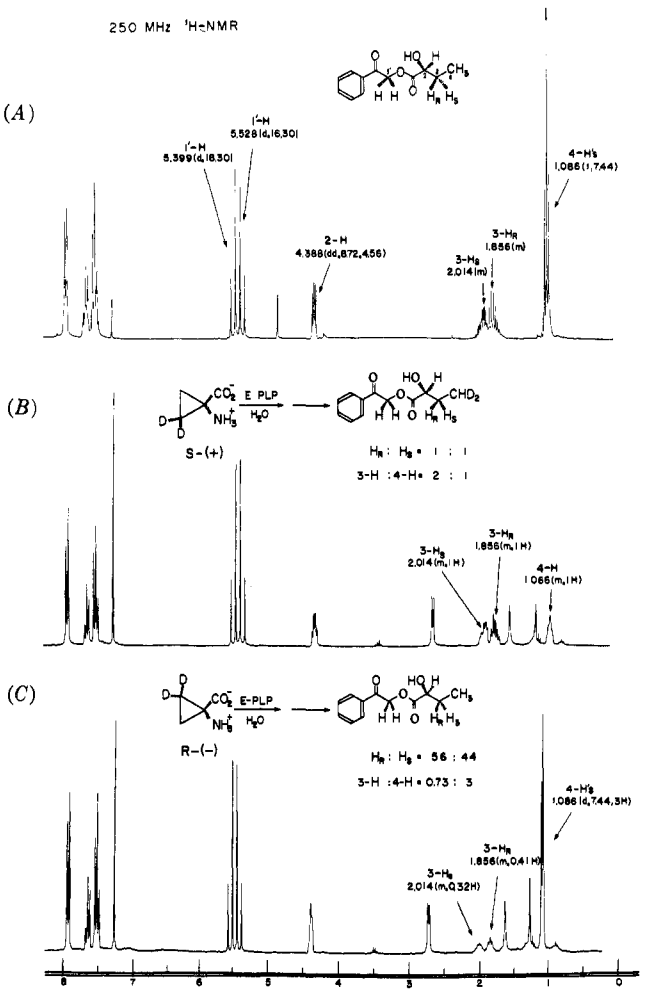

Figure 1. 250-MHz ^1H NMR of phenacyl esters from ACPC deaminase reactions: (A) standard, (B) incubation product from (*S*)-[$^2H_2$]-**10**, (C) incubation product from (*R*)-[$^2H_2$]-**10**.

followed by benzoylation gave the tribenzoate **14**, $[\alpha]_D$ +9.14° ($CHCl_3$). Comparison with authentic material, $[\alpha]_D$ +10.23°, prepared independently from (*S*)-citramalic acid (**15**)[11] confirmed the anticipated *S* configuration of **13** and established an enantiomeric purity of 90 ± 2%.

The *tert*-butyldimethylsilyl ether of **13** was converted in three steps (OH^-; TsCl; Bu_4NBr) into bromide **16**. The critical C–C bond was generated by an intramolecular displacement reaction when **16** was treated with lithium in THF to give the cyclopropane derivative **17**. Oxidation of **17** with RuO_4 to acid **18** and Curtius rearrangement of the latter furnished the oxazolidone **19**, which was converted by hydrolysis, trifluoroacetylation, and chromic acid oxidation to amide **9**, $[\alpha]_D$ −0.75° (acetone), subsequently hydrolyzed to (*R*)-(−)-**10**,[12] $[\alpha]_D$ −0.73° (*c* 3.5, H_2O); CD $\Delta\epsilon$ (205 nm) 4.13 × 10^{-3} L mol^{-1} cm^{-1} (H_2O) (Scheme III).

Enzymatic deamination of 6 mg of each labeled form of **10** was carried out as previously described,[4] coupling the ring opening with in situ reduction to (2*S*)-2-hydroxybutyric acid by NADH and L-lactate dehydrogenase. The resulting acid was converted to the crystalline phenacyl ester for NMR analysis. The NMR spectra in Figure 1 show unambiguously that ACPC deaminase differentiates sharply between enantiotopic methylene groups of its substrate. (*S*)-[$^2H_2$]ACPC (**10**) yields product deuterated exclusively in the methyl group (2.0 ^2H) whereas each of the independently synthesized *R*-[$^2H_2$] samples retains deuterium only in the product C-3 methylene group (1.27 and 1.29 ^2H, respectively). Thus, the methyl group of the product has its specific origin in the *pro-S* methylene of the substrate, and the regiospecificity of cleavage parallels that previously observed for the conversion of the higher homologue (1*S*,2*S*)-coronamic acid to 2-ketohexanoate.[13]

Partial loss of label (0.7 equiv), as in enzymic processing of (*R*)-[$^2H_2$]ACPC (**10**), is also observed in fragmentation of [$^2H_4$]ACPC (1.19 equivalents of ^2H at C-3 of product) and is not unexpected on the basis of earlier work with ACPC in ^2H_2O.[4] Accepting vinylglycyl-PLP imine equivalent (Scheme I) as a reaction intermediate,[4] this fractional exchange of label implies that the proton removed in the formation of that intermediate is shielded in the active site of the enzyme and therefore can return to C-3 in a subsequent step. The 56:44 distribution of label, evidenced in Figure 1C, among the stereoheterotopic positions at C-3 of the product indicates a nearly stererandom course for this reprotonation step.

Acknowledgment. This work has been supported in part by NIH Grants GM-28236 (R.K.H.) and GM 20011 (C.T.W.) and an NIEHS training grant (H.W.L.). Financial help from SANDOZ AG, Basel, is gratefully acknowledged. We thank Stephen E. Brown for large-scale resolutions and Richard Auchus for preparation of [$^2H_4$]ACPC.

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(12) For an independent assessment of the enantiomeric purity of the *R*-[$^2H_2$] acid, **10**, the compound was converted with (*S*)-phenylalanine to the diketopiperazine **20**, $[\alpha]_D$ + 71.9° (*c* 0.7, CH_3COOH). The ^1H NMR displayed two sharp doublets (δ 0.33 and 0.71, *J* = 5, 1 Hz) and two weak signals with less than 5% intensity (δ 0.95, 1.45) whereas in the ^1H spectrum of unlabeled **20**, the four methylene protons gave rise to four well-separated eight-line multiplets of equal intensity.

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Catalysis by Human Leukocyte Elastase. 2.[1] Rate-Limiting Deacylation for Specific *p*-Nitroanilides and Amides

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Received August 26, 1983

Serine proteases catalyze acyl-transfer reactions according to the kinetic mechanism of Scheme I.[2] In the absence of nucleophile, Nuc, k_c [$=k_2k_3/(k_2 + k_3)$] is commonly thought to be limited by deacylation, k_3, during ester hydrolysis and by acylation, k_2, during amide and anilide hydrolysis. Contrary to these generalities, Christensen and Ipsen have recently suggested[3] that reaction rates for highly specific peptide *p*-nitroanilides with certain serine proteases may be determined by deacylation and not acylation. Reported herein are steady-state kinetic experiments that address this problem for human leukocyte elastase. We report that *deacylation* is in fact rate limiting for the HLE[4]-catalyzed hydrolysis of specific-peptide esters, *p*-nitroanilides, and amides.

Comparisons of kinetic parameters for the HLE-catalyzed hydrolysis of peptide-derived substrates appear in Table I. Of

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(4) Abbreviations: MeOSuc, *N*-methoxysuccinyl; Ac, acetyl; pNA, *p*-nitroanilide; ONP, *p*-nitrophenyl ester; Phe-NH_2, L-phenylalaninamide; CHES, 2-(cyclohexylamino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; HLE, human leukocyte elastase.

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Scheme I

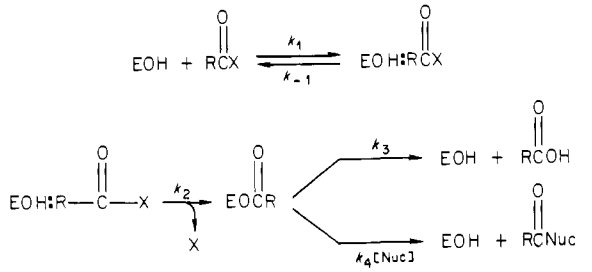

Table I. Kinetics for Reactions of Human Leukocyte Elastase

substrate	k_c, s^{-1}	$10^6 K_m$, M	k_c/K_m, $M^{-1}\ s^{-1}$	ref
MeOSuc-Ala-Ala-Pro-Val-pNA[b]	10	54	185 000	*a*
MeOSuc-Ala-Ala-Pro-Val-ONP[b]	9	3.4	2 600 000	*a*
MeOSuc-Ala-Ala-Pro-Val-pNA[c]	6.8	465	15 000	*a*
MeOSuc-Ala-Ala-Pro-Val-ONP[c]	6.4	30	210 000	*a*
Ac-Ala-Ala-Pro-Val-pNA[d]	8.1	310	27 000	12
MeOSuc-Ala-Ala-Pro-Val-pNA[e]	11	190	58 000	*a*
Ac-Ala-Ala-Pro-Val-NH_2[f]	2.4	6000	400	11
MeOSuc-Ala-Ala-Pro-Val-NH_2[f]	3.9	4400	910	11
Ac-Ala-Ala-Pro-Val-Ala-Ala-NH_2[f]	34	740	46 000	11
MeOSuc-Ala-Ala-Pro-Val-pNA[f]	29	88	330 000	*a*

[a] This work. Leukocyte elastase was purified as previously reported.[14] Initial velocities were determined and kinetic parameters calculated as described elsewhere.[1] Standard deviations deviations for the kinetic parameters are less than 10% in all cases. [b] 0.10 M phosphate, 0.50 M NaCl, pH 7.37, 3.3% Me_2SO, 25 °C. [c] 0.20 M Mes, 0.50 M NaCl, pH 6.03, 3.3% Me_2SO, 25 °C. [d] 0.05 M Tes, pH 7.5, 10% Me_2SO, 25 °C. [e] 0.10 M phosphate, pH 7.5, 10% Me_2SO, 25 °C. [f] 0.04 M phosphate, pH 7.5, 37 °C.

particular interest are hydrolysis kinetics for the *p*-nitrophenyl ester and *p*-nitroanilide of MeOSuc-Ala-Ala-Pro-Val. As anticipated from the relative reactivities of esters and anilides toward acylation, k_c/K_m [$=k_1k_2/(k_{-1} + k_2)$] is much larger for the ester than for the anilide. Unexpectedly, however, both substrates hydrolyze with *identical values* of k_c suggesting that the HLE-catalyzed hydrolysis of these substrates occurs with the same rate-determining step. We propose that this step is *deacylation*.[5]

If deacylation is in fact rate limiting for the hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA, then addition of an appropriate nucleophile to solutions of this substrate and HLE should, in accord with Scheme I, provide the acyl-enzyme with an alternate, low-energy pathway for decomposition and result in enhancement of k_c.[7] As shown in Figure 1, enhancement of k_c was indeed observed for reaction solutions containing the nucleophilic species L-phenylalaninamide.[8] Enhancement was more pronounced at pH 9 than at pH 7 indicating that these rate effects are due to the unprotonated, basic form of Phe-NH_2. From these data it is possible to calculate a pK_a of 7.7 for Phe-NH_2, identical with

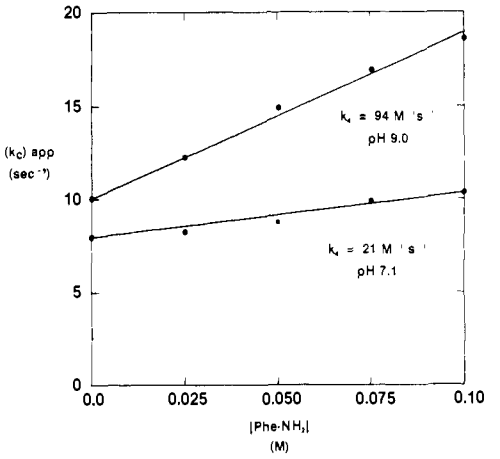

Figure 1. Dependence of k_c for the human leukocyte elastase catalyzed hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA on L-phenylalaninamide concentration. Initial velocites were determined with [MeOSuc-Ala-Ala-Pro-Val-pNA] = 2.71×10^{-4} M = $6K_m$ and [HLE] = 1.0×10^{-8} M in solutions containing 5.0×10^{-2} M CHES, 5.0×10^{-2} M HEPES, 5.0×10^{-1} M NaCl, and 3.3% Me_2SO buffered at either pH 7.08 or 9.03. $(k_c)_{app}$ vs. [Phe-NH_2] data were fit to the equation $(k_c)_{app} = k_c + k_4$[Phe-NH_2] where $(k_c)_{app}$ is the apparent k_c determined by dividing the initial velocity by [HLE].

pK_a values of aromatic amino acid amides.[9]

It appears from this work that HLE accelerates acylation for specific peptide *p*-nitroanilides to such an extent that deacylation becomes rate limiting. In contrast, less specific anilides, such as Suc-Ala-Ala-Ala-pNA, hydrolyze with rate-limiting acylation.[10] Deacylation may also be rate limiting for reactions of HLE and specific peptide *amides*. McRae et al. recently reported kinetics for the HLE-catalyzed hydrolysis of several peptides[11] (see Table I), including Ac-Ala-Ala-Pro-Val-Ala-Ala-NH_2 (hydrolysis occurs between the Val and Ala). Pertinent data are collected in Table I and indicate that substrates R-Ala-Ala-Pro-Val-pNA where R is acetyl[12] or methoxysuccinyl hydrolyze with similar values of k_c and, more significantly, that Ac-Ala-Ala-Pro-Val-Ala-Ala-NH_2 and MeOSuc-Ala-Ala-Pro-Val-pNA are hydrolyzed by HLE with *identical values* of k_c. Furthermore, when the dialanylamide leaving group of the substrate Ac-Ala-Ala-Pro-Val-Ala-Ala-NH_2 is replaced by ammonia, k_c falls off by an order of magnitude.

Results reported here for the leukocyte elastase catalyzed hydrolysis of tetrapeptide-based substrates are in contrast to earlier studies, which demonstrated without exception that serine protease catalyzed amide and anilide hydrolysis proceeds with rate-limiting acylation.[13] These earlier results are in accord with an enormous body of chemical literature for acyl-transfer reactions documenting that nucleophilic attack of an alcohol (active-site serine) on the carbonyl carbon of an amide to form an ester (acyl-enzyme) will have a much larger energy of activation than hydrolysis of that ester. The results of this study demonstrate that subsite interactions between a protease and polypeptide substrate can conspire

(5) The alternative that both substrates hydrolyze with rate-limiting acylation seems improbable to us. Due to large electronic differences between *p*-nitrophenol and *p*-nitroaniline, expressed at least in part by k_c/K_m, chemical processes involved in acyl-enzyme formation for their two substrates, such as the nucleophilic addition of the serine hydroxyl to the substrate carbonyl, will have quite different activation energies and thus cannot be considered as candidates for the common rate-limiting reaction. Conceivably, some physical processes such as a slow conformation change of the Michaelis complex limits k_c for both substrates. This explanation is also unlikely since identical, large solvent deuterium isotope effects on k_c were determined for the anilide[1] and the ester[6] (3.34 ± 0.05 and 3.2 ± 0.2, respectively).

(6) Unpublished results of one of the authors (R.L.S.).

(7) Enhancement of k_c occurs only if the rate of production of the leaving group (X of Scheme I) is monitored. For the classic, general treatment, see: Bender, M. L.; Clement, G. E.; Gunter, L. R.; Kezdy, F. *J. Am. Chem. Soc.* **1964**, *86*, 3679–3703.

(8) When these solutions were subjected to chromatography on Bio-Gel P-2 a material eluted at a position similar to that of the intact substrate. The UV–visible spectrum had none of the characteristics of a nitroanilide but was identical with that of PheNH_2 and thus suggest that MeOSuc-Ala-Ala-Pro-Val-Phe-NH_2 was formed.

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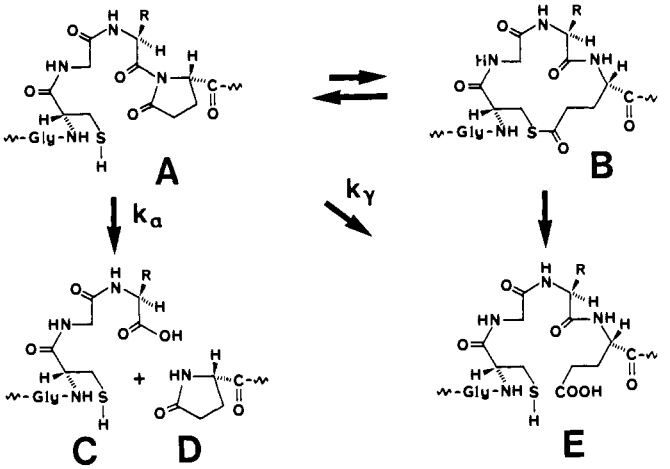
to override our chemical expectations. In the present case, interactions between HLE and the peptide moiety of MeOSuc-Ala-Ala-Pro-Val are sufficiently strong in the acylation transition state that acylation of HLE by the *p*-nitroanilide of this peptide proceeds faster than hydrolysis of the acyl-enzyme, MeOSuc-Ala-Ala-Pro-Val-HLE. Furthermore, while such interactions between HLE and MeOSuc-Ala-Ala-Pro-Val may not be strong enough to cause rate-limiting deacylation for the primary amide of this peptide, when leaving group subsite structural requirements are fulfilled sufficient stabilization of the transition state for amide acylation does occur, again resulting in rate-limiting deacylation.

Registry No. MeOSuc-Ala-Ala-Pro-Val-pNa, 70967-90-7; MeOSuc-Ala-Ala-Pro-Val-ONP, 88425-48-3; Phe-NH_2, 5241-58-7; elastase, 9004-06-2.

Scheme I



Synthesis and Regioselective Hydrolysis of Peptides Containing an Internal Residue of Pyroglutamic Acid

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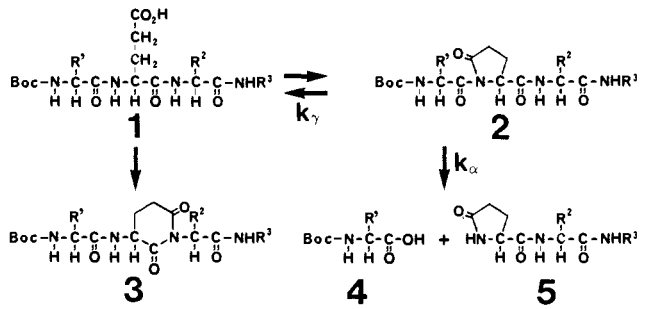
Received July 19, 1983

Efficient, mild, and rather general procedures are described for conversion of internal glutamic acid (Glu) residues into internal pyroglutamic acid (Glp; 5-oxoproline) residues and regioselective hydrolysis of the latter mainly with peptide chain fragmentation. The metastable binding sites of complement components C3[1] and C4[2] and protease inhibitor α_2-macroglobulin[3] evidently exist as macrocyclic thiolactone B, R = $CH_2CH_2CO_2H$ (Scheme I). Under denaturing conditions they undergo spontaneous hydrolysis[4] with fragmentation. Since product D contains a preformed N-terminal Glp residue, a plausible intermediate is lactam A containing an *internal* Glp residue. Synthetic hexapeptide models of lactam A having R = CH_3 or $CH_2CH_2CO_2H$ undergo chain fragmentation (k_α) to C and D in preference to ring opening (k_γ) to E.[5-7]

Several methods for preparing small peptides containing internal Glp residues (activation of Glu with thionyl chloride[8] or ethyl chloroformate;[9] permanganate oxidation[10] of proline) are reported to give low yields and byproducts. Conversion of an internal residue of Glu benzyl ester[11] or Glu sugar ester[6] to Glp in liquid HF, however, proceeds in moderate to good yield. Seven Boc-tripeptide amides (**1a-g**, R = Bzl, Scheme II) were synthesized. Typically, mixed anhydride coupling of Boc-Glu(OBzl) with Val-NH_2 followed by acidolysis of the Boc group gave Glu(OBzl)-Val-NH_2 (92% yield), which was coupled with Boc-Ala mixed anhydride to afford Boc-Ala-Glu(OBzl)-Val-NH_2 in 94% yield. Catalytic transfer hydrogenolysis[12] of the latter furnished Boc-Ala-Glu-Val-NH_2 in 95% yield. Half esters **1c,d**, R = Bzl, were made by the scheme[5] for Boc-Glu(OBzl)-Glu-Asn-NH_2.

Scheme II



a, $R^1 = CH_3$; $R^2 = CH_2CONH_2$
b, $R^1 = CH_2CH_2SCH_3$; $R^2 = CH_2CONH_2$
c, $R^1 = CH_2CH_2CO_2R$; $R^2 = CH(CH_3)_2$
d, $R^1 = CH_2CH_2CO_2R$; $R^2 = CH_2CONH_2$
e, $R^1 = H$; $R^2 = CH(CH_3)_2$
f, $R^1 = CH_3$; $R^2 = CH(CH_3)_2$
g, $R^1 = CH_2CH(CH_3)_2$; $R^2 = CH(CH_3)_2$
h, $R^1 = CH(CH_3)CH_2CH_3$; $R^2 = H$

Cyclization. Tripeptides **1** (Scheme II) were activated with *N,N'*-carbonyldiimidazole (CDI) and allowed to cyclize to give tripeptides **2**.[13] Typically, solid CDI (0.20 g, 1.2 mmol) was added to Boc-Gly-Glu-Val-NH_2 (**1e**, 0.40 g, 1.0 mmol) in DMF (2.0 mL) at -20 °C. The solution was stirred at -20 °C for 0.5 h and at 20 °C for 1.0 h and concentrated under reduced pressure. Addition of ether precipitated crude **2e**, which was purified by reprecipitation from DMF/ether. Half esters **1c,d**, R = Bzl, were treated with CDI to form ester lactams **2c,d**, R = Bzl, and deprotected by hydrogenolysis[12] to obtain acid lactams **2c,d**, R = H, which were purified by chromatography on octadecyl-silica. Yields of **2** were about 80% (Table I). Each of the peptides **1** and **2** showed the expected $(M + Na)^+$ ion in the ^{252}Cf fission

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(13) Cyclization using either *N,N'*-dicyclohexylcarbodiimide or isobutyl chloroformate and *N*-methylmorpholine gave lower yields and occasionally byproducts.

0002-7863/84/1506-0798$01.50/0