High-efficiency transpeptidation catalysed by clostripain and electrostatic effects in substrate specificity

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Clostripain catalyses the transpeptidation between benzoylarginine ethyl ester and amino acid amides, oligopeptides, insulin A- and B-chains and tryptic peptides of myoglobin at millimolar substrate concentrations. The reactions proceed with temporary accumulation of the products, followed by hydrolytic decomposition. The yield was not affected significantly by the type of *N*-terminal amino acid, but was diminished markedly by the negative charges of the amine components. The yields for natural peptides were linearly related to the charge density of the peptides.

INTRODUCTION

Proteinases are efficient tools for peptide synthesis [1,2]. However, they have been applied to the fragment condensation of long-chain peptides only in limited cases, usually assisted by specific interactions between acyl and amine components [1–5]. The main reason for the limited application is the low solubility of long-chain peptides in terms of molar concentration. Ordinary methods of enzymic peptide synthesis require substrate concentrations of the order of 0.1 M, which are difficult to achieve for most long-chain peptides. For this reason, a method of enzymic peptide synthesis applicable at low substrate concentrations is sought.

Usually thiol enzymes have a high amidase activity that is close to the level of their esterase activity [6,7]. This property of the enzymes has been thought to make them unsuitable for peptide synthesis by transpeptidation, since the product formed is hydrolysed rapidly. However, from the thermodynamic point of view of acyl-enzyme mechanisms, high amidase activity should be associated with high efficiency in the aminolysis of acyl-enzymes. Polymerization of dipeptide amides and amino acid esters by cathepsin C [8] and papain [9] suggests a high efficiency of cysteine proteinases in transpeptidation at low substrate concentrations. Clostripain has been used in the condensation of protein fragments by means of a reverse reaction [3,4,10] and in the preparation of oligopeptides by means of a transpeptidation [11,12]. However, high efficiency of transpeptidation at low substrate concentrations has not been demonstrated conclusively or utilized effectively. The present paper indicates that transpeptidation by clostripain is a good candidate for a method of fragment condensation for long-chain peptides. It also indicates that the main factor determining the yield of transpeptidation is electrostatic interactions between the enzyme and substrates. A linear dependence of the yield on the charge density of the substrates, found in the present study, may provide a clue not only for further improvement of the yield, but also for the interpretation

of various phenomena concerning interactions between proteinases and natural peptides.

MATERIALS AND METHODS

Materials

Benzoylarginine ethyl ester (Bz-Arg-OEt) and Gly-Gly-Tyr-Arg acetate were obtained from The Peptide Institute, Osaka, Japan. Amino acid amides, oligopeptides, clostripain, horse heart myoglobin (Mb) (type III), hog trypsin (type IX) and ovomucoid were obtained from Sigma. Electrophoretically homogeneous hog insulin was obtained from Shimizu Seiyaku Co., Shimizu, Japan. Ovomuroid-Sepharose (10 mg/g of Sepharose) was prepared by routine method as reported previously [13].

Insulin peptides. Hog insulin (1 g) oxidized according to the method of [14] was applied to a DEAE-Sephadex A-25 column (1.5 cm × 10 cm) equilibrated with 8 M-urea/40 mM-acetate/20 mM-Tris buffer, pH 4.8. Performic acid-oxidized insulin B-chain (Ins.B) passed through the column, whereas performic acid-oxidized insulin A-chain (Ins.A) was adsorbed and could be eluted with 8 M-urea/40 mM-acetate/Tris buffer, pH 4.8, containing 1 M-NaCl. When the separation was found incomplete by cellulose acetate electrophoresis (c.a.e.), the same chromatographic run was repeated. The Ins.B fraction (about 60 ml), dialysed against water, was made 0.01 M in Tris/acetate buffer, pH 8.2, 0.005 M in CaCl₂ and 0.03 M in NaCl, and incubated with 5 mg of trypsin for 7 h at 25 °C, then passed through a ovomuroid-Sepharose column (1 cm × 2 cm). The eluate was applied to a column of DEAE-Sepharose A-25 (1.5 cm × 8 cm) equilibrated with 0.01 M-Tris/acetate buffer, pH 8.2. The C-terminal threonine and Ins.B-(23–29)-peptide released by tryptic digestion passed through the column, whereas Ins.B-(1–22)-peptide was adsorbed on the column and eluted with 0.1 M-Tris/acetate buffer (pH 8.2)/0.3 M-NaCl. Ins.B-(23–29)-peptide in the eluate was adsorbed

Abbreviations used: Bz-Arg-OEt, benzoylarginine ethyl ester; Ins.A and Ins.B, performic acid-oxidized insulin A- and B-chains; (RCM)Ins.A, reduced and carboxymethylated insulin A-chain; Mb, myoglobin; cit, citraconyl; TNBS, trinitrobenzenesulphonic acid; c.a.e., cellulose acetate electrophoresis.

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on a column (1.5 cm $\times$ 5.5 cm) of Bio-Beads SM2 (Bio-Rad), washed with distilled water and eluted with methanol. The methanol was removed by evaporation. The solutions of Ins.A and Ins.B-(1-22)-peptide were dialysed against distilled water and freeze-dried. The yields were usually about 60% and the purities of the peptides were more than 90% as estimated from the amino acid compositions.

For the preparation of Leu-Ins.A, t-butoxycarbonyl-leucine succinimide ester (33 mg) was added to 27 mg of (RCM)Ins.A (prepared by a similar method) dissolved in 50% dioxan/0.02 M-borate buffer, pH 9.0, at room temperature. After 30 min the sample was dialysed against water and then freeze-dried. The Boc-Leu-(RCM)Ins.A prepared was deprotected by the method described in [15], using 10 ml of 2 M toluene-*p*-sulphonic acid/2% anisole in dioxan at room temperature. After 3 h the solution was neutralized with 20 ml of 1 M-NaHCO₃ and desalted with a column (2 cm $\times$ 40 cm) of Bio-Gel P2 (Bio-Rad). The Leu-(RCM)Ins.A sample showed only a leucine residue when analysed by the dinitrophenol method [16].

Mb peptides. Horse heart Mb (1 g) was dissolved in 60 ml of 0.1 M-borate buffer, pH 9.0, and 10 ml of 1 M-citraconic anhydride in tetrahydrofuran was added in several portions with an equivalent amount of 2 M-NaOH with vigorous stirring at room temperature. After dialysis against water, the solution was adjusted to pH 8.2 with NaOH and made 5 mM in CaCl₂, then digested with 10 mg of tosylphenylalanylchloromethane-treated hog trypsin at 35 °C for 3 h. The low salt concentration of the medium was effective in limiting the digestion to the two arginine residues (Arg-31 and Arg-139). The processes of the citraconylation and the tryptic digestion were monitored by a rapid trinitrobenzenesulphonic acid (TNBS) method [17] and c.a.e. The digested sample was applied to a DEAE-Sephadex A-25 column (1.6 cm $\times$ 18 cm) equilibrated with 0.01 M-Tris/HCl, pH 8.2. The trypsin passed through the column. The peptides adsorbed were eluted with the same buffer containing 0.5 M-NaCl, whereas the haem remained on the column. The peptide fractions were eluted as a broad peak and were divided into three portions and subjected to gel chromatography on a Sephadex G-50 column (2.2 cm $\times$ 110 cm) with 0.01 M-Tris/HCl, pH 8.2, by the method described in [18]. The chromatogram was similar to that obtained by Hagenmaier *et al.* [18], and amino acid analyses of the three main peaks gave satisfactory values for Mb-(1-31)-, Mb-(32-139)- and Mb-(140-153)-peptides. The citraconyl groups are so labile that they are hydrolysed by the lowering of the pH caused by dialysis of the acidic peptides, so the peptide solutions should be neutralized or frozen rapidly after dialysis. Free Mb-(1-31)-peptide was prepared by decitraconylation of cit-Mb-(1-31)-peptide in 30 mM-HCl for 15 h at room temperature and dialysed after neutralization. The h.p.l.c. patterns of the peptides are included in the Results and discussion section.

Methods

Assay of transpeptidation. In the standard method, 0.05 ml of clostripain (0.5 mg/ml) was added to a 3 ml mixture of 2.5 mM-Bz-Arg-OEt and 1 mM-amine component in 0.05 M-sodium borate buffer (pH 9.2)/3.3 mM-dithiothreitol/3.3 mM-CaCl₂, adjusted to pH 9.2 $\pm$ 0.1

with NaOH. At appropriate time intervals, 0.1 ml of the reaction mixture was withdrawn and quenched with 0.1 ml of 10 mM-*p*-chloromercuribenzoate and subjected to amino-group determination by the TNBS method [17]. For natural peptides, the reactions and the TNBS assay were carried out on a 1/5 or 1/10 scale. The amounts of the natural peptides were determined as dry weights for Mb-(1-31)-peptide and cit-Mb-(32-139)-peptide and by amino acid analysis for other peptides. Clostripain preparations of lot no. 49C-0087 (71 units/mg) and 76F-0604 (81 units/mg) were used. Pre-activation by thiol reagents [7] was not carried out, because the amount of the enzyme should not change the transpeptidation yield at the peak of the reaction profiles.

Identification of products. The transpeptidation products were separated by h.p.l.c., except for Mb-(140-153)-peptide, and identified by amino acid analyses (for small peptides) or enzymic digestion to the original components (for the natural peptides). H.p.l.c. was carried out with a CCPM pump equipped with a UV-8000 u.v. detector and columns of ODS 120A (4.6 mm $\times$ 150 mm), DEAE 3SW (8 $\times$ 75 mm) and DEAE 5PW (8 mm $\times$ 75 mm) all purchased from Toso Co. Tokyo, Japan. The eluent compositions are shown in the Results and discussion section. The flow rate was 1 ml/min. C.a.e. was carried out in 0.025 M-sodium tetraborate, pH 9.2, for the product identification and in 0.08 M-sodium Veronal buffer, pH 8.6, for the monitoring of the peptide preparation both on Separax membrane (Jookoo Co., Tokyo, Japan). The membrane was stained with nigrosin or Pauli reagents [for Ins.A and Mb-(140-153)-peptide].

RESULTS AND DISCUSSION

Representative time courses in Fig. 1 show that clostripain can catalyse the transpeptidation between 2.5 mM-Bz-Arg-OEt and 1 mM-amine components. The reactions

Fig. 1. Transpeptidation between 2.5 mM-Bz-Arg-OEt and 1 mM-amine components

The reaction mixtures contained 0.05 M-borate buffer (pH 9.2)/3.3 mM-dithiothreitol/3.3 mM-CaCl₂ at 10 °C, except for glycineamide (Gly-NH₂) (25 °C). The enzyme used were 7, 5, 8 and 13 μ g/ml for glycineamide, leucineamide (Leu-NH₂), Ins.B-(1-22)-peptide and Ins.A respectively.

Fig. 2. H.p.l.c. patterns of the transpeptidation at pH 9.2 and 25 °C

(a) 2.5 mM-Bz-Arg-OEt/5 mM-Leu-NH₂; 15 min ($\times 1$; 5 μ l); (b) 5 mM-Bz-Arg-OEt/0.5 mM-Ins.B-(1-22)-peptide; 40 min ($\times 1/10$; 20 μ l); (c) 5 mM-Bz-Arg-OEt/0.2 mM-Mb-(1-31)-peptide; 71 min ($\times 1$; 10 μ l); (d) 5 mM-Bz-Arg-OEt/0.5 mM-cit-Mb-(32-139)-peptide; 39 min ($\times 1/20$; 10 μ l). The enzyme concentration was 16 μ g/ml, except for (a), where it was 10 μ g/ml. The h.p.l.c. patterns were taken at the time indicated. The values in the parentheses above are the extents of dilution of the samples before injection and the injection volume. The conditions for h.p.l.c. are given in Table 2.

were carried out at about pH 9.2, because the deprotonated form of the amine components was assumed to be the reactive species in the reaction from the pH-dependence of the transpeptidation between Bz-Arg-OEt and leucinamide, which was represented by a normal titration curve with a middle point at pH 7.6. (The pH-yield profiles obtained at a constant reaction time showed a pH optimum [12], but this must be a result of the low enzyme activity at high pH.) Fig. 2 shows the h.p.l.c. patterns of the reactions carried out for the product identification. The substrate concentrations are changed from the standard method for the sake of easy detection of the products. In the early phase of the reactions, the product peaks increased with the concomitant decrease of the amine peaks, whereas the reverse phenomena took place in the later phase. The slower elution of Bz-Arg-peptide on anion-exchange chromato-

Table 1. Transpeptidation between 2.5 mM-Bz-Arg-OEt and 1 mM small amine components at pH 9.2

Amine	Yield (%)			Amino acid (mol/mol of Arg)
	25 °C	10 °C	t_R^* (min)	
Leu-NH ₂	72	78	7.95 ^b	Leu (0.96)
Val-NH ₂	—	62	4.55 ^b	Val (0.93)
Ala-NH ₂	—	53	5.18 ^c	Ala (0.90)
Gly-NH ₂	59	61	3.40 ^a	Gly (1.07)
Gly-Gly-NH ₂	53	—	3.35 ^a	Gly (2.30)
Leu	0	—	—	—
Gly-Gly	11	—	4.19 ^a	Gly (1.80)
Gly-Gly-Gly	14	—	3.80 ^a	Gly (2.92)
Gly-Gly-Gly-Gly	26	—	3.59 ^a	Gly (4.11)
Leu-Gly-Gly	37	—	7.72 ^b	{ Gly (2.17) Leu (0.96)
Gly-Gly-Tyr-Arg	61	—	5.75 ^a	{ Gly (2.14) Tyr (0.88)
Asp-Phe-NH ₂	0	—	—	—
Bz-Gly-Lys	0	—	—	—

* Retention times of Bz-Arg derivatives on an ODS 120A column eluted with: ^a25 % methanol/0.02 M-potassium phosphate, pH 2.6 [t_R = 14.20 (Bz-Arg-OEt), 4.65 (Bz-Arg)]; ^b40 % methanol/0.02 M-potassium phosphate, pH 2.6 [t_R = 4.62 (Bz-Arg-OEt), 2.67 (Bz-Arg)]; ^c25 % methanol/0.02 M-potassium phosphate, pH 7.6 [t_R = 10.50 (Bz-Arg-OEt), 4.50 (Bz-Arg)].

graphy may be due to hydrophobic interactions between the Bz-Arg group and the resin matrices.

Table 1 summarizes the results of the transpeptidation for amino acid amides and small peptides. The pK values of the amino acid amides are about 8, and those for the amino groups of diglycine (8.13), triglycine (7.91) and some tetrapeptides are similar to that of glycine (7.99) [19]. More than 90 % of the amino groups are in their deprotonated form at pH 9.2. The data for the amino acid amides indicate that the yield is not affected markedly by the kind of the acceptor amino acids, although there may be some preference for bulky amino acids, which is more evident in the yields for triglycine (14 %) and Leu-Gly-Gly (37 %). The similar yields for glycine and glycylglycine suggest the unimportance of the P₂' site in this reaction. The low yields for peptides with a free carboxy group and the stepwise increase of the yield from di- to tetra-glycine suggest a repulsive interaction between the charge on the peptide C-terminus and negative charge(s) on the enzyme. This idea is supported by the high yield for Gly-Gly-Tyr-Arg, which has a carboxy group but no net charge. The amino acids with an amino group with a carboxy group in the same residue namely leucine, N-terminal aspartic acid and C-terminal lysine showed no transpeptidation, although this may be partly accounted for by the ionization of the amino groups.

Table 2 shows the results of transpeptidation for natural peptides. The reactions were carried out at 10 °C, where the yields are slightly better than at 25 °C. The product for cit-Mb-(139-145)-peptide was confirmed only by c.a.e., because the h.p.l.c. pattern of the peptide is complicated by the two isomeric forms [20] of the citraconyl groups.

The reaction yields are linearly related to the charge density of the peptides, as shown in Fig. 3. The amino groups of the peptides may be principally in their un-

Table 2. Transpeptidation between 2.5 mM-Bz-Arg-OEt and 1 mM natural peptides at pH 9.2 and 10 °C

Peptide	N-Terminus	Charge density (charge/residue)	Yield (%)	t_R^* (min)	Column†
Ins.A	Gly	-0.286 (-6/25)	26	5.2 (2.5)	ODS 120T ^a
Leu-(RCM)Ins.A	Leu	-0.273 (-6/26)	25		
Ins.B-(1-22)-peptide	Phe	-0.182 (-4/22)	46	11.7 (7.8)	ODS 120T ^b
Ins.B-(23-29)-peptide	Gly	0 (0/7)	74	7.7 (2.4)	ODS 120T ^c
Mb-(1-31)-peptide	Gly	-0.129 (-4/31)	43	7.1 (6.2)	DEAE 3SW ^d
cit-Mb-(32-139)-peptide	Leu	-0.259 (-28/108)	35	10.1 (6.8)	DEAE 5PW ^e
cit-Mb-(40-153)-peptide	Asn	-0.357 (-5/14)	16	0.8 (1)	

* Retention times of Bz-Arg-peptides and peptides (in parentheses), except for the last line (Asn *N*-terminus), which are the relative mobilities in c.a.e.

† Eluents were as follows: ^a55 % (v/v) methanol/0.05 M-Na₂SO₄/0.01 M-potassium phosphate, pH 2.6; ^b60 % methanol/0.05 M-Na₂SO₄/0.01 M-potassium phosphate, pH 6.7; ^c50 % methanol/0.02 M-potassium phosphate, pH 2.6; ^d0.2 M-NaCl/0.01 M-Tris/HCl, pH 8.2; ^e0.29 M-NaCl/0.02 M-potassium phosphate, pH 6.8.

Fig. 3. Relationship between the transpeptidation yield and the charge density of the peptides

ionized form at pH 9.2, since the charges separated more than two amino acid residues on extended peptide chains [0.8 nm (8 Å)] has only a small electrostatic effect, as observed with the glycine peptides and angiotensin analogues [21], and should not change the extent of ionization of the terminal amino groups appreciably at pH 9.2. The finding that the Bz-Arg derivatives of Ins.A, Ins.B-(1-22)-peptide and Mb-(139-145)-peptide are separated from their original peptides by c.a.e. at pH 9.2 also supports the above assumption. The result shown in Fig. 3 may therefore be attributable to electrostatic interactions between the enzyme and the peptides. The electrostatic effect in protein reactions is a subject under active study [22]. Clostripain (M_r 50000), [7] can be approximated by a sphere of 5 nm (50 Å) diameter bearing a net charge of about -30 at pH 9, as estimated from the amino acid composition and the pI of 4.7. The dimensions of the natural peptides in their extended [0.38 nm (3.8 Å)/residue] or random-coiled forms are similar to that of the enzyme. The Debye length, $1/\kappa$, a measure of the range of electrostatic effects, is about

1.8 nm (18 Å). Under these circumstances many charges carried by the enzyme and the peptides must interact significantly when they approach each other. This may be the reason for the involvement of all the charges (in the form of the charge density) of the peptides in the interaction with the enzyme. The dependence of the yield on the charge density, not on the net charge, which is an important finding in the present study, may be related to the expanded conformations of the peptides. The electrostatic potential (represented by the activity coefficient of the counterion) around a molecule of poly(vinyl sulphate) esterified to various extents depends on its charge density and is nearly independent of its M_r , which represents the total charge [23]. Quantitative interpretation of the electrostatic effect in the present case will not be tried here, because the transpeptidation yield is not an adequate parameter for precise discussion. The result in Fig. 3, however, is the first example of the quantitative relationship found in the reactions of proteinases with natural peptides and gives a measure of the electrostatic effects to be considered in such cases. It also provides important information on the enzymic fragment condensation of long peptides. Electrostatic interactions between acyl and amine components also should be taken into account in such cases.

There are at least three procedures for overcoming electrostatic repulsion. One is addition of salts. The yield for 1 mM-Ins.A and 2.5 mM-Bz-Arg-OEt increased from 26 % (0 M-NaCl) to 35 % (1 M-NaCl) and to 50 % (2 M-NaCl) at 25 °C. Others are to change the enzyme charge by chemical modification and to change the charge of peptides. Esterification of the *C*-terminal carboxy group of long peptides is also possible in principle [13].

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Received 25 October 1989; accepted 3 January 1990