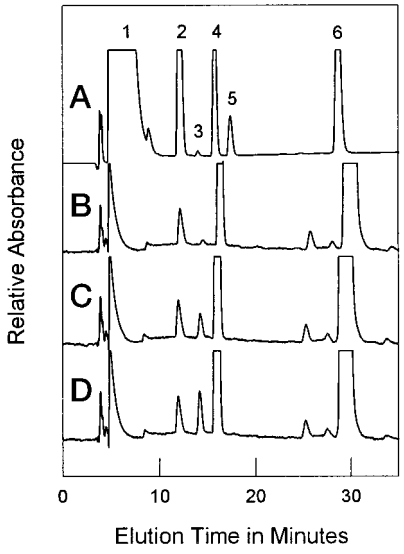

FIG. 1. **HPLC elution profiles of transpeptidation incubations.** Absorbencies in profile *A* were monitored at 214 nM at 16 h; profiles *B*, *C*, and *D* were monitored at 254 nm at 4, 8, and 12 h, respectively, on a different scale. Peaks were identified as: *1*, solvent front; *2*, Leu-Leu; *3*, Leu-Ser-*p*-nitro-Phe-Leu-Leu; *4*, Leu-Ser-*p*-nitro-Phe; *5*, Nle-Ala-Leu-OMe; *6*, Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe; unidentified peaks preceding 6 were impurities.

MATERIALS AND METHODS

Leu-Ser-p-nitro-Phe-Nle[3]-Ala-Leu-OMe, Leu-Leu(-OH), Leu-Leu-NH_2 and Boc-Leu-Leu were purchased from Bachem and D_2O from Cambridge Isotope Laboratories. Porcine pepsin was purchased from Sigma and purified on a Bio-Scale Q2 column (7 × 52 mm) equilibrated with 0.1 M acetate buffer, pH 4.0. A 0–1 M NaCl gradient was used with a flow rate of 2 ml/min. The purified enzyme was found to be homogenous by electrophoresis. Separation of reactant and product peptides was accomplished by reverse-phase HPLC using a μ-Bondapack C-18 HPLC column (3.9 × 300 mm, particle size 10 μm, pore size 125 Å) obtained from Millipore and a Hitachi L-6200 chromatographic system. Transpeptidation reaction mixtures were run at 25 °C in 40 mM formate buffer pH or pD 4.0 and contained 1.15 nM pepsin, 1.2 mM Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe and 6.43 mM acceptor peptide, Leu-Leu. During the course of the reaction, aliquot parts were taken at time intervals up to 16 h and assayed by HPLC reverse-phase chromatography, monitored at either 214 or 254 nm, using a 0–70% acetonitrile gradient at a flow rate of 5 ml/min.

Electrospray ionization mass spectra were collected in the positive-ion mode using a VG Platform II mass spectrometer (VG Analytical, Manchester, UK) and a methanol mobile phase. Samples were dissolved in 98% formic acid and diluted methanol to 0.1 mM. The diluted sample was pumped into the mass spectrometer source (60 °C) at a flow rate of 0.01 ml/min. Progress curves of the formation of peptide products were fitted to the following equation describing a hysteretic burst or lag as described by Frieden (10) using a BASIC computer program employing the nonlinear regression routine of Duggleby (11),

$$P = i + v_s t + \frac{(v_o - v_s)(1 - e^{-kt})}{k} \qquad \text{(Eq. 1)}$$

where P is the amount of product formed, i is the intercept (subtracted from the progress curves below), v_o is the initial velocity at time zero, v_s is the steady-state velocity, k is the rate constant controlling the hysteretic transition between v_o and v_s, and t is the elapsed time.

RESULTS

Fig. 1 shows the HPLC optical absorption elution profiles, which document a transpeptidation reaction between Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe and Leu-Leu in the presence of porcine pepsin. Peak 3 could not be detected in the absence of either peptide. A similar peak was observed with Leu-Leu-NH_2 as the accepting peptide, but not with Boc-Leu-Leu, which suggests a P_2-N transpeptidation product. The product was more absorptive at 254 nm than at 214 nm, which is further suggestive of P_2-N, because the P_1 product, Nle-Ala-Leu-OMe identified as peak 5, is transparent at the higher wavelength. A transpeptidation product of P_2-N in the form of Leu-Ser-*p*-nitro-Phe-Leu-Leu was confirmed by mass spectrometry of material from peak 3, giving a measured mass of 636.1 daltons (data not shown) as compared with a calculated value of 636.

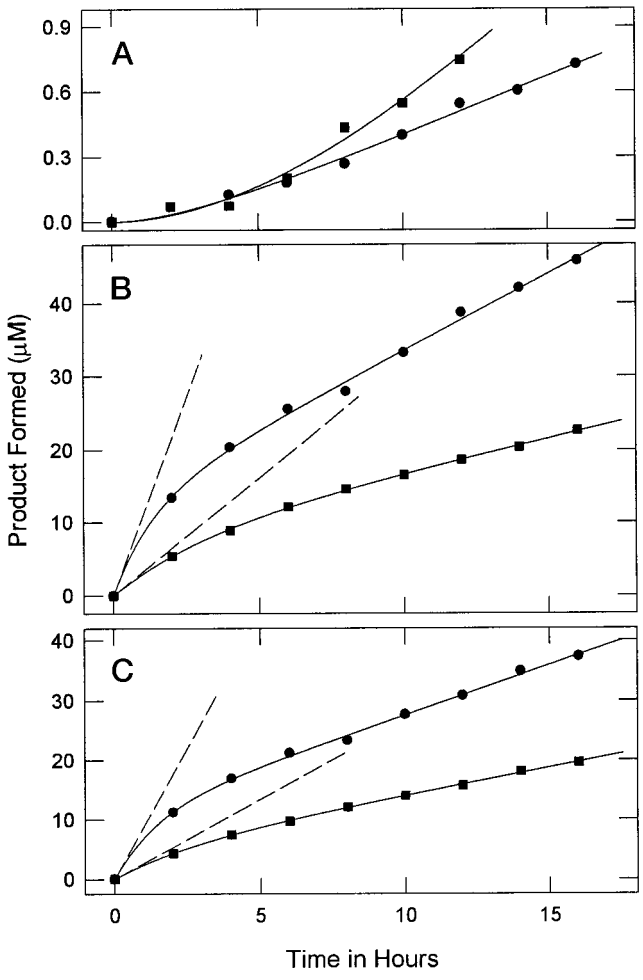

FIG. 2. **Time course of transpeptidation reactions.** Areas of peaks of Fig. 1 and other absorbance profiles were measured in H_2O (●) and in D_2O (■), compared with areas at the beginning (*A*) or the end (*B* and *C*) of the reaction representing 1.2 mM to calculate peptide concentrations and plotted as a function of reaction time. *A*, Leu-Ser-*p*-nitro-Phe-Leu-Leu; *B*, Nle-Ala-Leu-OMe; *C*, Leu-Ser-*p*-nitro-Phe.

The increase in areas of peak 3 in profiles *B–D* of Fig. 1 and other profiles not shown were converted to peptide concentration and are plotted as a function of time as shown in Fig. 2. A distinct lag is apparent in Fig. 2*A*, the progress of the formation of the transpeptidation product that is even more pronounced in D_2O, leading to a steady-state velocity that is more than twice as fast in D_2O than in H_2O and generating an inverse solvent isotope effect. Coincident with the lag is a burst in the progress of formation of the proteolytic products Nle-Ala-Leu-OMe and Leu-Ser-*p*-nitro-Phe shown in Fig. 2, *B* and *C*, respectively. These bursts are not seen in the absence of the accepting peptide or in the presence of Boc-Leu-Leu and are less pronounced in D_2O. The progress curves in both Fig. 2*B* and 2*C* develop more slowly in D_2O than in H_2O, thus generating normal solvent isotope effects, but the effects are not the same for both products.

[3] The abbreviations used are: Nle, norleucine; OMe, methoxy; Boc, *t*-butoxycarbonyl; HPLC, high performance liquid chromatography.

TABLE I
Reaction velocities and solvent isotope effects of proteolysis and transpeptidation

Product formed		Reaction velocities (s^{-1})		Solvent isotope effects	
		H_2O	D_2O	v_H/v_D	k_H/k_D[a]
Leu-Ser-*p*-nitro-Phe	v_o	3.2 ± 0.7	0.97 ± 0.06	3.29 ± 0.71	
	v_s	0.63 ± 0.04	0.28 ± 0.002	2.23 ± 0.18	2.24 ± 0.76
Nle-Ala-Leu-OMe	v_o	2.1 ± 0.4	0.64 ± 0.06	3.31 ± 0.59	
	v_s	0.40 ± 0.02	0.23 ± 0.01	1.78 ± 0.11	1.78 ± 0.55
Leu-Ser-*p*-nitro-Phe-Leu-Leu	v_o	0	0		
	v_s	0.014 ± 0.001	0.036 ± 0.007	0.40 ± 0.09	3.6 ± 1.8

[a] This new isotope effect is on the curvature of the plots in Fig. 2 (see Equation 1).

The results of fitting the progress curves to Equation 1 are listed in Table I. Proteolysis of the substrate was severely inhibited as compared with the k_{cat} of 133 s^{-1}, presumably due to competitive inhibition from Leu-Leu. Fitted initial velocities from Fig. 2*A* did not significantly differ from zero, so fittings were repeated with v_o masked to enhance the precision of v_s. The solvent isotope effects on initial velocities are the same for formation of Leu-Ser-*p*-nitro-Phe and Nle-Ala-Leu-OMe, but the effect on steady-state velocities is larger for the formation of Leu-Ser-*p*-nitro-Phe than for Nle-Ala-Leu-OMe *by an amount that matches the inverse solvent isotope effect on the formation of the transpeptidation product* (*cf.* 2.23 − 1.78 = 0.45 and 0.40). Moreover, the normal isotope effect on the steady-state formation of Leu-Ser-*p*-nitro-Phe (v_H/v_D = 2.23) *matches the reciprocal of the inverse solvent isotope effect on the formation of the transpeptidation product* (v_D/v_H = 1/0.4 = 2.5).

DISCUSSION

The lag in the transpeptidation reaction is consistent with a dissociation of Leu-Ser-*p*-nitro-Phe from pepsin followed by a dependence upon the accumulation of this proteolytic product in the reaction medium. In contrast, Kasche (2) describes the progress curves of transpeptidation by covalent acyl-enzyme intermediates as having a maximal rate at time 0 and progressively decreasing rates as products accumulate. The burst kinetics in the formation of proteolytic products similarly support a dissociative mechanism and are not consistent with any dependence upon an enzyme-bound product mechanism, covalent or otherwise. The correlation between lags and bursts shows a linkage between proteolysis and transpeptidation that effectively rules out participation of a contaminant enzyme or of a putative secondary reaction mechanism.

The linkage between lags and bursts is dramatically evidenced in the numerical symmetry of the data in Table I. The inverse isotope effect on transpeptidation rules out the traditional interpretation (that is, effects originating in a substrate bond-breaking chemical step, which are normal) and instead requires that the effect originate from some change in equilibria. Equilibrium isotope effects, termed "fractionation factors" by Kresge (12), have a long history and are rarely this small. Moreover, the linkage to normal isotope effects with a reciprocal magnitude is inconsistent with the origins described by Kresge. Instead, the mix of solvent isotope effects in Table I constitutes a new type of isotope effect, with a kinetic origin but with similarities to fractionation factors; it arises from a change in the quasi-equilibrium distribution of intermediate enzyme forms associated with the steady-state phase of enzymatic catalysis. The diversion of the Leu-Ser-*p*-nitro-Phe away from proteolytic product accumulation and toward transpeptidation places the isotopically sensitive step "downstream" from the steps participating in transpeptidation. It was previously reported that solvent isotope effects were observed on V_{max} but not on V_{max}/K_m (9), which only placed the isotopically sensitive step downstream from the first irreversible step, the release of the first product during initial velocity measurements. A reaction mechanism consistent with all solvent isotope effects is shown in Scheme II,

$$E + P_2\text{-}P_1 \rightleftharpoons E \cdot P_2\text{-}P_1 \xrightarrow{-P_1,\ -P_2} F \rightleftharpoons G \xrightarrow{+H_2O} E$$
$$F \xrightarrow{+N} F \cdot N \xrightarrow{+P_2} E \cdot P_2\text{-}N \rightarrow E + P_2\text{-}N$$

SCHEME II

where E = (H_2O)-pepsin$^{COOH}_{COO^-}$, F = pepsin$^{COO^-}_{COOH}$, and G = pepsin$^{COOH}_{COO^-}$. The nomenclature is similar to that used in Scheme I, but Leu-Ser-*p*-nitro-Phe is considered the second product, P_2, only by analogy. Because both products dissociate from the enzyme, the order of their dissociation is unknown and not designated. The dissociation of products is shown as being irreversible, because initially the concentration of products is zero. The starting form of enzyme, *E*, has water tightly bound at the active site and the two aspartic carboxyl groups are in the proper protonation state for catalysis. The product form of enzyme, *F*, is dehydrated, and the carboxyls are in the reverse state of protonation, having acted as a general acid and a general base during catalysis. Converting *F* to *G*, with the proper protonation, is the isotopically sensitive step. The final step in proteolysis is re-hydration of the re-protonated enzyme, shown here as being irreversible, because in 55 M H_2O, the equilibrium would be very far to the right. *F* can bind the accepting peptide, N, and after sufficient product has accumulated, *F*·N can bind P_2. Further support for this mechanism can be found in the solvent isotope effects associated with the onset of inhibition of pepsin by pepstatin (13), which also placed the isotopically sensitive step after the release of both products but before rehydration, with rapid binding of pepstatin to the *G* form of enzyme. In the new isotope effect, the analogy to the fractionation factor is actually a D_2O-dependent diversion of *F* away from the upper rehydration pathway back to *E*, toward the lower transpeptidation pathway.[4]

[4] By this analogy, the inverse isotope effect on transpeptidation might be termed a "diversion factor." Although new to isotope effects, these reaction kinetics themselves have been described before in the form of the induced transport kinetics described by Britton (16) and used to unequivocally establish iso-mechanisms. During induced transport, an *F* form of enzyme reacts with an isotopically labeled acceptor that is chemically identical with one product. For example, in the presence of an excess of $H^{12}CO_3^-$, bovine carbonic anhydrase type II reacts with $^{13}CO_2$ and catalyzes the synthesis of $H^{13}CO_3^-$ (17). Curiously, pepsin and carbonic anhydrase have similar isomerization segments in that both involve an intramolecular proton transfer step that is isotopically sensitive plus a step involving the binding of a molecule of water.

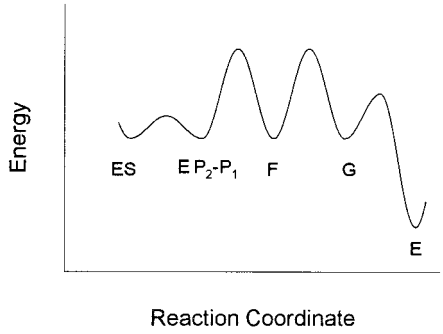

FIG. 3. **Free energy diagram for proteolysis catalyzed by pepsin.** Enzyme is saturated with substrate S, and the reaction effectively begins with *E*S. The first transition state represents peptide bond cleavage and is estimated to be much lower than the transition state for the release of products because of the lack of an isotope effect on *V*/*K*. The isotopically sensitive reprotonation step is between enzyme forms *F* and *G*. The large negative free energy for proteolysis is hypothesized to be focused on the rehydration of *G* to form *E*, the starting form of enzyme, which can then bind another molecule of substrate.

The chemical mechanism for pepsin is postulated to involve general acid catalysis by the carboxyl group of Asp-215, and general base catalysis by the carboxyl group of Asp-32 (5), which leaves the active site groups in the reverse state of protonation. Reprotonation of the carboxyls in a partially rate-limiting isomerization step of free enzyme is reminiscent of the iso-mechanism of proline racemase (14) in which two thiols in opposite states of protonation act as a general acid and general base. The racemization of proline, however, has an equilibrium constant of one and the reaction runs freely in both directions. For pepsin, the reverse reaction (transpeptidation) must be initiated before the drop in free energy associated with the overall proteolysis is expressed. Hence, the origin of the free energy needed to synthesize a peptide bond between free peptides is the tightness of the binding of a water molecule in the active site. As illustrated in Fig. 3, the energy level of enzyme form *F* and enzyme with a reactant containing an intact peptide bond, such as *E*S, must be fairly equivalent, but the energy levels of the hydrated and dehydrated forms of free enzyme, *E* and *G*, respectively, must differ, with the hydrated *E* form lying much lower than *G* in 55 M H_2O. It follows, that a similar negative free energy must be associated with the enzymatic removal of a water molecule from between neighboring carboxyl and amino groups in the formation of a peptide bond as an alternative pathway for hydrating the *F* form of enzyme and converting it back to *E* (see Equation 2).

$$\text{R-COOH}\cdot\boldsymbol{F}\cdot\text{H}_2\text{N-R} \rightleftharpoons \text{H}_2\text{O}\cdot\boldsymbol{E} + \text{R-CO-NH-R} \qquad \text{(Eq. 2)}$$

For this mechanism to account for the kinetics and solvent isotope effects of transpeptidation, it must be further postulated that the conversion of *F* to *G* be accompanied by a conformational change, which allows water to enter the active site. Hydration of the product form of enzyme, *F*, must await reprotonation, which is slower in D_2O and which in turn raises the steady-state concentration of *F*. The increase in [*F*] drives the increase in the rate of transpeptidation in D_2O. This change in [*F*] is the origin of the inverse solvent isotope effect and why it is analogous to an equilibrium isotope effect.

Pepsin-catalyzed transpeptidation is extremely sensitive to pH and other reactant conditions. For example, although peptide bond cleavage of slow substrates has a maximum rate at pH 3.5 (15), and therefore one might expect that the isomerization of free enzyme would be most rate-limiting, the transpeptidation described below could barely be detected at this pH. Equally confusing, at pH 5, where the chemical step is not at all favorable and therefore significantly rate-limiting, the original solvent isotope effect suggesting an iso-mechanism was first measured; nevertheless, transpeptidation was elusive and difficult to repeat at pH 5. But at pH 4, enzyme plus Leu-Ser-*p*-nitro-Phe-Nle-Ala-Leu-OMe and Leu-Leu consistently generate a large and reproducible peak on HPLC analysis, evidenced by the precision of the progress curves of Fig. 2. The sketchy and uncertain history of transpeptidation by aspartic proteases can now be appreciated with a more sympathetic understanding, especially for studies employing slow substrates, which we can now see as being very poor catalysts for transpeptidation because of the very low levels of *F* that would be present when the chemical segment is slow and rate-limiting. These reaction conditions may be exploited to develop practical methods of synthesizing specific peptide bonds. Kasche (2) noted the necessity for operating at a pH between the p*K* values of the carboxyl and amino groups to be joined together, that in many cases this occurs at acidic values of pH where many enzymes are unstable, and that "until better enzymes are found, this equilibrium-controlled synthesis is of no practical interest." Pepsin is a "better enzyme" with both stability and catalytic activity at acidic pH, plus a kinetic mechanism ideally suited to join *free* carboxyl and amino groups in peptide bonds provided one drives the peptide synthesis kinetically with a sacrificial peptide that is a fast enough substrate to ensure that most of the pepsin is in the *F* form.

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