Pergamon

Tetrahedron Vol. 51, No. 6, pp. 1827-1844, 1995
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0040-4020/95 $9.50+0.00

0040-4020(94)01060-9

Chemoselectivity of Chemically Modified α-Chymotrypsin.

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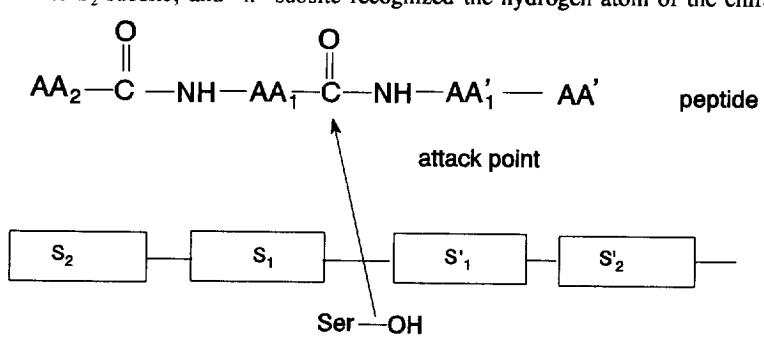
Abstract: The enzymatic activity of α-chymotrypsin chemically modified with monomethoxypolyethylene glycol (α-CT-PEG) is discussed using as test reactions the hydrolysis of esters different from the natural substrates of the enzyme , and the peptidic synthesis. These reactions allowed the analysis of the dimensions of "ar", "h", "n" and "am" subsites of α-CT-PEG. Using 2-aminoethanol esters and derivatives of glycine we have proved that the depth of the "n" subsite of the chemically modified α-CT (α-CT-PEG) must be lower than 14.455 Å because the synthesis of peptides is not observed using 2-aminoethanol octanoate as nucleophile. α-CT-PEG is chemoselective towards the aromatic esters because unnatural aliphatic esters are not hydrolyzed in the presence of N-benzoyl-L-Tyrosine ethyl ester. Unnatural aliphatic acyl-donors with a distance H-C.....C=O smaller than 8.8 Å are recognized by the chemically modified enzyme as acyl-donors. From these results we can conclude that the presence of N-acyl group in the acyl-donor molecule is not necessary to be recognised by α-CT-PEG. The presence of bulky alkyl chain both in the acyl donor and in the nucleophile, reduces the enzymatic activity of the biocatalyst both in the hydrolysis of esters and in the synthesis of peptides.

INTRODUCTION.

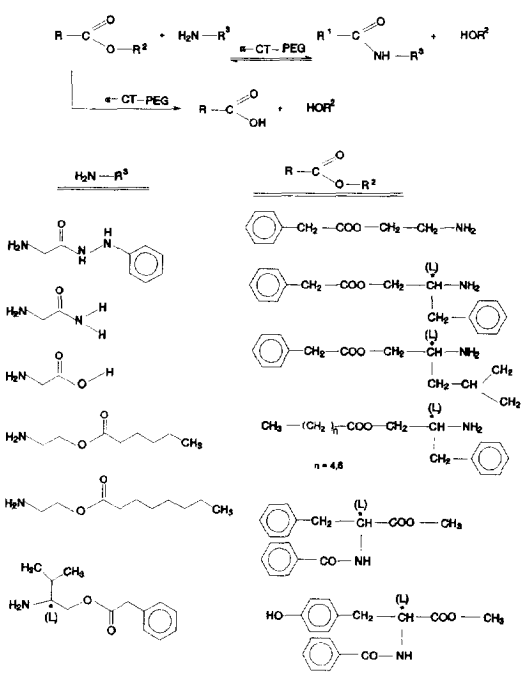
Although α-chymotrypsin chemically modified with monomethoxypolyethylene glycol (α-CT-PEG) is one of the modified enzymes most extensively studied [1-4] from a chemical point of view there is a lack of the information about the interaction enzyme - substrate in the case of unnatural substrates. The alteration of the chemoselectivity respect to the native enzyme has been reported when natural aminoacids are used as acyl donors[3,5,6]. West et al[7] have reported a more detailed structure of the active site of this protease. In this sense three different subsites have been postulated into the S_1 site (part of the active site that recognizes the acyl donor molecule): "ar" subsite recognizes the aromatic ring of Phe or Tyr; "am" subsites recognizes the amide bond and can be related to S_2 subsite; and "h" subsite recognized the hydrogen atom of the chiral carbon.



Using phenylalanine analogs, we have reported the presence of two previously undescribed cavities in the aromatic subsite "ar"[6], placed in front of the ortho- and meta- position of the aromatic ring of phenylalanine (natural substrate of the native enzyme). Besides, we have described the active conformation of the acyl donor molecule which interacts with the "ar", "am" and "h" subsites[7] of S_1 position in the kinetically controlled synthesis of peptides. Aliphatic aminoacid derivatives such as Arg or Lys can be accepted by the native enzyme (α-CT)[8] but aminoacids possessing -COOH groups such as Asp or Glu are not accepted in the "ar" subsite. Besides esters of some locked compounds such as 1-keto-3-carboxytetrahydroisoquinoline[9] or hydrocoumarin derivatives[10,11] are susceptible to be hydrolyzed by the native enzyme. Nevertheless, the geometry of the nucleophile subsite "n" (corresponding to the S_1' and S_2' position in the traditional biochemical nomenclature)[7] has not been analyzed as far as we are concerned. From synthetic results, it has only been reported that native enzyme (α-CT) is not chemoselective towards the nature of the nucleophile because water, amino alcohols, and D- or L-aminoacids can be used as nucleophiles[7]. Furthermore it has not systematically been analyzed if the modified enzyme (α-CT-PEG) can accept natural or unnatural acyl donors as nucleophiles as in the case of the native enzyme[12,13].

In the present paper we have studied the chemoselectivity of the α-CT-PEG with respect to some unnatural nucleophiles and acyl donors, in order to analyze the geometry of S_1 site ("ar", "am" and "h" subsites) and of S_1' and S_2' ("n" subsite) sites to compare them with the dimensions of the native α-CT[7,8].

Scheme 1



RESULTS and DISCUSSION

1. Influence of the structure of the nucleophile in the synthesis of peptides.

The synthesis of the peptidic bond Tyr-AA was carried out using several natural and unnatural substrates. This reaction allows us to explore the chemoselectivity of the modified enzyme (α-CT-PEG), and the dimensions of "n" subsite corresponding to S_1'and S_2' subsites in the classical biochemical nomenclature.

In Table 1, we compare the synthesis of the peptide Tyr-AA catalyzed by the modified enzyme (α-CT-PEG) and by the enzyme immobilized on a hydrophilic support such as agarose (α-CTag), in Cl_3C-CH_3 with 1% (v/v) of 50 mM sodium phosphate buffer (pH=7.8). In all cases, N-benzoyl-L-tyrosine ethyl ester (BTEE) was used as acyl donor, employing several different L-aminoacid amides as nucleophiles. This ester is the best acyl-donor both for the native chymotrypsin and for the modified enzyme (α-CT-PEG)[6,14]. α-CT-ag is a well described enzymatic derivative whose chemoselectivity - in the hydrolysis of esters in water - is analogous to that observed for the native α-chymotrypsin[15]. Therefore α-CT-ag was used as the reference system in both the synthesis of peptides and in the hydrolysis of esters, because under the experimental conditions used with α-CT-PEG (1,1,1-trichloroethane/water 99%/1%, v/v), the reaction yields obtained with the native enzyme were lower than 4%, so that native enzyme cannot be used as the reference enzyme in these experimental conditions. In Table 1, we can observe that α-CT-PEG lead to higher yields in peptide with the hydrophobic nucleophiles (L-Phe-NH_2; L-Tyr-NH_2 and L-Thr-NH_2) instead of with the hydrophilic aminoacid amides such as L-Ser-NH_2 or L-Gly-NH_2 (Table 1), according to the nucleophile specificity of the S_1' site of the native α-CT[16]. Good yields are still obtained with small aminoacid amides (L-Ser-NH_2 and Gly-NH_2). When using charged acyl-donor (L-Arg, L-Lys and L-Glu) - very hydrophilic nucleophiles - the yield obtained using α-CT-PEG was very low. This behaviour is different to that observed in the case of the immobilized derivative α-CTag (Table 1) which accepts these molecules as acyl-donors. This different behaviour can be explained taking into account the amount of water retained by each support. Both enzymatic derivatives (α-CT-PEG and α-CTag) were lyophilized for 8 h. Due to the hydrophilic characteristics of the agarose[15],α-CTag retains higher amounts of water than α-CT-PEG. These water molecules favour the diffusion of the hydrophilic nucleophiles towards the active site (S_1') and so, hydrophilic amides can be used by α-CTag as nucleophiles . In the case of the chemically modified α-CT, the water molecules are stripped off the microenvironment of the protein by the amphyphylic polymer giving a hydrophobic microenvironment around the active site, which hinders the diffusion of the charged nucleophiles towards the active site and leads to poor yields with charged nucleophiles. This explanation is supported by previous results[15] where we show that the peptide synthetase activity of α-CT-ag can be related to the lyophilization time of the enzymatic derivative ,in other words, to the different amount of water in the microenvironment of the enzymatic derivative. Recently, Mattiasson et al[4] have reported that the enzymatic activity of chemically modified α-CT with PEG changes with the amount of water in the microenvironment of the enzyme.

Table 1.- Influence of the structure of the natural nucleophile in the enzymatic activity of α-CT-PEG and α-CTag in the synthesis of peptides Tyr-AA.

Nucleophile	**α-CT-PEG Yield % at 20h**	**α-CT-ag Yield % at 20h**
L-Phe-NH_2	95	75
L-Tyr-NH_2	91	74
L-Thr-NH_2	94	94
L-Ser-NH_2	89	89
L-Gly-NH_2	69	64
L-Arg-NH_2	8	68
L-Lys-NH_2	4	67
L-Glu-NH_2	2	25

Schellenberger et al[8] and Keil et al[17] showed that L-Arginine and L-Lysine amides (L-Arg-NH_2 and L-Lys-NH_2) are excellent nucleophiles for native α-CT because there is one specific ionic interaction in the S_1' and S_2'subsites (nucleophile subsite) of α-CT between the positively charged aminoacid amides and some negative charges in S_1'and S_2'subsites [8,18]. These interactions have been confirmed by crystallographic data[19], which show the presence of two acidic residues - Asp 34 and Asp 64 -located near the surface of the S^1'and S_2'region in the native α-CT. The electrostatic potential of these residues (COO^-) seems to play a crucial role in the interactions of L-Arg-NH_2 and L-Lys-NH_2 (with H_3N^+ group in the reaction conditions) with the enzyme. Therefore L-Arg-NH_2 and L-Lys-NH_2 are recognized as nucleophiles by the "n" subsite by the electrostatic inrteraction COO^- (Asp residues)...H_3N_+ (nucleophile) in the case of native α-CT. L-Arg-OEt and L-Lys-OMe are excellent acyl donors in the synthesis of the peptide AA-Phe-NH_2 (Bz-L-Arg-OEt 95-98% and Bz-L-Lys-OMe 95-98%)[20]and good yields are obtained with these aminoacids as acyl-donor using α-CTag (Table 1), in the same experimental conditions (1,1,1-trichloro ethane/H_2O 99%/1% (v/v)) used in the case of α-CT-PEG (Table 1), so that diffusional problems of these charged nucleophiles trough out the medium towards the active site must be rejected because of the diffusional problems would be the same for L-Arg-OEt than for L-Arg-NH_2. Therefore, we may conclude that the low reaction yield obtained with these aminoacids using chemically modifed enzyme α-CT-PEG as catalyst (Table 1) must be related to the burying of Asp-35 and Asp-64 of the α-CT of "n" subsite, produced by a slight modification of the structure of α-CT-PEG respect to the structure of the native enzyme that takes place during the chemical modification of the protein. This burying of

Asp-35 and Asp-64 avoids the interaction of these residues with the positively charged nucleophiles, giving poor results because the "n" subsite becomes very hydrophobic. The very poor yields obtained with L-Glu-NH_2 using both biocatalysts α-CT-PEG and α-CT-ag, confirm the reported data in the literature[8] and can be explained by the increase of hydrophobicity of the "n" subsite produced by the chemical modification (α-CT-PEG).

Table 2.- Influence of the structure of the unnatural nucleophile in the synthesis of the peptide Bz-L-Tyr-Nu using α-CT-PEG in 10ml of 1,1,1-trichloroethane. 1% H_2O. T=37°C

Nucleophile	d(Å)[a]	Yield (%)	time (min)
H_2N-CH_2-$CONH_2$[b]	4.428	69	1200
H_2N-CH_2-CONHNHPh[b]	9.491	91	5
H_2N-CH_2-$CONHCH_2CONH_2$[b]	8.145	86	30
$H_2NCH_2CH_2$-OH[c]	4.343	69	1200
$H_2NCH_2CH_2$-OOC-$(CH_2)_4CH_3$[c]	11.95	16	1200
$H_2NCH_2CH_2$-OOC-$(CH_2)_6CH_3$[c]	14.455	0.5	1200
H_2NCH-CH_2.$OOCCH_2$-Ph[c,d] | CH_2-iPr	8.57	4.2	1200
H_2NCH-CH_2.$OOC(CH_2)_6CH_3$[c,e] | CH_2-iPr	15.55	0.3	1200

[a] Distance between H_2N and the end of the nucleophile in the conformer of the minimum energy.
[b] 10mM of nucleophile; 10mM of BTEE; 40 μl of solution of α-CT-PEG (2mg protein/ml)
[c] 40mM of nucleophile; 10mM of BTEE; 40 μl of solution of α-CT-PEG (2mg protein/ml)
[d] L-leucinol phenyl acetate
[e] L-leucinol octanoate

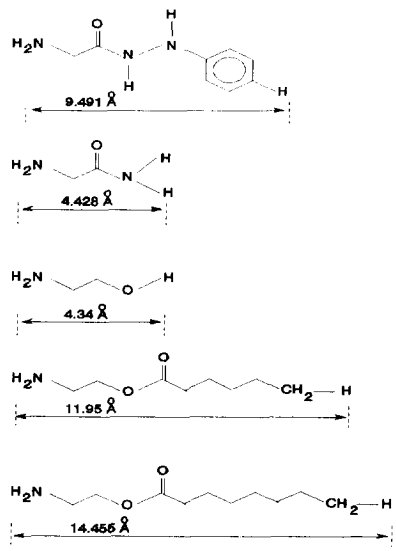
The influence of the structure of some unnatural nucleophiles in the synthesis of amidic bonds (Tyr-Nu), using α-CT-PEG as biocatalyst is shown in Table 2. In all cases, N-benzoyl-L-tyrosine ethyl ester (BTEE) was used as acyl donor. We can observe that the presence of the structure H_2N-CH_2-CONH- (α-aminoacidic structure) in the nucleophile favours the synthesis. This finding can be explained because C=O of Ser-214 in subsite S_1' interacts with HN-CO bond by H-bonding[18,21]. The best results are obtained with N-substituted amides H_2N-CH_2-CONHNHPh and H_2N-CH_2-$CONHCH_2CONH_2$ as can be expected from the endopeptidase nature of the enzyme which favours the interaction of these "peptides" with S_1' and S_2' subsites. The best yield and the

smallest reaction time are obtained with H_2N-CH_2-CONHNHPh (Table 2). Therefore we should conclude that the nucleophile region must, at least, possess the dimensions permitting the acceptance of the phenylhydrazide of glycine (N...C_6H_4-H = 9.491 Å) (see Scheme 1) as nucleophile. Besides the more hydrophobic the nucleophile (H_2N-CH_2CONHNHPh), the greater the reaction yield and the lower the reaction time (H_2N-CH_2CONH_2). These results agree with the lipidic nature of "n" subsite of the α-CT-PEG discussed above.

If we compare the behaviour of the 2-aminoethanol derivatives with the behaviour of the glycine amide we can observe how similar yields - at the same reaction time - are obtained with 2-aminoethanol and glycinamide (Table 2). This finding can be explained because both molecules have similar size and one hydrogen atom at the same distance from the nucleophile group (H_2N) (scheme 1) that can interact with O=C of Ser-214 by H-bonds.

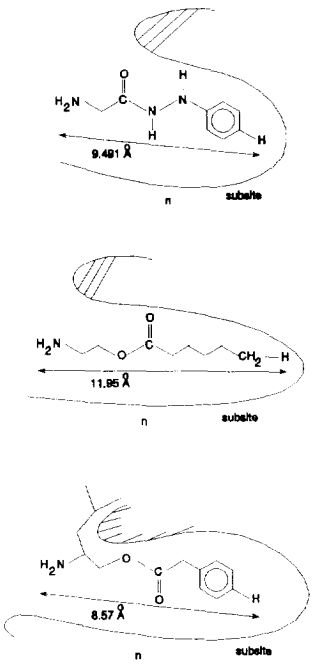
If this hydrogen atom is not present, as in the case of the esters of 2-aminoethanol, the yield diminishes as observed in Table 2 for 2-aminoethanol hexanoate and octanoate. We can see that the larger the size molecule, the lower the reaction yield. In this way, we can conclude that the dimensions of the 2-aminoethyl octanoate (14.455 Å) (Scheme 1) are greater than the nucleophile pocket because no conversion is obtained when this molecule is used as nucleophile. So, we can conclude that the dimensions of the nucleophile pocket must be lower than 14.455 Å. 2-Aminoethanol octanoate or hexanoate are not hydrolyzed in the presence of N-benzoyl-L-tyrosine ethyl ester. Therefore, modifed α-CT is strongly chemoselective towards the aromatic acyl-donors respect to the alkyl acyl- donors. This chemoselectivity is analogous to that described in the literature for the esters of the aminoacids, where L-Phe and L-Tyr esters are described as the best acyl donors[3].

Scheme 2



The presence of an alkyl group in β-position respect to the nucleophile nitrogen atom difficults the progress of the reaction as we can observe in the case of L-leucinol esters (Table 2). The L-leucinol octanoate is not suitable for peptide synthesis, as can be expected from the dimensions of the octanoic acid. Nevertheless the phenyl-acetate of L-leucinol, which distance, N....C_6H_5-H =8.57 Å is lower than 14.555 Å, does produce the reaction. Therefore we should conclude that the isopropyl group is located outside the "n" subsite of the protein giving a small steric hindrance. According to our results we can postulate the nucleophile subsite as pictured in Scheme 3.

Scheme 3

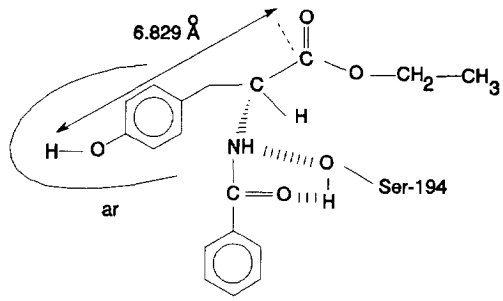


As in the other cases, these esters of L-Leucinol are not hydrolyzed in the presence of BTEE, instead of the presence of the aromatic ring - phenyl acetate. Therefore we should conclude that α-CT-PEG is chemoselective respect to the aromatic aminoacids as the native α-CT.(Scheme 3).This result must be explained by the positive effect for the interaction acyl-donor enzyme complex created by the presence, in the benzamide group of the acyl-donor molecule, of the group NH-CO which interacts with Ser-194[21] (Scheme 4).

2. Influence of the structure of the acyl-donor in the hydrolysis of esters.

In Table 3 we show the kinetic constants obtained for the hydrolysis of some unnatural esters using α-CT-PEG. These results are compared to those obtained in the hydrolysis of the esters of L-Phe and L-Tyr, which are the best substrates for the native enzyme. We can observe that α-CT-PEG hydrolyzes esters from phenyl acetic or hexanoic acids and L-phenylalaninol. In all cases, the initial reaction rate values are reasonably fitted to a Michaelis-Menten equation. We can observe that the ester of the highly hydrophilic alcohol - 2-aminoethanol- gives very low value of k_{ap}. L-phenylalaninol phenylacetate and hexanoate provide k_{ap} values similar to those obtained with the methyl ester of the N-benzoyl-L-phenylalanine.

Scheme 4



A plausible explanation for this fact is that the distance from CO-OR (attack point of Ser-195) and the end of the substrate located in "ar" subsite is similar in the case of the natural substrate of the enzyme (N-benzoyl-L-Phenylalanine methyl ester (end of the aromatic ring = 6.264 Å) (Figure 1) and in the case of the L-leucinol hexanoate (end of the alkylic chain = 6.653 Å) (Figure 2) and in the L-phenylalaninol phenylacetate (end of the aromatic ring = 6.780 Å) (Figure 3). Therefore all these acyl donor molecules could be accepted in the "ar" pocket allowing the hydrolysis reaction (Table 3). In the case of L-phenylalaninol octanoate the interaction between the acyl-donor and the pocket must place the COOMe from the serine (Ser-195) position, because the distance between COOMe and the chain is 8.815 Å (Figure 4), and the hydrolysis is not observed.

Therefore, the attack of Ser-195 must take place in similar conditions in the case of the L-Phenylalaninol hexanoate and phenylacetate , and in the case of N-benzoyl-L-Phe-OMe. This fact explains why the k_{ap} value (Table 3) is similar in these cases. From these results we deduce that the critical distance between CO-OEt and the end of the chain must be lower than 8.815 Å (the distance in the case of the L-phenylalaninol octanoate). The presence of isopropyl group - L-leucinol phenylacetate - avoids the hydrolysis reaction. So, the presence of branched alkyl chains in the nucleophile (Table 2) or in the acyl donor (Table 3) difficults the synthesis of peptides and the hydrolysis of esters because of L-leucinol phenylacetate -with a molecular size in the "ar"

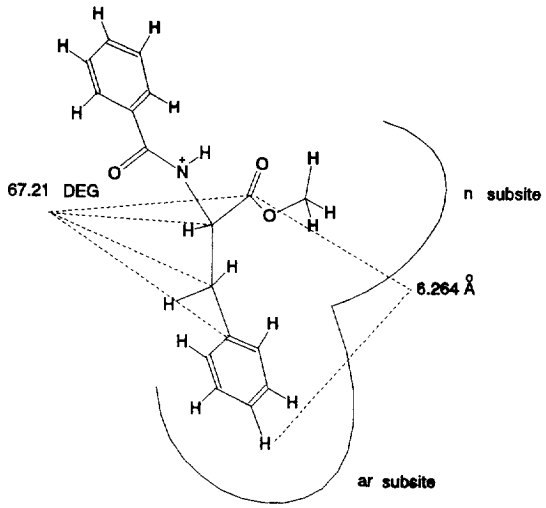


Figure 1. Interaction with active site of α-CT-PEG of the conformer of minimum energy of methyl ester of the N-benzoyl L-Phenylalanine.

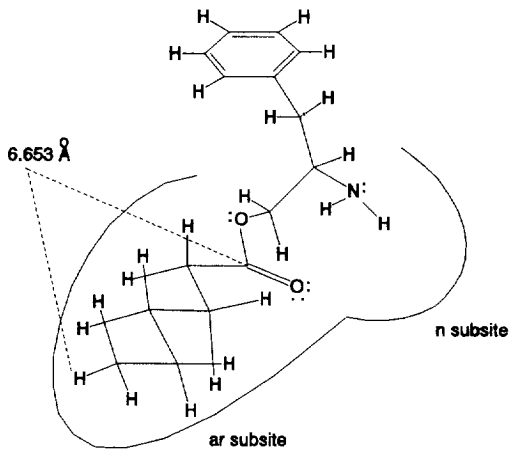


Figure 2. Interaction with active site of α-CT-PEG of the conformer of minimum energy of hexanoate of the L-leucinol.

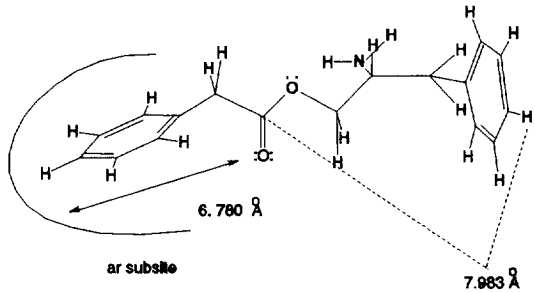


Figure 3. Postulated interaction of phenyl acetate of L-leucinol with the active site of α-CT-PEG.

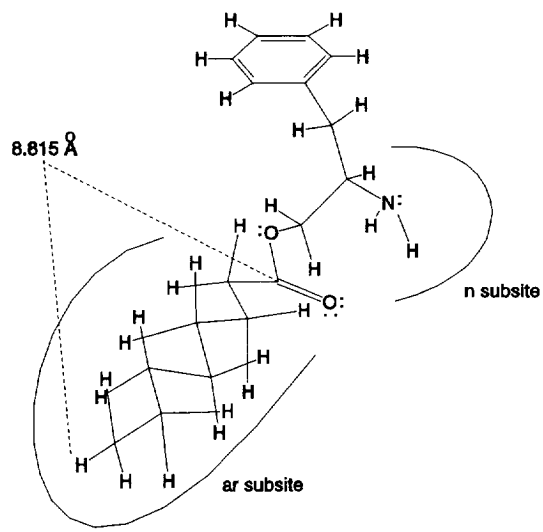


Figure 4. Interaction of the conformer of minimum energy of octanoate of L-leucinol and the active site of α-CT-PEG.

subsite -lower than N-benzoyl-Tyrosine ethyl ester- leads to null conversion.

In conclusion we must assume the presence of an undescribed sterical restriction in the nucleophile subsite that rejects the presence of the isopropyl group in the nucleophile. Nevertheless these problems have not been observed with small nucleophiles with the same structure such as L-leucineamide or L-leucine esters[8]. Therefore, the transformation of -COOR (ester of L-leucine) or -$CONH_2$ (amide of L-leucine) in -CH_2-O-C changes the interaction of the molecule with residues of the enzyme pocket (Scheme 5).

Table 3.- Hydrolysis of different esters catalyzed by α-CT-PEG

Ester	k_{ap} (mmol/(min x mg enz))	Km_{ap} (mM)
$PhCH_2$-$COOCH_2CH_2NH_2$	0.082	0.65
$PhCH_2$-$COOCH_2CH(CH_2Ph)$-NH_2[a]	14.7	0.20
$PhCH_2$-$COOCH_2CH(CH_2iPr)$-NH_2[b]	g	g
CH_3-$(CH_2)_4COOCH_2CH(CH_2Ph)$-$NH_2$[c]	13.4	8.4
CH_3-$(CH_2)_6COOCH_2CH(CH_2Ph)$-$NH_2$[d]	g	g
$PhCH_2$-$CH(PhCO\text{-}NH)$-$COOCH_3$[e]	15.45	0.19
4-HO-C_6H_4-CH_2-$CH(PhCO\text{-}NH)$-$COOCH_3$[f]	43.52	0.14

[a] L-penylalaninol phenylacetate

[b] L-leucinol phenylacetate

[c] L-phenylalaninol hexanoate

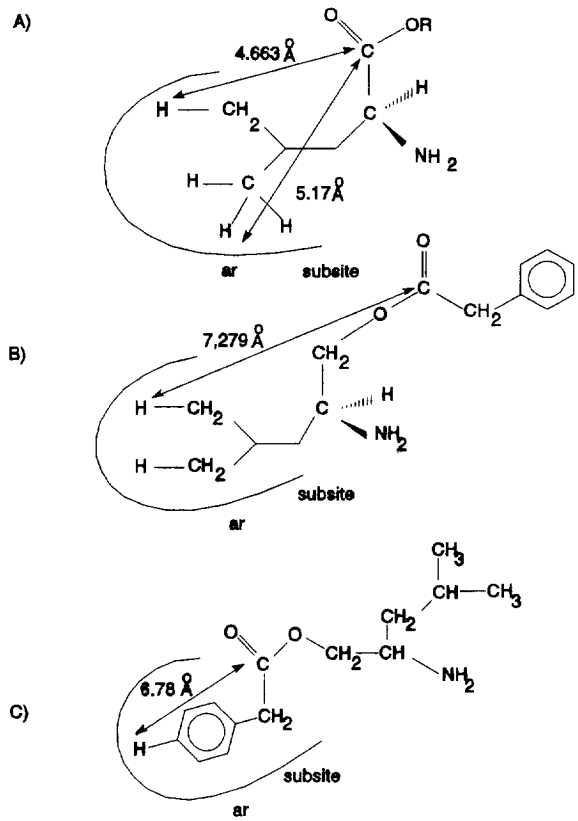
[d] L-phenylalaninol octanoate

[e] N-benzoyl-L-phenylalanine methyl ester

[f] N-benzoyl-L-tyrosine methyl ester

[g] No reaction was observed

Scheme 5



A possible explanation could be that the protease interacts better with L-α-aminoacids or its amides or esters than with other structures. In this way the interaction of L-Leucine esters with the enzyme can be pictured according to the geometry described by Hansch et al[22] (Scheme 5A). L-Leucinol phenylacetate will interact with the S_1 position of the active site better as depicted Scheme 5B than Scheme 5C. In this case the interaction described as Scheme 4B, is preferred to the interaction described for Scheme 5C, probably by the presence of the amino group in the "natural position" for this endopeptidase that could interact with Ser-214 of the recognition subsite by H-bonding. So, the distance between the end of the chain (located in the "ar" subsite) and the CO (atacking point of Ser-195) is larger (7.279 Å) than the critical distance in the substrates [L-Phe-OMe ≡ 6,364 Å (Figure 1)] [n-hexanoate ≡ 6,653 Å (Figure 2)] and the hydrolysis of this ester does not occurrs.

The Km values are different depending on the structure of the acyl donor (Table 3). Aromatic acyl-donors (L-Phe, L-Tyr or phenyl-acetate), give lower Km values than the aliphatic

acyl-donors (hexanoate) according to the specificity of α-CT for aromatic aminoacids (L-Phe or L-Tyr) in S_1 subsite[3,5,6]. This finding can be explained because the chain of the hexanoate (Figure 2) has similar length to that of the phenylacetate (Figure 3), but no π-bonds are present in the first case. Therefore the stabilization of the acyl donor - enzyme complex in the "ar" subsite, by means of the π-π interaction is not present in that case. This finding can explain the increase in the Km value observed in the case of the derivative of the hexanoic acid (Table 3) respect to the L-Phenylalanine ethyl ester. The recognition of the alkyl chains by α-CT has been reported in the literature e.g: N-acetyl-alkyl-α-aminoacids[12] or in the case of the basic acyl donors: Arg-OEt and Lys-OEt[3].

The chemically modified enzyme can interact with acyl donors with structures different to the natural aminoacids, as the native enzyme. The only steric request is that CO-OEt must be located over Ser-195 and that the distance between the end of the "ar" subsite and the CO must be between 6.26 and 6.8 Å. This distance is 6.26 Å in the case of the subtrate of native enzyme L-Phe (Figure 1) and 6.829 in the case of L-Tyr, for which interaction of the acid hydrogen of the phenol with the end of the pocked has been described as a positive effect.[18,20,21]. Thus we can conclude that hexanoate and, obviously, phenylacetate chains are introduced into the "ar" subsite in the same conformation as the phenylalnine residue. The absence of reaction in the case of the octanoate - with a distance O=C....CH_2-H - of 8,815 Å - in the minimum energy conformer (Figure 3) - cannot be explained using only sterical reasons because the N-benzoyl-p-methyl-phenylalanine ethyl ester - with a distance of 8.63 Å between the H-CH_2-C_6H_4-....CO-OEt gives hydrolysis and peptide synthesis using the same enzyme and the same experimental conditions[20]. Therefore we should conclude that both effects i) the distance H-CH_2.....C=O and ii) the stabilization produced by the interaction π-π of the aromatic ring with the "ar" pocket should be analyzed in order to explain the reactivity of α-CT-PEG respect to unnatural esters. In this way we can affirm that p-substituted phenylalanine homologs, with a distance H-CH_2-C_6H_4-...C=O ≤ 8.87 Å can be used as acyl-donor because π- π interaction favours the recognition of the acyl donor by the "ar" pocket, but in the case of alkyl chain, only acyl-donor with a distance H-CH_2-....C=O ≤8.8Å can be used because in this case there is not any π - π stabilization. This distance is lower than that described by Steiz et al[23], who assume a distance of 10 - 12 Å for the "ar" pocket because the hydrolysis of p-Iphenylalanine ethyl ester is not observed.

Finally, the presence of bulky alkyl chains such as iBu(L-leucinol derivatives) reduces the reactivity by sterical reasons compared to the presence of the aromatic ring (L-Phenylalaninol derivatives).

EXPERIMENTAL

Materials

α-chymotrypsin (α-CT), monomethoxypolyethylene glycol (Mw 5,000), 1,1,1-trichlorotriazine and natural aminoacids were obtained from Sigma Chemical (St. Louis Mo, USA).

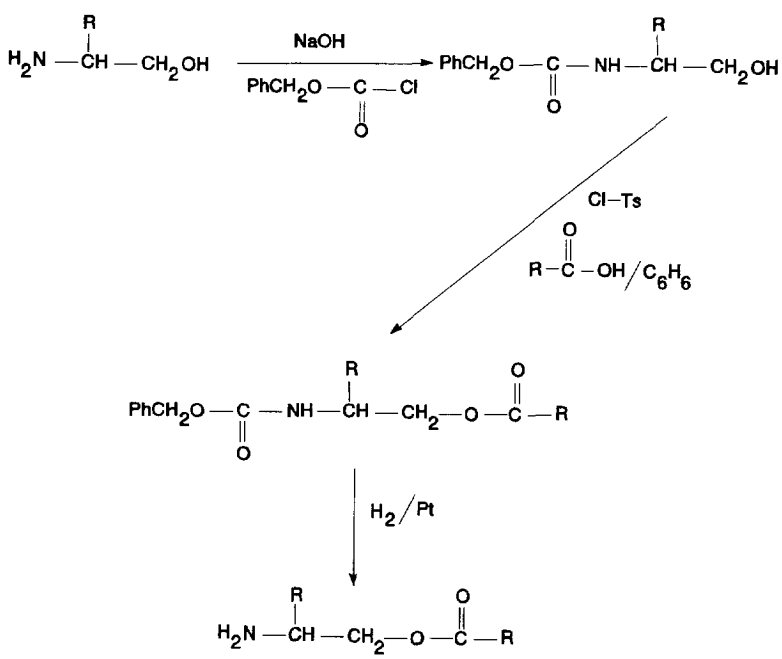
Anhydrous 1,1,1-trichloroethane was purchased from Merk and was dried by storage over a 0.4 mm molecular sieve.

Methods

Synthesis of unnatural substrates. ^{1}H-NMR spectra were obtained using a Varian VXR-300, a Bruker AC-250 or a Hitachi-Perkin-Elmer R-24 spectrometers and they were recorded in ppm downfield from the internal standard of tetramethylsilane in deuteriochloroform or hexadeuteriodimethylsulfoxide. The IR spectra were obtained either from thin films or potassium bromide pellets with a Buck Scientific-500 spectrophotometer. Elemental analyses were obtained by the Service of Microanalysis using a Perkin-Elmer 2400 CHN instrument. TLC monitoring reactions was carried out using aluminium plates coated with silica gel and a fluorescent indicator (Scharlau Cf 530). Purifications by flash chromatography were performed on silica gel (SDS 60 ACC 230-400 mesh). All reagents were of commercial quality (Aldrich, Fluka, Merk, SDS, Probus) and were used without further purification.

The synthetic scheme was:

Scheme 6



General procedure for the N-amino protection[24].

A cold solution (0°C) of aminoalcohol (0.1 mol) in water (12.5 cm^3) was added dropwise under stirring to a mixture of benzyl chloroformate (0.105 mol) and sodium hydroxide 4N (25 cm^3) for 40 min. The stirring was continued for 30 min. at 0°C and 30 min. at room temperature.

The solution was acidified with hydrochloric acid. The resulting aqueous solution was extracted with diethyl ether (3 x 30 cm^3) and the combined extracts were dried over magnesium sulfate. Solvent removal under vacuum afforded a crude product, which was purified by crystallization from ethylacetate-hexane.

N-benzyloxycarbonyl-ethanolamine **1**.-Yield 81%; m.p. 60°C. (lit. 62-62.5°C).(Found: C, 61.25; H, 6.68; N, 7.17. $C_{10}H_{13}NO_3$ requires C, 61.52;H, 6.71;N, 7.18); ν_{max} 1695 cm^{-1}; δ_H(90MHz;$CDCl_3$) 7.50 (5H,s,C_6H_5); 5.20 (2H,s,CH_2-C_6H_5); 4.00 - 3.75 (2H,m,$^1CH_2$); 3.70 - 3.45 (2H,m,$^2CH_2$)

N-benzyloxycarbonyl-L-leucinol **2**.-Yield 37%. m.p. liquid.. Found: C, 71.15;H, 8.90; N, 5.87; $C_{14}H_{21}NO_2$ requires C, 71.45; H, 8.99;N, 5.95); ν_{max} 1720 cm^{-1}; δ_H(250 MHz;$CDCl_3$) 7.34 (5H,s,C_6H_5); 5.13 (2H,s,CH_2-C_6H_5); 3.78 (1H,dd,CH_2-O<u>H</u>); 3.65 (1H,dd,CH_2-OH); 2.90(1H,m,CH-N); 1.65 (1H,m,C<u>H</u>$(CH_3)_2$); 1.23 (2H,t,CH-CH_2-CH); 0.92 (6H,d,$CH(CH_3)_2$).

N-benzyloxycarbonyl-L-phenyalaninol **3**.-Yield 46%. m.p. 88-89°C. Found: C, 71.12; H, 6.79; N, 4.48; $C_{17}H_{18}NO_2$ requires C, 71.57, H, 6.66;N, 4.91); ν_{max}=1710 cm^{-1}; δ_H(250 MHz;$CDCl_3$) 7.28 (10H,m,$(C_6H_5)_2$); 5.09 (2H,s,O-CH_2-C_6H_5); 3.96 (1H,m,CH-N); 3.69 (2H,dd,CH_2-OH); 3.58 (1H,dd,CH_2-O<u>H</u>);2.87 (2H,d,CH_2-C_6H_5).

General procedure for the synthesis of esters.

A mixture of **1**, **2** or **3** (1.0 mol), the corresponding carboxylic acid (1.10mol), p-toluensulfonic acid (0.10 mol) and benzene (250 cm^3) were heated under reflux for 24h. The benzene solution was neutralized with 2N $NaHCO_3$ and washed with water and then dried over magnesium sulfate. Solvent was removed under vacuum and afforded a residue that was chromatographed on silica gel eluting with ethyl acetate- hexane.

N-benzyloxycarbonyl-2-aminoethylhexanoate **4**.- Yield 79%. Found: C, 68.10; H, 7.92;N, 4.84;$C_{16}H_{23}NO_4$ requires C, 68.25; H, 8.22; N, 4.92%; ν_{max}=1740 and 1690 cm $^{-1}$; δ_H(90MHz;$CDCl_3$) 7.45 (5H,s,C_6H_5); 5.10 (2H,s,CH_2-C_6H_5); 4.15 (2H,t,$^1CH_2$); 3.40(2H,t,$^2CH_2$); 2.35(2H,t,CH_2-CO).

N-benzyloxycarbonyl-2-amino-4-methyl-penthyl-hexanoate **5**.- Yield 62%. Found: C, 68.16; H, 8.45; N, 4.09; $C_{20}H_{31}NO_4$ requires C, 68.80; H, 8.90; N, 4.00; ν_{max} 1740 and 1715 cm^{-1}; δ_H (300MHz;$CDCl_3$) 7.35 (5H,s,C_6H_5); 5.10 (2H,s,CH_2-C_6H_5); 4.70 (1H,b.s.,NH); 4.06(2H,s,CH_2-OH); 0.92 (6H,d,CH-$(CH_3)_2$CH); 0.82 (3H,t,CH_3-CH_2).

N-benzyloxycarbonyl-2-amino-3-phenylpropylhexanoate **6**.- Yield 73%.m.p. 66-68 °C; Found: C, 72.17; H, 7.08; N, 3.57; $C_{23}H_{29}NO_4$ requires C, 74.40; H, 7.08; N, 3.67; ν_{max} 1730 and 1695 cm^{-1}; δ_H (250MHz;$CDCl_3$) 7.25 (10H,m,$(C_6H_5)_2$); 5.09 (2H,s,OCH_2-C_6H_5); 4.17 (1H,m,CH-N); 4.06 (2H,d,CH_2-O); 2.32 (2H,t,CH_2-CO); 0.91 (3H,t,CH_3).

N-benzyloxycarbonyl-2-amino-etyloctanoate **7**.-Yield 94%.; Found: C, 67.05; H, 8.75; N,

4.03; $C_{18}H_{27}NO_4$ requires C, 67.28; H, 8.41; N, 4.36; ν_{max} 1730 and 1690 cm^{-1}; δ_H (90MHz;$CDCl_3$) 7.40 (5H,s,C_6H_5); 5.20 (2H,s, CH_2-C_6H_5); 4.25 (2H,t,$^1CH_2$); 3.55 (2H,c,$^2CH_2$); 2.35 (2H,t,CH_2-CO).

N-benzyloxycarbonyl-2-aminoethyl-phenylacetate **8**.-Yield 40%. m.p. 48-50°C. Found: C, 68.68; H, 6.01; N, 4.45; $C_{18}H_{19}NO_4$ requires C, 69.00; H, 6.01; N, 4.47; ν_{max} 1722 and 1700 cm^{-1}. δ_H (90MHz;$CDCl_3$) 7.35 (10H,m,$(C_6H_5)_2$); 5,20 (2H,s, CH_2-C_6H_5);4.30 (2H,t,$^1CH_2$); 3.50 (2H,c,$^2CH_2$).

N-benzyloxycarbonyl-2-amino-3-phenylpropylphenylacetate **9**.- Yield 61%; Found: C, 74.29; H, 5.99; N, 3.47; $C_{25}H_{25}NO_4$ requires C, 74.44; H, 6.20; N, 3.50; ν_{max} 1725 and 1685 cm^{-1}; δ_H (250MHz;$CDCl_3$) 7.80 (10H,m, $(C_6H_5)_2$); 5.09 (2H,s,O-CH_2-C_6H_5); 4.06 (2H,m,CH_2-O); 3.67 (2H,s,CH_2-CO);3.55(1H,m,CH-NH); 2.79 (2H,m,C_6H_5-CH_2-CH).

General procedure for catalytic hydrogenolysis of benzyloxycarbonyl group.

A solution of **4**, **5**, **6**, **7**, **8** or **9** in ethyl acetate was hydrogenolyzed at room temperature and 40 psi using 10 % Palladium on charcoal for 24 h in a hydrogenator Parr 3920. The catalyst was filtered and the solvent was removed under vacuum. The residue was chromatographied on silica gel using ethyl acetate-hexane (10:1) as eluant.

2-Aminoethylhexanoate **10**.-Yield 95%;m.p. 40-50°C; Found: C, 60.75; H, 11.04; N, 8.58; $C_8H_{17}NO_2$ requires C, 60.33; H, 10.76; N, 8.79; ν_{max} 1730 cm^{-1}; δ_H (300MHz;$CDCl_3$) 3.80 (2H,s,NH_2); 3.69 (2H,t,$^1CH_2$); 3.39 (2H,c,$^2CH_2$); 2.18 (2H,t,CH_2-CO);1.61 (2H,q,βCH_2); 1.28 (4H,m,γ,δ-CH_2); 0.87 (3H,t,CH_3).

2-Amino-4-methylpentyl-hexanoate **11**.-Yield 84%; Found C, 66.2; H, 10.89; N, 6.96; $C_{12}H_{25}NO_2$ requires C, 67.0; H, 11.4; N, 6.50; ν_{max} 1720 cm^{-1}; δ_H (250MHz;$CDCl_3$) 3.90 (2H,d,CH_2-O);2.65(2H,bs,NH_2);2.30(2H,t,CH_2-CO);1.30(2H,t,CH-CH_2CH);0.90(6H,d,$(CH_3)_2$-CH); 0.83 (3H,t,CH_3-CH_2).

2-Amino-3-phenylpropylhexanoate **12**.-Yield 89%; Found: C, 70.79; H, 9.70; N, 5.85; $C_{15}H_{23}NO_2$ requires C, 70.85; H, 9.76; N, 5.90; ν_{max} 1645 cm^{-1}; δ_H (250MHz;$CDCl_3$) 7.23 (5H,m,C_6H_5); 4.17 (2H,m,NH_2); 3.71 (1H,dd,CH_2-O); 3.60 (1H,dd,CH_2O);2.86 (2H,m,CH_2-C_6H_5);2.10 (2H,t,CH_2-CO); 0.87 (3H,t, CH_3-CH_2).

2-Aminoethyloctanoate **13**.-Yield 95%;m.p.69-70°C (lit 69-70°C); Found: C, 64.46; H, 11.30; N, 7.48; $C_{10}H_{21}NO_2$ requires C, 64.13; H, 11.43; N, 7.53; ν_{max} 1740 cm^{-1}; δ_H (90MHz;$CDCl_3$) 4.8 (2H,t,NH_2); 4.30 (2H,t,$^1CH_2$); 3.50 (2H,c,$^2CH_2$); 2.30 (2H,t,CH_2-CO).

2-Aminoethyl-2-phenylacetate **14**.-Yield 71%;m.p. 53-55°C; Found: C, 66.96; H, 7.32; N, 7.79; $C_{10}H_{13}NO_2$ requires C, 67.03; H, 7.26; N, 7.82; ν_{max} 1670 and 1659 cm^{-1}.
δ_H (90MHz;$CDCl_3$) 7.40 (5H,m,$(C_6H_5)_2$); ;4.35 (2H,t,$^1CH_2$); 3.60 (2H,c,$^2CH_2$).

2-Amino-3-phenylpropyl-2-phenylacetate **15**.-Yield 83%; m.p. 71-72 °C; Found: C, 74.33; H, 6.97; N, 4.76; $C_{17}H_{19}NO_2$ requires C, 75.80; H, 7.06; N, 5.20; ν_{max} 1645 cm^{-1}; δ_H (250MHz;$CDCl_3$) 7.23 (10H,m,$(C_6H_5)_2$); 3.70 (2H,dd,CH_2-O); 3.62 (2H,s,CH_2-CO); 3.50 (1H,m,CH-NH_2);3.38 (2H,dd,CH-CH_2-C_6H_5); 1.64 (2H,bs,NH_2).

Preparation of α-CT-PEG

The chemically modified enzyme was prepared according to the procedure previously described[3]. The modified enzyme was lyophilized for 8 h.

The determination of the amount of protein in the chemically modified enzyme was carried out by Lowry methodology[25], taking into the account the modifications proposed by Hartree[26].

Immobilization of α-chymotrypsin on agarose

The enzyme was immobilized on Sepharose-CL6B (Pharmacia). The activation of Sepharose was carried out according to the tosylation methodology, previously described by Sinisterra et al[27] for the activation of polysacharides. The enzymatic derivative was lyophilized for 8 h.

Enzymatic hydrolysis of esters

The enzymatic hydrolysis of esters was performed in a pHstat Crisson Micrott 2022 using 10^{-3} M NaOH solution as the titrating agent. The reactions were carried in a 10^{-2} M phosphate buffer (pH=8.0).

Synthesis of peptides

The synthesis of peptides was carried out in 1,1,1-trichloroethane according to the methodology previously described by Sinisterra et al[3]. The reaction course was monitorized by HPLC using a HPLC Chromatograph LDC model CM4000 and a C_8 5 μ column using an isocratic program with 0.1 M phosphate buffer (pH=7.0)/acetonitrile (50/50 v/v) as eluant. Flux=1.0 ml/min. λ=270 nm in the case of N-benzoyl-tyrosine ethyl ester (BTEE) and λ=254 nm in the other cases.

Conformational analysis

The conformational analysis was studied by the Molecular Mechanics Methodology[28] using MMX 88 program[29]. PCMODEL program[30] was used to measure spatial distances between atoms in the minimized conformation.

ACKNOWLEDGMENTS

This paper has been supported by a grant of the Ministerio de Educación y Ciencia of Spain. Grant PB 090-C010. We are grateful to Dr. A.R.Alcántara for critical reading of the manuscript.

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(*Received in UK* 11 *October* 1994; *revised* 29 *November* 1994; *accepted* 2 *December* 1994)