Acyl and Amino Intermediates in Reactions Catalysed by Pig Pepsin

ANALYSIS OF TRANSEPTIDATION PRODUCTS

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The action of pig pepsin on a variety of small peptides including Leu-Trp-Met-Arg, Leu-Trp-Met, Leu-Leu-NH₂, benzyloxycarbonyl-Phe-Leu and Gly-Leu-Tyr was studied. Leu-Leu-Leu was found to be the major product from the substrates Leu-Trp-Met-Arg and Leu-Trp-Met, indicating that the predominant reaction at pH 3.4 was a transeptidation of the acyl-transfer type. Leu-Leu-Leu was also formed in high yield by amino transfer from benzyloxycarbonyl-Phe-Leu. Like the amino-transfer reactions the acyl transfer proceeded via a covalent intermediate, since [¹⁴C]leucine was not incorporated into transeptidation products and did not exchange with enzyme-bound leucine in the presence of acceptors. With Leu-Trp-Met both acyl and amino transeptidation products, namely Leu-Leu, Leu-Leu-Leu, Met-Met and Met-Met-Met, were formed in addition to methionine and leucine. With Leu-Trp-Met-Arg (1 mM) the pH optimum for the rates of hydrolysis and acyl transfer is about pH 3.4. At this pH the rate of acyl transfer exceeds that of hydrolysis; at pH 2, however, hydrolysis was faster than transfer. A comparison of the effect of the length of substrates and products on the reaction rates allows the conclusion that the binding site can extend over eight to nine amino acid residues. Although the experiments provide no conclusive evidence for or against the involvement of amino and/or acyl intermediates in the hydrolysis of long peptides and proteins, the high yield of transeptidation reactions of both types observed with some substrates suggests a major role for the intermediates in pepsin-catalysed reactions. The results also show that when pig pepsin is used for the digestion of proteins for sequence work, the likelihood of the formation of transeptidation products is considerable. In this way peptides not present in the original sequence could easily form in a reasonably good yield.

We have described (Takahashi *et al.*, 1974; Takahashi & Hofmann, 1975) some initial studies of the action of pig pepsin and penicillopepsin on small substrates. We showed that the enzymes catalysed transeptidation reactions that involved the transfer of an *N*-terminal amino acid via a covalent acyl intermediate. On the basis of these experiments it was proposed that catalytic mechanisms of the action of pepsin described so far do not account for all the observed reactions. Takahashi & Hofmann (1975) proposed that the pathway of pepsin- and penicillopepsin-catalysed reactions is determined by the nature of the substrate and that reactions may proceed via an amino intermediate, an acyl intermediate, or possibly no covalent intermediate. To evaluate further the significance of the covalent intermediate we decided to extend the study of substrates that show a transeptidation of either amino- or acyl-transfer type. Work published so far has described only amino transeptidation reactions in which the amino acid that was transferred was either phenylalanine, tyrosine or tryptophan (Neumann *et al.*, 1959; Fruton *et al.*, 1961; Kitson & Knowles,

1971b; Silver & Stoddard, 1972). The yields in these transfer reactions were generally low (less than 10%) compared with the yield of hydrolysis. This contrasts with some of the acyl transeptidation reactions described previously (Takahashi *et al.*, 1974). When pig pepsin or penicillopepsin acted on Leu-Tyr-NH₂ about 50 or 66% respectively of the *N*-terminal leucine was found in the transeptidation products Leu-Leu and Leu-Leu-Leu. In the present paper we describe in detail transeptidation reactions catalysed by pig pepsin. The reactions described are of both the amino- and the acyl-transfer type. Emphasis was placed in particular on obtaining quantitative data for the rate of formation and the distribution of the major transeptidation and hydrolysis products.

Materials and Methods

Materials

Pig pepsin (3× crystallized) was from Calbiochem, San Diego, Calif., U.S.A. (lot 300001). The following chemicals, purchased from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A. were used as received:

Cbz*-Leu-Leu, Cbz-Tyr-Leu, Cbz-Phe-Leu, Cbz-Leu-Met, Phe-Tyr-NH₂, Leu-Tyr-NH₂, Tyr-Phe-NH₂, Tyr-Leu-NH₂, Leu-Leu-NH₂, Met-Tyr-Phe-NH₂, Ala-Tyr-Phe-NH₂, Met-NH₂, Tyr-NH₂, Phe-NH₂, Leu-Leu-Leu, Gly-Leu-Tyr, Tyr-Tyr, Phe-Phe, Leu-Leu and Leu-NH₂ were obtained from Schwarz-Mann, Orangeburg, N.Y., U.S.A. Leu-Trp-Met, Leu-Trp-Met-Arg, Met-Met-Met and Met-Tyr were purchased from Research Plus Laboratories, Den-ville, N.J., U.S.A. All amino acids and peptides used were the L isomers. [¹⁴C]Leucine (10mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Analysis of reaction products by amino acid analyser. The modified amino acid analyser methods for the quantitative determination of amino acids and oligopeptides in the reaction mixture were described previously (Callahan *et al.*, 1970; Takahashi *et al.*, 1974). In the present study, the mixture of methionine, leucine, Met-Met, Leu-Leu and Leu-Leu-Leu was separated on a column (0.9cm×60cm) of UR 30 resin at 60°C with a Beckman-Spinco model 120C amino acid analyser. The buffers were 0.2M-sodium citrate, pH4.3, and 0.35M-sodium citrate, pH5.25. The flow rate was 70ml/h and the buffer change occurred at 50min. The elution times are shown in Table 1. The reaction products from Gly-Leu-Tyr were separated with the same buffers on a shorter column (0.9cm×20cm) with a buffer change at 40min. Tyrosine was eluted at 39min, Gly-Leu at 51min, Gly-Leu-Tyr at 75min, and Tyr-Tyr at 98min. Tyr-NH₂ was separated on a column (3.0cm×0.9cm) of PA 35 resin at 60°C, with a 0.35M-sodium citrate buffer, pH5.25, and eluted at 80min.

General procedure for transpeptidation and hydrolysis studies. Pig pepsin was incubated at 37°C with various substrates in 0.05M-sodium citrate at the pH indicated (final vol. 0.5ml). The reaction was stopped by immersion in boiling water for 10min. The precipitate was spun down and the supernatant was applied to the amino acid analyser. The quantitative evaluation of each component was based on the colour values obtained by running standard amino acids and oligopeptides. Met-Met and Met-Met-Met could not be determined accurately because they partially overlapped.

Analysis of reaction products by high-voltage electrophoresis. The reaction mixtures were separated as described previously (Takahashi & Hofmann, 1975) by high-voltage electrophoresis on Whatman 3MM paper in volatile buffer (pyridine/acetic acid/water, 1:10:89, by vol.) at pH 3.6 (Bennett, 1967) and Methyl Green was added as a marker (Stevenson, 1971). Cadmium/ninhydrin reagent (Heilmann *et al.*,

* Abbreviation: Cbz, benzyloxycarbonyl.

Table 1. Separation of amino acids and peptides on amino acid analyser

Standard mixtures of amino acids and peptides (0.1 μmol of each) were separated on a column (0.9×60cm) of UR 30 resin at 60°C with a Beckman-Spinco model 120C amino acid analyser. The buffers used were sodium citrate, 0.2M at pH4.3, and 0.35M at pH5.25; the buffer change occurred at 50min. The flow rate was 70ml/h.

Compound	Elution time (min)
Methionine	52
Leucine	61
Tyrosine	85
Phenylalanine	93
Met-Met	104
Met-Met-Met	106
Leu-Leu	112
Leu-Leu-Leu	120
Leu-Phe	145
Phe-Leu	168
Tyr-Tyr	184
Phe-Phe	237

1957) was used to locate the products containing free amino groups and the standard peptides. For the analysis of reaction mixtures containing [¹⁴C]leucine, guide strips were stained and the unstained part of the electrophoretogram was cut into 2 cm-wide bands. These were put into scintillation bottles containing *p*-bis-2-(5-phenyl-2,5-phenyloxazolyl)benzene (0.01%) and 2,5-diphenyloxazole (0.4%) in toluene, and their radioactivity was measured in a Nuclear-Chicago liquid-scintillation counter.

Results

Substrates involving acyl intermediates

The progress of the action of pig pepsin on Leu-Leu-NH₂ and Leu-Trp-Met-Arg is shown in Figs. 1 and 2 respectively. As we observed with Leu-Tyr-NH₂ (Takahashi *et al.*, 1974) the *N*-terminal leucine is converted into the products leucine, Leu-Leu and Leu-Leu-Leu. The action on Leu-Leu-NH₂ (Fig. 1) is comparable with that on Leu-Tyr-NH₂ observed by Takahashi *et al.* (1974) in both the rate of reaction and in the approximate distribution of products. The sum of the two transpeptidation products is about equal to that of the hydrolysis products. On the other hand, Leu-Trp-Met-Arg (Fig. 2) is a much better substrate. The approximate specific rate constant was $k_{sp.} = 5 \times 10^{-2}$ mol of substrate/s per mol of enzyme as compared with approximate specific rates of 7×10^{-4} mol of substrate/s per mol of enzyme for Leu-Leu-NH₂. (Because many of the substrates do not obey Michaelis-Menten kinetics, no attempt was made to measure K_m and k_{cat} values. It was nevertheless considered desirable, for comparative purposes,

Fig. 1. Progress curve of cleavage of *Leu-Leu-NH₂* (4 mM) by pig pepsin (2 mg/ml) at pH 3.4 and at 37°C in 0.05 M-sodium citrate buffer

The products were analysed by amino acid analyser (see the Materials and Methods section). ○, Leucine; ●, Leu-Leu; □, Leu-Leu-Leu; and Δ, Leu-NH₂.

Fig. 2. Progress curve of cleavage of *Leu-Trp-Met-Arg* (1 mM) by pig pepsin (0.6 mg/ml) at 37°C in 0.05 M-sodium citrate buffer, pH 3.4

○, Leu; ●, Leu-Leu; □, Leu-Leu-Leu.

to determine approximate rate constants, termed k_{sp} . These are expressed in the same units as k_{cat} , but apply only to the conditions of the particular experiments. Where lag periods were observed, rate constants were measured from the slopes at their maxima.) In a separate experiment we determined $k_{cat} = 5.6 \times 10^{-4} \cdot s^{-1}$ for the cleavage of *Leu-Leu-NH₂* by measuring the initial rate of formation of *Leu-NH₂*. The former rate is comparable with the rate of hydrolysis of a wide variety of small substrates studied by Fruton (1970). A striking feature is that some 80% of the *N*-terminal leucine on the substrate is converted into a transpeptidation product. There was no evidence for a cleavage at the *Trp-Met* bond under initial rate conditions. This is in accordance with the studies on the specificity which indicate that the binding site *S'*₁ (as defined by Berger & Schechter, 1970) has a strong preference for aromatic amino acids, especially tyrosine and tryptophan. In this context it is noteworthy that pepsin failed to cleave the substrates *Tyr-Leu-NH₂* and *Tyr-Phe-NH₂* at a detectable rate, even after prolonged incubation and with a high enzyme concentration. This can also be explained by assuming a preference of *S'*₁ for the tyrosine residue. The favourable rate of cleavage of *Leu-Trp-Met-Arg* as compared with the shorter substrates with *N*-terminal leucine is probably due to the effects of secondary binding on catalytic rates as discussed by Fruton (1970). Similarly the increased yield of transpeptidation product is probably also due to the secondary binding effect.

The effect of pH on the initial rates of cleavage of two substrates is shown in the first two columns of

Table 2. Both peptides are hydrolysed with an optimum of approx. pH 3.4. However, there is a difference in the optimum of the transpeptidation reaction. *Leu-Tyr-NH₂* yields considerably more transpeptidation product at pH 4.7 than at pH 3.4 and no transpeptidation is observed at pH 2. *Leu-Trp-Met-Arg* (1 mM), on the other hand, shows an optimum of formation of *Leu-Leu* and *Leu-Leu-Leu* at pH 3.4. *Leu-Leu* was formed at pH 2 but not the tripeptide, whereas at pH 4.7 only tripeptide and no dipeptide was formed. The rate of hydrolysis at pH 4.7 was lower than at the other two pH values. Because of the complexity of the reaction and the assay system, no attempt has been made to determine the kinetic parameters (k_{cat} , K_m) separately for the hydrolysis and the transpeptidation. It is not possible, therefore, to state whether the pH optima refer to k_{cat} , or k_{cat}/K_m . In the following paper (Wang & Hofmann, 1976) we have determined apparent k_{cat} , and K_m values for the overall cleavage reaction (hydrolysis + transpeptidation) for *Leu-Tyr-NH₂*. Apparent K_m values of 4 and 5 mM, at pH 4.7 and 3.4 respectively, were obtained.

We have shown (Takahashi *et al.*, 1974) that leucine cannot act as acceptor when *Leu-Tyr-NH₂* is used as a substrate. An extensive study of the effect of adding free leucine to *Leu-Trp-Met* and *Leu-Trp-Met-Arg* as well as potential acceptors is shown in Table 3.

Table 2. *Effect of pH on the hydrolysis and transpeptidation reactions catalysed by pepsin*

Substrates: Leu-Tyr-NH₂ (2 mm), Leu-Trp-Met-Arg (1 mm) and Cbz-Phe-Leu (2 mm) were incubated at 37°C with pepsin in 0.05 M-sodium citrate buffers at the pH indicated. The concentration of pepsin with Leu-Tyr-NH₂ and Leu-Trp-Met-Arg was 0.6 mg/ml, with Cbz-Phe-Leu 1.06 mg/ml. Reaction time of Leu-Tyr-NH₂ (2 mm) is 1 h. The products were analysed by the amino acid analyser as described in the Materials and Methods section and products formed are expressed as leucine on the basis of the initial concentration of substrate.

Substrate	pH	Products (mol of Leu/100 mol of substrate)			Tyr-NH ₂ (% of Substrate)
		Leucine	Leu-Leu	Leu-Leu-Leu	
Leu-Tyr-NH ₂	2.0	1.1	0	0	1.4
	3.4	3.9	1.0	2.1	8.9
	4.7	1.7	2.6	8.1	12.3
Leu-Trp-Met-Arg	2.0	1.8	1.0	0	
	3.4	2.4	2.8	37.8	
	4.7	0.3	0	8.4	
Cbz-Phe-Leu	2.0	1.1	0	0	
	3.4	1.3	0	1.5	
	4.7	0.9	0	4.5	

Table 3. *Action of pepsin on [¹⁴C]leucine and Leu-NH₂ or Tyr-NH₂ in the presence and absence of substrate at pH 3.4, 37°C*

Pig pepsin (0.6 mg/ml) was incubated with amino acids and derivatives as indicated in column headed 'Reactions'. The following concentrations were used: [¹⁴C]leucine (4 μmol, 2.5 × 10⁵ c.p.m.), Tyr-NH₂ (2 μmol), Leu-NH₂ (2 μmol), Leu-Leu (2.5 μmol), Leu-Leu-Leu (0.5 μmol), Leu-Trp-Met (0.5 μmol) and Leu-Trp-Met-Arg (0.5 μmol) in a total volume of 0.5 ml sodium acetate buffer (0.05 M), pH 3.4. Reaction times were, for Expts. 1–3, 24 h; for Expts. 4–10, 2 h. The reactions were stopped by dipping into boiling water for 10 min, and removing the precipitate. The solutions were freeze-dried, and the residues dissolved in 100 μl of water. Samples (10 μl) were taken for high-voltage electrophoresis at pH 3.5, 3 kV for 2 h. After staining with ninhydrin the transpeptidation products Leu-Leu-NH₂ and Leu-Tyr-NH₂ respectively were identified in Expts. 5–10. In a separate high-voltage electrophoresis run at pH 3.6, all the products listed were eluted after separation and counted for radioactivity. Their positions were identified by comparison with standard amino acids and peptides run on the same sheet.

Experiment	Reactions	Product radioactivity (c.p.m.)			
		Leucine	Leu-Leu or Leu-Leu-Leu	Leu-Leu-NH ₂ or Leu-Tyr-NH ₂	Leu-NH ₂ or Tyr-NH ₂
1	Leu + Leu-NH ₂	61	57	76	102
2	Leu + Leu-NH ₂ + [¹⁴ C]Leu	9057	55	71	134
3	Leu + Leu-NH ₂ + [¹⁴ C]Leu + Leu-Leu	10908	51	76	117
4	Leu + Leu-NH ₂	36	35	40	52
5	Leu + Leu-NH ₂ + [¹⁴ C]Leu + Leu-Leu-Leu	11262	39	48	65
6	Leu + Leu-NH ₂ + [¹⁴ C]Leu + Leu-Trp-Met	12350	44	43	69
7	Leu + Leu-NH ₂ + [¹⁴ C]Leu + Leu-Trp-Met-Arg	12401	43	58	69
8	Leu + Tyr-NH ₂ + [¹⁴ C]Leu + Leu-Leu-Leu	12193	43	43	48
9	Leu + Tyr-NH ₂ + [¹⁴ C]Leu + Leu-Trp-Met	12929	45	46	50
10	Leu + Tyr-NH ₂ + [¹⁴ C]Leu + Leu-Trp-Met-Arg	12345	46	47	51

These experiments provide strong evidence for the involvement of a covalent intermediate in the transpeptidation reactions, as well as for the failure of free leucine to act as acceptor and for the absence of any exchange reaction between peptides such as Leu-Leu and Leu-Leu-Leu and free leucine in solution. Expt. 2 (Table 3) shows that neither Leu-Leu nor Leu-Leu-NH₂ can form, even after prolonged incubation of leucine with Leu-NH₂. Expt. 3 shows that no exchange reaction between leucine and Leu-NH₂

on one hand, and the dipeptide Leu-Leu on the other hand, occurs. Similar results are obtained when the dipeptide Leu-Leu is replaced by Leu-Leu-Leu (Expt. 5). When a substrate, Leu-Trp-Met (Expt. 6) or Leu-Trp-Met-Arg (Expt. 7), is added to a mixture of leucine and Leu-NH₂, no radioactive leucine is incorporated into any of the products. Expts. 8–10 were similar to Expts. 5–7 except that Leu-NH₂ was replaced by Tyr-NH₂. Again [¹⁴C]leucine was not involved in the reaction.

Table 4. *Pepsin-catalysed transfer of acyl groups to non-substrate acceptors*

Leu-Leu (1.8 μ mol), Leu-Tyr-NH₂ (1.0 μ mol) or Tyr-Tyr (1.0 μ mol) and pig pepsin (0.4 mg) were incubated with acceptor Leu-NH₂ (1.2 μ mol), Tyr-NH₂ (1.0 μ mol), Met-NH₂ (1.0 μ mol), Phe-NH₂ (1.0 μ mol) as shown, in acetate buffer, pH 3.4, total volume 0.375 ml, for 18 h at 37°C except where noted. The reaction products were separated by high-voltage electrophoresis at pH 3.6 and located by ninhydrin. The mobility of products at pH 3.6 relative to Leu-NH₂ were: Leu (0.29); Tyr (0.27); Leu-Met-NH₂ (0.74); Leu-Leu-NH₂ (0.74); Leu-Leu (0.43); Tyr-Tyr (0.57); Leu-Tyr-NH₂ (0.70); Leu-Phe-NH₂ (0.71); Tyr-Leu-NH₂ (0.69); Tyr-Phe-NH₂ (0.66); Leu-Leu-Tyr-NH₂ (0.63); Tyr-NH₂ (0.89); Met-NH₂ (1.0); Phe-NH₂ (0.97). Identification of the products was by comparison with standard peptides.

Reaction no.	Substrate	Acceptor	Product
1	Leu-Tyr-NH ₂ *	Leu-NH ₂	Leu-Leu-NH ₂ (Leu-Leu-Tyr-NH ₂ , Leu-Leu-Leu)
2	Leu-Leu	Leu-NH ₂	Leu-Leu-NH ₂
3	Leu-Leu	Tyr-NH ₂	Leu-Tyr-NH ₂
4	Leu-Leu	Met-NH ₂	Leu-Met-NH ₂
5	Leu-Leu	Phe-NH ₂	Leu-Phe-NH ₂
6	Tyr-Tyr	Leu-NH ₂	Tyr-Leu-NH ₂
7	Tyr-Tyr*	Phe-NH ₂	Tyr-Phe-NH ₂

* Incubated 6 h instead of 18 h.

Fig. 3. Progress curve of cleavage of Cbz-Phe-Leu (2 mM) by pig pepsin (1 mg/ml) at 37°C in 0.05 M-sodium citrate buffer at pH 4.7

○, Leu; ●, Leu-Leu; and □, Leu-Leu-Leu.

Although the experiments with [¹⁴C]leucine clearly show that the free amino acid cannot act as an acceptor, amino acid amides can. Table 4 shows the products that were formed when substrates such as Leu-Tyr-NH₂ or Leu-Leu were incubated with either Leu-NH₂, Tyr-NH₂ or Met-NH₂. Although

Reaction 1 definitely involves a transpeptidation where Leu-NH₂ acts as acceptor, the possibility cannot be excluded that in Reactions 2–7 a condensation, rather than a transpeptidation reaction followed by hydrolysis, is responsible for the product formation. Condensation reactions of the type Ac-Phe + TyrOEt $\rightleftharpoons$ Ac-Phe-Tyr OEt have been observed by Kozlov *et al.* (1966). No evidence is available as to whether covalent intermediates are involved in these reactions.

Substrates involving amino intermediates

Although transpeptidation reactions involving amino transfer have been studied by a number of workers, only one experiment had been carried out with substrates in which the C-terminal group, the group which is transferred, was not an aromatic residue (Mal'tsev *et al.*, 1966). In the latter case leucine appeared to be transferred in low yield and at a slow rate from acetyl Phe-Leu to Cbz-Phe. Because of the ease by which leucine is transferred via acyl transfer by pepsin, it was decided to see whether substrates could be found in which leucine was transferred efficiently to a suitable acceptor via an amino intermediate. The first example is shown in Fig. 3, which shows the progress curve of the action of pepsin on Cbz-Phe-Leu at pH 4.7. As in the transfer reactions involving N-terminal leucine, the tripeptide Leu-Leu-Leu is the major product followed by the dipeptide Leu-Leu and free leucine. The effect of pH is shown in Table 2. As with previously studied systems (Denburg *et al.*, 1968; Jackson *et al.*, 1969) no transpeptidation was observed at pH 2.0. It is noteworthy that there is a considerable lag period before a maximum rate of cleavage is reached. Similar results were obtained with three other

Fig. 4. Progress curve of cleavage of *Leu-Trp-Met* (2mM) by pig pepsin (0.6mg/ml) at 37°C in 0.05M-sodium citrate buffer at pH3.4

○, Leu; ●, Leu-Leu; □, Leu-Leu-Leu; ■, methionine; ▽, Met-Met; ▲, Met-Met-Met.

substrates, Cbz-Tyr-Leu, Cbz-Leu-Leu and Cbz-Leu-Met. In the latter case the transpeptidation products were Met-Met and Met-Met-Met. With these peptides too, lag periods were observed. In contrast, however, when Gly-Leu-Tyr was used as a substrate no lag was distinguishable. The products here were tyrosine, Tyr-Tyr and Gly-Leu. No evidence for Tyr-Tyr-Tyr was obtained.

An attempt to obtain semi-quantitative values for the rates of cleavage of all these substrates was made by measuring the maximum slope obtained with the substrates. The values for $k_{sp.}$ were between $5 \times 10^{-4} s^{-1}$ and $2.5 \times 10^{-3} s^{-1}$. These rates are comparable with those observed for the acyl-transfer reactions described in the preceding chapter. These rates are also comparable with the $k_{cat.}$ values for the cleavage of Cbz-Glu-Tyr [$1 \times 10^{-3} s^{-1}$ at pH4 (Casey & Laidler, 1950)], Ac-Tyr-Phe [$5 \times 10^{-3} s^{-1}$, at pH2 (Jackson *et al.*, 1966)] or the leucine-containing peptide Cbz-His-Phe-Leu-OMe [$5.2 \times 10^{-3} s^{-1}$, pH4 (Fruton, 1970)].

Substrate involving both amino and acyl transfer

The progress curve for the action of pepsin on *Leu-Trp-Met* at pH3.4 is shown in Fig. 4. As with the substrate *Leu-Trp-Met-Arg*, *Leu-Leu-Leu* and *Leu-Leu* are readily formed by an acyl transpeptidation.

At the same time, however, the C-terminal methionine is also transferred via an amino transfer to yield both Met-Met and Met-Met-Met. Not surprisingly, the formation of *Leu-Leu-Leu* is slower and levels off at a considerably lower value than with *Leu-Trp-Met-Arg* (Fig. 2) in spite of the fact that the substrate concentration used was higher. This is undoubtedly because the substrate undergoes two different reactions and thus each reaction is inhibitory for the other. Here again a lag is observed in the acyl-transfer reactions, in the hydrolysis and in the amino-transfer reaction. This lag is most likely due to an activation of the reactions by the transpeptidation products. The absence of a lag in the formation of *Leu-Leu-Leu* from *Leu-Trp-Met-Arg* (Fig. 2) contrasts with the experiment in Fig. 4 and can possibly be ascribed to the effect of arginine in the substrate.

Discussion

The experiments reported in the present paper are an extension of observations on pepsin-catalysed acyl transpeptidations (Takahashi *et al.*, 1974; Takahashi & Hofmann, 1975) and on amino transpeptidations studied by a number of workers (Neumann *et al.*, 1959; Fruton *et al.*, 1961; Kozlov *et al.*, 1966; Mal'tsev *et al.*, 1966; Denburg *et al.*, 1968; Silver & Stoddard, 1972; Kitson & Knowles, 1971b; Jackson *et al.*, 1969; Terada *et al.*, 1971). In particular, an attempt was made to obtain quantitative data for the transpeptidation and hydrolysis reactions. Unfortunately the kinetics for many of the substrates are complicated owing to a lag in the formation of the products. The most likely explanation for this lag is that some of the peptides produced cause an activation. [The lag in the formation of *Leu-Leu-Leu* from Cbz-Phe-Leu (Fig. 3) and *Leu-Trp-Met* (Fig. 4) may also in part be due to a lag in the formation of the intermediate acceptors Cbz-Phe-Leu-Leu and *Leu-Leu-Trp-Met* respectively.] Such an activation phenomenon was first observed with penicillopepsin (Wang *et al.*, 1974). A detailed study of the activation of pepsin by a variety of peptides appears in the following paper (Wang & Hofmann, 1976). Where possible, an estimate has been made of the maximum rate of product formation. The specific rates [$k_{sp.}$ = mol of product formed/s per mol of enzyme (defined above)] allow a semiquantitative comparison with $k_{cat.}$ (= $k_{sp.}$ under conditions where the enzyme is saturated with substrate) measured for the hydrolysis of a wide variety of substrates (Fruton, 1970; Clement, 1973). The values obtained for most of the transpeptidation substrates are in the range 1×10^{-4} – $1 \times 10^{-2} s^{-1}$ and thus compare favourably with $k_{cat.}$ values published for the hydrolysis of short peptides. This comparison adds weight to the significance of the transpeptidation reactions and of the formation of covalent intermediates for the mechanism of

Scheme 1. *A proposed minimal reaction scheme*

action. In addition, the high yields of some of the transpeptidations (i.e. Leu-Leu and Leu-Leu-Leu formation from Leu-Trp-Met-Arg by acyl transfer and from Cbz-Phe-Leu by amino transfer) suggests that covalent intermediates are also compulsory for hydrolysis. The high yields observed in our studies contrast with the low yields of transpeptidation observed previously. For example, Kitson & Knowles (1971*b*) found with Ac-Phe-Phe-Gly as substrate and acetyl[^3H]phenylalanine as acceptor 7.5% transpeptidation and 85% hydrolysis. Although the specificity studies with small substrates (Fruton, 1970) show a preference for aromatic residues in the primary binding sites S_1 and S'_1 , the highest yields of transpeptidation are obtained when a leucyl residue is transferred, whereas both the rates of reaction and the yields are lower when phenylalanine and tyrosine residues are transferred.

The substrate Leu-Trp-Met demonstrates clearly the multiple binding that occurs in the binding site of pepsin. A minimal reaction scheme is given in Scheme 1.

It is probable that some of the dipeptides formed also act as acceptors, and give rise to small amounts of new products that have not been detected. The fact

that Leu-Leu-Leu and Met-Met-Met respectively are formed faster than Leu-Leu and leucine or Met-Met and methionine is consistent with the increase in catalytic efficiency when longer substrates bind in the extended binding site, as postulated by Fruton (1970). Thus the putative intermediates Leu-Leu-Leu-Trp-Met and Leu-Trp-Met-Met-Met are better substrates for hydrolysis than Leu-Leu-Trp-Met and Leu-Trp-Met-Met respectively. Newmark & Knowles (1975) have since described experiments which are analogous to our experiments with Leu-Trp-Met. They incubated [^{14}C]Leu-Tyr-[^3H]Leu with pepsin, and found that Leu-Leu was formed both by acyl and by amino transpeptidation. As with Leu-Trp-Met, acyl transpeptidation exceeded amino transpeptidation. The product Leu-Leu-Leu was not reported, but the possibility that it had been formed in these experiments was not excluded (J. R. Knowles, personal communication).

From the observations with Leu-Trp-Met and the fact that Leu-Trp-Met-Arg is an excellent substrate for the formation of Leu-Leu-Leu one can attempt to estimate the length of the extended binding site. We define the individual binding sites of the amino acid residues as proposed by Berger & Schechter (1970):

and designate S_2 – S_n as secondary site A and S'_2 – S'_n as secondary site B. We also assume that all reactions, hydrolysis and acyl and amino transfer take place on the same site, with the amino acid residues involved in the peptide-bond cleavage being bound to S_1 and S'_1 . As we noted above, the putative Leu-Leu-Leu-Trp-Met-Arg appears to be a better substrate than Leu-Leu-Trp-Met-Arg. This indicates that the minimum length of the secondary site A is equivalent to two amino acid residues. A similar conclusion can be drawn from the experiment with Cbz-Phe-Leu, where the formation of the putative intermediate Cbz-Phe-Leu-Leu-Leu requires binding in subsites S_3 , S_2 , S_1 and S'_1 (and possibly in S_4 if the benzyloxy-carbonyl group is also bound). Sampath-Kumar & Fruton (1974) showed that in the series Cbz-(Gly) $_n$ -Phe-Phe-3-(4-pyridyl) propyl ester the highest rate of hydrolysis at the Phe-Phe bond was observed with $n = 2$. This peptide, too, would bind in subsites S_3 to S'_1 and possibly S_4 . For the formation of the putative intermediate Leu-Trp-Met-Met-Met from Leu-Trp-Met, the peptide Leu-Trp-Met-Met acting as acceptor would bind in subsites S_4 to S'_1 . Similar estimates can be made for the secondary site B. For the formation of intermediate Leu-Leu-Leu-Trp-Met-Arg, the pentapeptide Leu-Leu-Trp-Met-Arg binds to the leucyl-enzyme in subsites S'_1 to S'_5 . Since this reaction proceeds readily, it is likely that at least four if not all five amino acids bind to the enzyme. The secondary binding site B therefore would be as long as a tri- or tetra-peptide chain. From these estimates the total binding site of pig pepsin would be equivalent to an octa- or nonapeptide and would be between 2.9 and 3.2 nm (29–32 Å) long. It is noteworthy that Andreeva *et al.* (1971) identified a marked depression in the three-dimensional structure of pepsin at 0.5–0.55 nm (5–5.5 Å) resolution. A similar deep groove of about 3 nm (30 Å) length is clearly visible in the 0.6 nm (6 Å) resolution structure of penicillopepsin and has been tentatively identified as the active site on the basis of heavy-metal-inhibition studies (I.-N. Hsu, A. DeJong, M. N. G. James, T. Hofmann & S. C. Nyburg, unpublished work).

A further discussion of the nature of the amino acids required for binding in sites A and B is given in the following paper (Wang & Hofmann, 1976). Suffice it to say here that there is evidence that binding in the secondary sites appears to increase the hydrophobic environment near the catalytic site. This could explain the high yields of the acyl transpeptidations, which are a measure of the stability of the acyl enzyme. This latter is presumably an anhydride (Takahashi & Hofmann, 1975) and therefore should be highly unstable at a low pH if it was exposed on the enzyme surface. We envisage that binding of substrates and acceptors leads to an expulsion of water from the active site and stabilization of the acyl enzyme.

The effect of pH on the amino-transpeptidation reactions has been studied by several workers. It has been found generally that transpeptidation reactions are appreciable above pH 4 and insignificant or undetectable at pH 2 or below (Denburg *et al.*, 1968; Jackson *et al.*, 1969; Kitson & Knowles, 1971b). It was concluded that the substrate and/or the acceptor peptide (i.e. acetylphenylalanine) must be in the carboxylate form (Clement, 1973). The amino-transfer reactions thus exhibit a different pH-dependence from the hydrolysis reactions, which show maxima for k_{cat}/K_m and k_{cat} , in the range of 1.9–3.1 (Clement, 1973). The experiments with Leu-Tyr-NH₂ and Cbz-Phe-Leu as substrates are similar in that transpeptidation is maximum at pH 4.7 and not observable at pH 2.0. With the better substrate Leu-Trp-Met-Arg, however, optimum transpeptidation as well as optimum hydrolysis is observed at pH 3.4. The failure of the amino-transpeptidation to occur at low pH (2–3), where hydrolysis is readily observable, has been used by Silver & Stoddard (1972) as an argument against the amino intermediate in the pathway of hydrolysis. [It should be noted, however, that Terada *et al.* (1971) observed transpeptidation with Gly-Tyr-Tyr at pH 2.5.] The acyl intermediate cannot be dismissed on these grounds, because of the pH-dependence of the cleavage reactions of Leu-Trp-Met-Arg. The higher transpeptidation and hydrolysis rates are probably due to the increased length of the substrate. It is noteworthy that Terada *et al.* (1971) found an increase in transpeptidation with increase in n (to 3) in the peptide series (Gly) $_n$ -Tyr-Tyr at pH 4.

The present experiments and those reported in the following paper (Wang & Hofmann, 1976) have a bearing on the use of pepsin in sequence studies. With simple substrates neither acyl- nor amino-transpeptidation is very significant at pH 2.0 where peptic digestions of proteins are usually carried out. However, as the accompanying paper (Wang & Hofmann, 1976) shows, Leu-Leu-Leu is formed in significant amounts in the presence of Cbz-Leu-Met even at pH 2.0. It is therefore possible that in a peptic digest of a protein peptides could be released which could act as modifiers and induce significant transpeptidation reactions.

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