Anion Activation of Angiotensin Converting Enzyme: Dependence on Nature of Substrate†

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ABSTRACT: Anion activation of pulmonary angiotensin converting enzyme has been examined by using 23 furanacryloyl- and 3 benzoyl-tripeptides as substrates. Chloride stimulates hydrolysis of all substrates at least 24-fold. However, the kinetic mechanism, the amount of chloride required, and the effect of pH on activation, plus the relative activating potencies of various anions, are all strongly dependent on the substrate employed. Three substrate classes have been identified. Class I substrates appear to be hydrolyzed at pH 7.5 by an ordered bireactant mechanism in which anion must bind before substrate. The apparent activation constant (K_A') for Cl^- ranges from 75 to 150 mM at pH 7.5, doubles at pH 9.0, and decreases to about 3 mM at pH 6.0. Class II substrates, in contrast, are hydrolyzed by a nonessential activator mechanism. The kinetically determined K_A' for Cl^- at pH 7.5 ranges from 2.9 to 5.0 mM and changes only slightly with pH. Class III substrates are also hydrolyzed by a nonessential kinetic mechanism but one different from that followed by class II peptides. K_A' values for Cl^- at pH 7.5 measured with class III substrates are 18–30 mM. Class II substrates have Arg or Lys at the ultimate or penultimate position. The features distinguishing class I and III peptides are less clear, although all class III substrates identified have penultimate alanine residues. Possible explanations for this substrate dependence are offered.

Angiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (ACE)[1] has been given considerable attention during the past decade because of its key role in the regulation of blood pressure: it catalyzes both the conversion of the decapeptide angiotensin I to the potent vasoconstricting octapeptide angiotensin II and the inactivation of the vasodilating nonapeptide bradykinin (Soffer, 1976; Erdös, 1976; Peach, 1977). Studies with synthetic substrates have revealed that ACE can act on an extremely wide range of oligopeptides, its principal requirements being a free COOH terminus and a primary amide at the scissile bond (Elisseeva et al., 1971; Stevens et al., 1972; Cheung et al., 1980; Rohrbach et al., 1981).[2] Thus, while angiotensin I and bradykinin are its only well-documented physiological substrates, the enzyme could potentially hydrolyze many peptides in vivo. The existence of such additional substrates, and of further functions for ACE beyond control of blood pressure, is suggested by the presence of the enzyme in a large number of tissues. Although the association between lung ACE and blood pressure regulation has been established, the role of converting enzyme in loci such as kidney, testis, pancreas, stomach, intestine, eye, and brain (Roth et al., 1969; Cushman & Cheung, 1971b, 1972; Igić & Kojović, 1980) remains unknown.

In view of the broad specificity of the enzyme, it seems critical that a variety of substrates be employed in any study attempting to gain understanding of its physiological roles, molecular mechanism, and mode of regulation. A striking feature of ACE which may relate to all of these is its activation by monovalent anions, notably chloride. Significantly, widely different effects of chloride on activity have been reported, dependent on the particular substrate employed. Substantial activation has been found to occur with angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) (Skeggs et al., 1954) and its synthetic analogue (Bz-Gly-His-Leu) (Cheung et al., 1980) but relatively little with bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) (Dorer et al., 1974) and its analogue (Bz-Gly-Phe-Arg) (Cheung et al., 1980). On the other hand, the hydrolysis of the bradykinin potentiating peptide 5a (BPP_{5a}) (<Glu-Lys-Trp-Ala-Pro) (Cheung & Cushman, 1973) and its analogue (Bz-Gly-Ala-Pro) (Cheung et al., 1980) has been reported to be inhibited rather than activated by chloride.

The present study critically examines the substrate dependence of anion activation by using a series of benzoyl- and furanacryloyl-blocked tripeptides as substrates. In contrast with earlier reports, we find hydrolysis of all substrates to be strongly (at least 24-fold) activated by chloride. However, detailed analysis of the activation patterns reveals a marked substrate dependence in the mechanism of activation, the amount of chloride required for activation, the effects of pH on activation, and the relative potencies of various anions. On the basis of these patterns, the substrates can be assigned to three general classes.

Materials and Methods

ACE was isolated from rabbit lung acetone powder (Pel-Freez Biologicals, Inc., Rogers, AR) as described previously (Bünning et al., 1983). The final preparation was at least 98% pure as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis. Enzyme concentrations were determined from the absorbance at 280 nm, by using a molar absorptivity of 204 000 M^{-1} cm^{-1} (Bünning et al., 1983).

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[1] Abbreviations: ACE, angiotensin converting enzyme; BPP_{5a}, bradykinin potentiating peptide 5a (<Glu-Lys-Trp-Ala-Pro); $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; DCC, *N*,*N*′-dicyclohexylcarbodiimide; FA, 2-furanacryloyl; Bz, *N*-benzoyl; *N*-Cbz, *N*-carbobenzoxy; ONSu, succinimidooxy; *t*-Boc, *tert*-butyloxycarbonyl. In describing the position of an amino acid on a peptide relative to the scissile peptide bond, the terminology of Schechter & Berger (1967) was employed: for angiotensin converting enzyme, this corresponds to P_1, antepenultimate, P'_1, penultimate, and P'_2, ultimate residues, respectively.

[2] ACE also catalyzes the hydrolysis of ester analogues of peptide substrates (Keung et al., 1980).

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Adventitious metal ions were removed from all buffer solutions by extraction with a fresh solution of 0.01% dithizone in carbon tetrachloride. Levels of chloride contamination in buffers and substrates were determined by using a Model 91100 chloride electrode from Graphic Controls (Buffalo, NY).

Assays. With FA-tripeptide substrates, enzyme activities were measured as described previously (Holmquist et al., 1979). An appropriate wavelength between 328 and 348 nm was chosen, depending on the substrate concentration, and the decrease in absorbance was continuously monitored with a Varian Model 219 spectrophotometer. A 2-, 10-, or 50-mm cuvette was employed as required to give initial absorbance readings between 0.15 and 2.0. This allowed use of a substrate concentration range extending from 2.5×10^{-6} to 10^{-2} M. With Bz-tripeptide substrates, hydrolysis was followed spectrophotometrically by continuous monitoring of the increase in absorbance at 257 nm (Whitaker et al., 1966).

The kinetic parameters K_m and k_{cat} were obtained from Lineweaver–Burk plots. Initial velocities were measured during the first 10% of hydrolysis. K_i values were determined by the method of Dixon (1953).

The standard assay conditions were 25 °C, 50 mM Hepes, pH 7.5. Where velocities at other pH values were measured, Mes (from pH 5.1 to 6.9), Hepes (from pH 6.9 to 8.3), or borate (pH 8.3–10.0) was used, all at 50 mM. Because zinc begins to dissociate from the enzyme below pH 7 (Bünning & Riordan, 1981), SpecPure (Johnson-Matthey, Royston, England) $ZnCl_2$ was added as follows: 10^{-2} M between pH 5.0 and 5.5, 10^{-3} M between pH 5.5 and 6.0, 10^{-4} M between pH 6.0 and 7.0, 10^{-5} M between pH 7.0 and 7.5, and 10^{-6} M at pH 7.5 and above.

Chemical Modifications. All modification reactions were carried out at 20 °C with approximately 1 μM enzyme. Modification by 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) was performed in 50 mM Mes, pH 6.0, containing 10^{-4} M Zn^{2+} with 20 mM reagent for 1 h. Modification by 2,3-butanedione was carried out in 0.05 M borate, pH 8.3, for 2.5 h with 10 mM reagent. The tetranitromethane reaction was in 50 mM Tris-HCl, pH 8.0, for 20 min with 8 mM reagent.

Substrates. Dipeptides were either purchased from Vega Biochemicals (Tucson, AZ) or Sigma Chemical Corp. (St. Louis, MO) or synthesized by standard procedures in which *N*-carbobenzoxy-blocked amino acids were activated to the *N*-hydroxysuccinimide esters by using DCC. The carbobenzoxy group was removed after coupling by catalytic hydrogenolysis using 10% Pd/charcoal at 1 atm pressure.

The *N*-2-furanacryloyl (FA)-blocked tripeptides FA-Phe-Gly-Gly, -Phe-Ala-Phe, -Phe-Ala-Gly, -Ala-Ala-Ala, -Phe-Leu-Gly, -Leu-Ala-Gly, -Gly-Leu-Phe, -Leu-Leu-Gly, -Ala-Leu-Ala, -Phe-His-Leu, and -Gly-Gly-Gly were from earlier studies (Bünning et al., 1983; Holmquist et al., 1979; Blumberg & Vallee, 1975), as was Bz-Gly-Gly-Phe (Auld & Vallee, 1970). In addition, synthesis of the intermediates FA-ONSu, FA-Phe-ONSu, FA-Gly-ONSu, and Bz-Gly-ONSu have been described previously (Holmquist et al., 1979; Blumberg & Vallee, 1975). FA-Phe-Ala-ONSu and Bz-Gly-Ala-ONSu were prepared as for FA-Gly-ONSu and recrystallized from 2-propanol. The synthesis of other substrates and intermediates is presented in the supplementary material (see paragraph at end of paper regarding supplementary material). All amino acids are in the L configuration. Compositions of all substrates were confirmed by amino acid analysis. Thin-layer chromatography on fluorescent silica gel (Eastman) in 4:1:1 1-butanol:acetic acid:water in every case revealed a single fluorescent quenching spot.

Although the final purification step for most substrates involved crystallization, with those peptides containing lysine this was not possible. Instead, these substrates were separated from starting materials and byproducts by cation-exchange chromatography (described in detail in the supplementary material). Since it is critical for this study that substrates do not contain significant amounts of activating anions, any such anions remaining after this procedure were removed as follows: The peptide (in water) was applied to a column of AG50W-X2 (H^+ form), washed copiously with water, and eluted with 5% ammonium hydroxide.

Table I: Kinetic Parameters for ACE-Catalyzed Hydrolysis of Various Class I, II, and III Substrates[a]

	K_m (μM)	k_{cat} (min^{-1})	K_A' (mM)
Class I Substrates			
FA-Phe-Gly-Gly[b]	300	19000	90
FA-Phe-Leu-Gly[b]	250	3800	100
FA-Phe-Ala-Glu	1100	13900	100
FA-Phe-(N^ϵ-*t*-Boc)Lys-Ala	160	12300	90
FA-Leu-Ala-Gly[b]	410	17300	115
FA-Leu-Leu-Gly[b]	490	5200	150
FA-Ala-Leu-Ala[b]	380	1900	85
FA-Gly-Ala-Gly[b]	4100	3200	145
FA-Gly-Leu-Phe[b]	810	3100	130
FA-Lys-Gly-Gly	2500	22700	75
FA-(N^ϵ-*t*-Boc)Lys-Gly-Gly	720	16500	150
Bz-Gly-Gly-Phe[c]	2500	37000	90
Class II Substrates			
FA-Phe-Phe-Arg	15	3260	3.6
FA-Phe-Ala-Lys	23	5680	4.0
FA-Phe-Ala-Arg	6.5	1700	4.0
FA-Phe-Lys-Ala	68	6200	2.9
Bz-Gly-Phe-Arg	110	6650	5.0
Class III Substrates			
FA-Gly-Ala-Pro	77	9600	19
FA-Phe-Ala-(N^ϵ-*t*-Boc)Lys	97	8680	18
FA-Lys-Ala-Phe	200	9400	25
FA-(N^ϵ-*t*-Boc)Lys-Ala-Phe	200	14000	18
FA-Phe-Ala-Phe	120	8400	22
FA-Phe-Ala-Gly[b]	500	20700	30
FA-Ala-Ala-Ala[b]	520	12400	24
Bz-Gly-Ala-Pro	49	6160	20
Not Assigned[d]			
FA-Phe-His-Leu[b]	68	4100	30

[a] Assay conditions: 50 mM Hepes, pH 7.5, 25 °C. K_m and k_{cat} values were determined with 300 mM NaCl present. K_A' values were determined in most cases from plots of $1/V_0$ vs. $1/[Cl^-]$ at $[S] << K_m$, where $-1/K_A'$ = intercept with $1/[Cl^-]$ axis. Since these plots become nonlinear at low $[Cl^-]$ with class II and III peptides (see text), the linear portions at $[Cl^-] > K_A'/2$ were utilized with such substrates. In some cases, plots of K_m/k_{cat} (instead of $1/V_0$) vs. $1/[Cl^-]$ were used, where K_m and k_{cat} were derived from Lineweaver–Burk plots. [b] K_m and k_{cat} values are from Bünning et al. (1983). [c] K_m and k_{cat} values are from Keung et al. (1980). [d] See text.

Results

Profiles of V_0 vs. $[Cl^-]$. The dependence of initial reaction velocity on chloride concentration at pH 7.5 was examined by using 26 N-blocked tripeptides, 24 of which were at substrate concentrations well below K_m (Table I) under conditions where initial velocities are directly proportional to k_{cat}/K_m. For the two other substrates, the K_m values were too low to allow such measurements, and hence, the dependence of k_{cat}/K_m on $[Cl^-]$ was determined from Lineweaver–Burk plots. Three distinct patterns were obtained for the different peptides, typified by

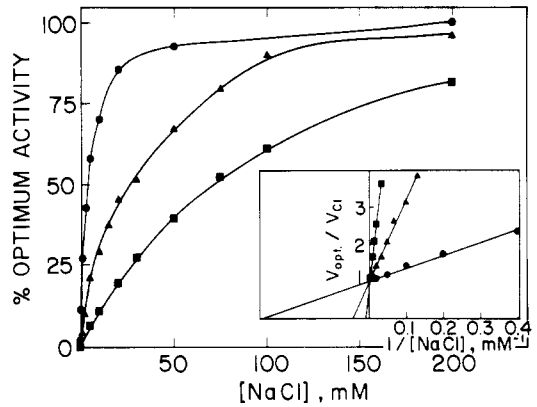


FIGURE 1: Effect of chloride concentration on the activity of ACE. Activity was measured with 50 μM FA-Phe-Gly-Gly (■), 23 μM FA-Lys-Ala-Phe (▲), or 3 μM FA-Phe-Phe-Arg (●) as substrates in 50 mM Hepes buffer, pH 7.5, at 25 °C. 100% activity is that measured at 300 mM NaCl with FA-Lys-Ala-Phe and FA-Phe-Phe-Arg and at 600 mM NaCl with FA-Phe-Gly-Gly. Higher chloride concentrations did not increase activity further. Inset: Replot of data in double-reciprocal form.

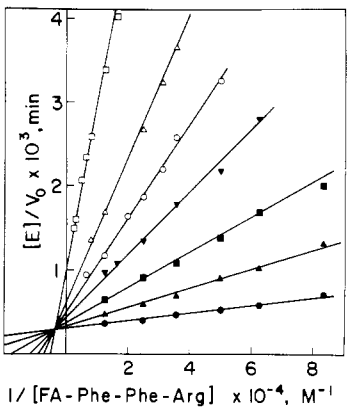


FIGURE 2: Lineweaver–Burk plots for the ACE-catalyzed hydrolysis of FA-Phe-Phe-Arg at various chloride concentrations. The following molarities of NaCl were added in 50 mM Hepes, pH 7.5: 2.0×10^{-2} (●); 2.5×10^{-3} (▲); 1.0×10^{-3} (■); 5.0×10^{-4} (▼); 2.5×10^{-4} (○); 1.25×10^{-4} (△); 0 (□).

those shown in Figure 1 for FA-Phe-Gly-Gly, FA-Lys-Ala-Phe, and the bradykinin analogue, FA-Phe-Phe-Arg. The apparent activation constants (K_A') for chloride, obtained from double-reciprocal replots of these data ($-1/K_A'$ = intercept with $1/[Cl^-]$axis), are 90, 25, and 3.6 mM, respectively.

Table I lists the 26 substrates examined along with K_A' values and the kinetic parameters K_m and k_{cat}. Hydrolysis of 12 of the substrates studied followed the pattern seen with FA-Phe-Gly-Gly, with K_A' values of 75–150 mM. These "class I" substrates all contain ultimate (P'_2) and penultimate (P'_1) amino acids with either neutral or negatively charged side chains. Five substrates, all having positively charged side chains at P'_1 or P'_2, exhibit the pattern observed for FA-Phe-Phe-Arg, and these are designated "class II". They exhibit a range of K_A' values from 2.9 to 5.0 mM. Eight peptides, including the two BPP_{5a} analogues, FA-Gly-Ala-Pro and Bz-Gly-Ala-Pro, followed the pattern shown for FA-Lys-Ala-Phe with K_A' values of 18–30 mM. Structurally, these "class III" substrates are similar to class I, although all thus far identified contain alanine at the P'_1 position.

FA-Phe-His-Leu, an analogue of angiotensin I, gives a pattern similar to that for class III substrates, with K_A' = 30 mM. Unlike the other peptides studied, however, it has a penultimate histidine that may be partially charged at the assay pH. Hence, this peptide may not be a true class III substrate, but rather a mixture of classes I and II, producing an apparent class III type profile. Data pertinent to this question will be presented below.

Extent of Chloride Activation. Although the ACE-catalyzed hydrolysis of all substrates examined is strongly activated by chloride, the exact extent of activation differs among the three classes. There is no detectable hydrolysis of class I substrates in the absence of chloride and under conditions where the assay method could measure velocities as small as 0.1% of maximal. Class II substrates can undergo hydrolysis, albeit slowly, even in the absence of added anion, but velocity enhancements of 24-, 25-, 45-, 55-, and 89-fold are observed for FA-Phe-Ala-Arg, FA-Phe-Lys-Ala, FA-Phe-Phe-Arg, Bz-Gly-Phe-Arg, and FA-Phe-Ala-Lys, respectively, when chloride is adjusted to its optimal concentration. Class III substrates are also hydrolyzed in the absence of added anion, and with these, the extent of activation varies from 45-fold (FA-Phe-Ala-Phe) to 160-fold (FA-Gly-Ala-Pro). In all cases, the extents of activation must be taken as minimum values since it is impossible to be certain that some amount of an activating anion is not present as a contaminant when the velocities are measured "without added anion" (see below).

Kinetic Mechanisms. A detailed study of the kinetics of anion activation of ACE with FA-Phe-Gly-Gly (class I) as substrate has recently been reported (Bünning & Riordan, 1983). The hydrolytic reaction appears to follow an ordered bireactant mechanism (Scheme I)

Scheme I

$$E + A \overset{K_A}{\rightleftharpoons} EA + S \overset{K_S}{\rightleftharpoons} EAS \xrightarrow{k_{cat}} EA + P$$

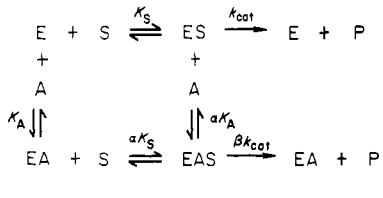
in which chloride must bind to the enzyme before substrate. Thus, only K_m is affected by chloride. With this mechanism, a plot of K_m vs. $1/[Cl^-]$ yields a straight line which intersects the $1/[Cl^-]$ axis at $-1/K_A$. The value for K_A at pH 7.5 is 90 mM when FA-Phe-Gly-Gly is the substrate.

Lineweaver–Burk plots at various chloride concentrations with FA-Phe-Phe-Arg, a class II substrate, demonstrate that in this case hydrolysis follows a different mechanism (Figure 2). Both K_m and k_{cat} are altered by chloride. In addition, hydrolysis can be measured without added chloride,[3] indicating a nonessential activator mechanism is followed, shown in its general form in Scheme II.

If rapid equilibrium assumptions are applied to Scheme II, values for α, β, and K_A can be obtained from the variations of K_m and k_{cat} with $[Cl^-]$, as described by Segel (1975) and modified by Rohrbach et al. (1981). $K_m^0/\Delta K_m$ and $V_{max}^0/\Delta V_{max}$ are plotted against $1/[Cl^-]$, where K_m^0 and V_{max}^0 are

[3] The possibility that the "zero" chloride velocities are due to contaminating anions has been considered. Measurements with a chloride-specific electrode put an upper limit of about 10 μM on the chloride concentration in the assay buffer and indicate that there is no more than 0.07 mol of chloride per mol of FA-Phe-Phe-Arg. A much larger amount of chloride would be necessary to account for the observed velocities. While chloride may be the most ubiquitous and, hence, most likely anionic contaminant, other anions can also activate ACE (Skeggs et al., 1954; Bünning & Riordan, 1983). Their presence in the Hepes buffer is unlikely since lowering the Hepes concentration from 50 to 0.5 mM in the absence of added chloride does not decrease the velocity. The substrates are also improbable sources of such contaminants since the zero chloride Lineweaver–Burk plots are linear. In addition, FA-Phe-Phe-Arg was purified by crystallizing it twice as the zwitterion from water, and FA-Phe-Ala-Lys and FA-Phe-Lys-Ala were subjected to cation-exchange column chromatography in order to remove extraneous anions (see Materials and Methods for details). Thus, it would appear that anion contamination does not contribute significantly to the velocities in Figure 2.

Scheme II



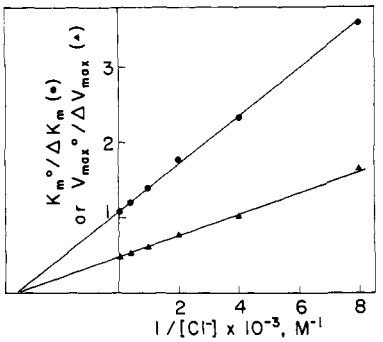


FIGURE 3: Replots of $K_m^0/\Delta K_m$ and $V_{max}^0/\Delta V_{max}$ vs. $1/[Cl^-]$ using the data from Figure 2.

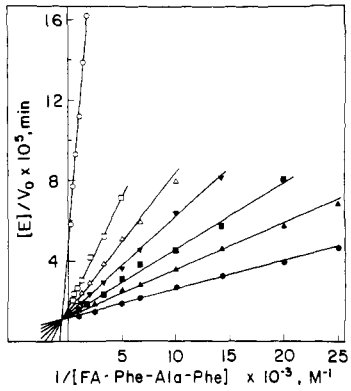


FIGURE 4: Lineweaver–Burk plots for hydrolysis of FA-Phe-Ala-Phe at various chloride concentrations. The following molarities of NaCl were added in 50 mM Hepes, pH 7.5: 3.0×10^{-1} (●); 5.0×10^{-2} (▲); 2.0×10^{-2} (■); 1.0×10^{-2} (▼); 5.0×10^{-3} (△); 2.0×10^{-3} (□); 0 (○).

the values in the absence of chloride and ΔK_m and ΔV_{max} are the differences between these values and those at a given chloride concentration. Both lines will intersect the $1/[Cl^-]$ axis at $-1/(\alpha K_A)$. The intersection of the K_m plot with the ordinate will give $1/(1-\alpha)$, and the V_{max} plot will yield $1/(\beta - 1)$. Such replots of the data in Figure 2 for FA-Phe-Phe-Arg are shown in Figure 3. The linearity of the plots demonstrates that the data obtained with this substrate are consistent with Scheme II. Values determined for K_A, α, and β are 4.2 mM, 0.071, and 3.1, respectively. This K_A value is in close agreement with that obtained by less rigorous means (Table I). It should be noted that while "K_A" represents the dissociation constant for chloride and *free enzyme* in both Schemes I and II, the values found by using class I and class II substrates differ by a factor of 16–20. Possible resolutions of this paradox are suggested under Discussion.

The kinetic plots for class III substrates are complex and do not fit either Scheme I or (a rapid equilibrium treatment of) Scheme II. The Lineweaver–Burk plots for FA-Phe-Ala-Phe at different chloride concentrations (Figure 4) demonstrate that both K_m and k_{cat} are altered by chloride, as they are with class II substrates. In addition, chloride is a nonessential activator with these substrates as well. However, the mechanism differs from that followed by class II substrates

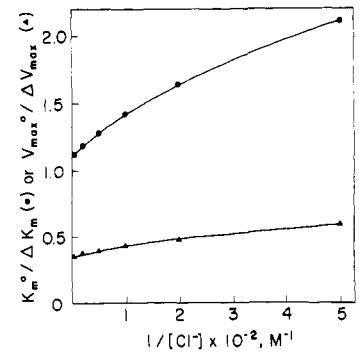


FIGURE 5: Replots of $K_m^0/\Delta K_m$ and $V_{max}^0/\Delta V_{max}$ vs. $1/[Cl^-]$ using the data from Figure 4.

Table II: K_A' Values for Chloride at pH 6.0, 7.5, and 9.0[a]

substrate	K_A' (mM) pH 6.0	pH 7.5	pH 9.0
FA-Phe-Gly-Gly (class I)	3.3	90	200
FA-Phe-Phe-Arg (class II)	1.8	3.6	6.2
FA-Phe-Ala-Phe (class III)	2.8	25	52

[a] Assay conditions: At pH 6.0, 50 mM Mes; at pH 7.5, 50 mM Hepes; at pH 9.0, 50 mM borate. Substrate concentrations were 50 μM for FA-Phe-Gly-Gly, 3.2 μM for FA-Phe-Phe-Arg, and 18 μM for FA-Phe-Ala-Phe. These concentrations are all well below K_m. The K_A' values in the table are derived from plots of $1/V_0$ vs. $1/[Cl^-]$, where the intersection with the $1/[Cl^-]$ axis is at $-1/K_A'$.

since the relationship between $K_m^0/\Delta K_m$ and $1/[Cl^-]$ (Figure 5) is nonlinear.

Effect of pH on K_A' for Chloride. Apparent activation constants for chloride were obtained by using class I, II, and III substrates at pH 6.0, 7.5, and 9.0 (Table II). It has been reported previously (Bünning & Riordan, 1983) that the K_A' with FA-Phe-Gly-Gly as substrate is strongly pH dependent, ranging 60-fold from 3.3 mM at pH 6.0 to 200 mM at pH 9.0. A similar pH effect is observed with other class I substrates. In contrast, when class II substrates are employed, K_A' is altered only slightly by pH. Thus, over the same range, the K_A' obtained by using FA-Phe-Phe-Arg increases by about 3-fold from 1.8 to 6.2 mM. With FA-Phe-Ala-Phe and other class III substrates, an intermediate, 19-fold change in K_A' is observed. The K_A' for hydrolysis of the angiotensin I analogue, FA-Phe-His-Leu, is 30 mM at pH 7.5 and increases to 280 mM at pH 9.0. This indicates that when the P'_1 histidine is uncharged, the peptide is a class I substrate, but when it is charged, it most likely behaves as a class II substrate.

Activation by Anions Other than Chloride. The relative activating potencies of anions other than chloride are also substrate dependent. The values listed in Table III reflect anion concentrations giving maximal velocities. At pH 7.5, bromide is somewhat less effective with class I than with class II and III substrates. Greater differences are seen when nitrate and iodide are used. While both of these anions are relatively poor activators for FA-Phe-Gly-Gly hydrolysis, they are almost half as potent as chloride with FA-Phe-Phe-Arg. Intermediate values are observed with the class III substrate. Acetate, a poor activator, seems to be slightly more effective with class II and III peptides than with class I. However, when velocities in the absence of activators are subtracted, the degree of activation for all three classes is similar.

At pH 6.0, the effects of the various anions change. Nitrate and iodide become better activators of hydrolysis of class I and III substrates than they were at pH 7.5. Bromide becomes as effective as chloride in activating FA-Phe-Gly-Gly hy-

Table III: ACE Activity with Various Anions[a]

	substrate					
	FA-Phe-Gly-Gly		FA-Phe-Phe-Arg		FA-Phe-Ala-Phe	
anion	pH 7.5	pH 6.0	pH 7.5	pH 6.0	pH 7.5	pH 6.0
chloride	100	100	100	100	100	100
bromide	52	103	72	73	74	81
nitrate	14	49	46	55	26	55
iodide	10	43	40	51	31	54
acetate	2.3	2.6	4.3		4.0	

[a] Assays are in 50 mM Hepes at pH 7.5 and 50 mM Mes at pH 6.0. Substrate concentrations are 50 μM for FA-Phe-Gly-Gly, 3.2 μM for FA-Phe-Phe-Arg, and 18 μM for FA-Phe-Ala-Phe, well below K_m in all cases. Activity with chloride is defined as 100. Anion concentrations for FA-Phe-Gly-Gly and FA-Phe-Ala-Phe at pH 7.5 are 300 mM. All other anion concentrations are 50 mM. All anions were added as sodium salts.

Table IV: Effects of Chemical Modification of ACE[a]

	activity [$(v/v_c) \times 100$]	
reagent	FA-Phe-Gly-Gly	FA-Phe-Phe-Arg
tetranitromethane	18.3	16.1
butanedione	16.2	16.2
CMC	24.0	21.0

[a] Assays were at pH 7.5, 25 °C. Modifications were performed as described in the text.

drolysis, while its potency with the other two substrates remains about the same as that at pH 7.5.

Substrate-Independent Features of ACE. Class I and II substrates, which kinetically are the most dissimilar, share several features that suggest a single enzyme active site is involved in their hydrolysis. First, chemical modifications of ACE with CMC, butanedione, and tetranitromethane have identical effects on activity toward FA-Phe-Gly-Gly and FA-Phe-Phe-Arg (Table IV). Second, the metal-chelating agent 1,10-phenanthroline (10^{-4} M) completely abolishes activity toward all substrates. Third, the pH–activity profiles measured in the presence of 0.3 M NaCl have virtually identical acidic limbs for class I and II substrates (Figure 6). Fourth, at pH 6, high concentrations of chloride inhibit hydrolysis of the two types of substrate to the same extent. In a previous report (Bünning & Riordan, 1983), it was noted that as the chloride concentration is increased at low pH, ACE-catalyzed hydrolysis of FA-Phe-Gly-Gly is first activated but then inhibited above 50 mM chloride. At pH 6, the velocity of FA-Phe-Gly-Gly hydrolysis at 0.3 M NaCl drops to 74% and at 1 M to 47% of the velocity at 50 mM NaCl. With FA-Phe-Phe-Arg, the respective percentages are almost identical: 77% at 0.3 M and 46% at 1 M NaCl.

Finally, Bz-Gly-Gly-Phe, a class I substrate, inhibits hydrolysis of FA-Phe-Phe-Arg, although not in a strictly competitive manner. A K_i value of 2.25 mM is obtained (for binding to free enzyme), which is similar to the K_m of 2.5 mM (Keung et al., 1980).

Discussion

Virtually all review articles published on angiotensin converting enzyme during the past several years [e.g., see Soffer (1976), Erdös (1976), Peach (1977), and Ondetti & Cushman (1982)] have noted that anion activation of the enzyme depends upon the particular substrate being examined. Thus, with different substrates, activation by chloride has been said to be strong, weak, absent, or, in fact, replaced by inhibition (Dorer et al., 1974; Cheung et al., 1980). However, despite the unusual nature of this feature in mechanistic terms and its possible implications for in vivo regulation of ACE, it has not yet been examined critically, systematically, or in depth.

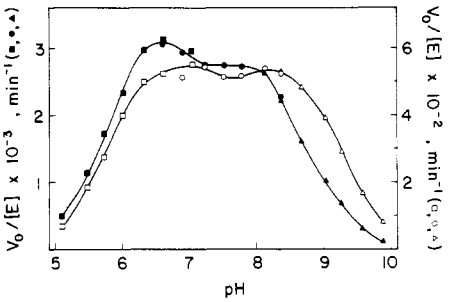


FIGURE 6: pH–activity profiles for hydrolysis of 3 μM FA-Phe-Phe-Arg (□, ○, △) and 50 μM FA-Phe-Gly-Gly (■, ●, ▲) with 300 mM NaCl present. Assays were performed in 50 mM Mes (□, ■), 50 mM Hepes (○, ●), or 50 mM borate (△, ▲). Zinc chloride was added as described under Materials and Methods.

The present studies were undertaken to better define this substrate dependence and to identify the structural features of the substrates that are associated with the differences in behavior.

In contrast with the prevailing view, we find that hydrolysis of all substrates examined, including the same peptides studied by Cheung et al. (1980), is greatly stimulated by chloride. However, we have also found that the kinetic mechanism of activation, the apparent K_A for chloride, the effect of pH on K_A, and the relative potencies of other anions as activators are all strongly dependent on the nature of the substrate. Based on the activation behavior observed with the 26 N-blocked tripeptides tested, we have divided these substrates into three "classes". When the molecular basis of this empirical classification is understood, it can likely be replaced by mechanistic descriptions.

The kinetics of class I peptide hydrolysis are consistent with an essential activator mechanism (Scheme I) in which anion must bind before substrate (Bünning & Riordan, 1983). The K_A for chloride in this scheme is about 90 mM at pH 7.5. In contrast, class II substrates appear to be hydrolyzed by a nonessential activator mechanism (Scheme II), although the degree of activation is still large. A K_A value of about 4 mM at pH 7.5 is obtained through an equilibrium treatment of this kinetic scheme. For class III peptides, there is a small but measurable rate of hydrolysis in the absence of added anions, suggesting a nonessential mechanism. However, the relationship between K_m and [Cl^-] is not that predicted by the equilibrium treatment of Scheme II (Figure 5). The apparent K_A at low substrate concentration is 18–30 mM at pH 7.5. Thus, three distinctive "K_A" values are observed with the three classes of substrates (see below).

The effect of pH on the anion activation properties of ACE is also strikingly different with the three types of substrates. The most dramatic changes are observed with class I sub-

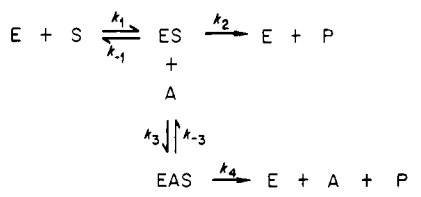
strates, while with class II they are relatively small (Table II). In addition, the potencies of bromide, nitrate, and iodide (relative to chloride) as activators change considerably with pH with class I substrates but remain virtually unchanged with class II (Table III). A similar phenomenon is seen with respect to the kinetic mechanism of activation with class I substrates (Bünning & Riordan, 1983), but with the class II substrate FA-Phe-Phe-Arg, there is no significant alteration in the kinetic mechanism at pH 6.0 (data not shown). A consequence of this differential sensitivity to pH is that activation of ACE becomes much less substrate dependent at pH 6.0 than at pH 7.5 and above.

In structural terms, class II substrates are distinguished from those of classes I and III by the presence of a positively charged side chain at the P'_1 or P'_2 position. The correlation is demonstrated most convincingly by the two substrate pairs with free vs. N^{ϵ}-blocked lysines at P'_1 and P'_2: elimination of the positive charge by *t*-BOC groups changes the substrate from class II to either class I or class III, depending on the other residues. A positive charge at the P_1 position does not induce class II properties: both FA-Lys-Gly-Gly and FA-(N^{ϵ}-*t*-Boc)Lys-Gly-Gly are class I, and both FA-Lys-Ala-Phe and FA-(N^{ϵ}-*t*-Boc)Lys-Ala-Phe are class III substrates. By these criteria, the physiological substrate bradykinin would be a class II substrate, as are the two peptides tested that share the same P'_1 and P'_2 residues as this nonapeptide. The structural features that distinguish class I and III substrates are less clear. Of the peptides examined, all in class III have P'_1 alanine. However, FA-Phe-Ala-Glu,[4] FA-Leu-Ala-Gly, and FA-Gly-Ala-Gly are all class I substrates, indicating that the residues at both P_1 and P'_2 are also relevant. Two peptides (Bz-Gly-Ala-Pro and FA-Gly-Ala-Pro) which share the same COOH-terminal dipeptide as BPP_{5a} fall into class III. The angiotensin I analogue, FA-Phe-His-Leu, represents a special case in that the penultimate histidine may be partially charged at pH 7.5. Thus, on the basis of the previously described classification, this peptide would be expected to be a class I substrate at high pH, a class II substrate at low pH, and a mixture of the two at intermediate pH. Measurements of K_A' at pH 7.5 and 9.0 are consistent with this interpretation.

Although the 26 tripeptides used in this study fall into three discrete classes, it is possible that additional classes, or a continuum rather than three groupings, will be observed as the range of substrates examined is expanded. Peptides containing uncharged hydrophilic residues or more than a single charged side chain have not been included. Such structural features may result in different anion activation patterns from those seen thus far. Further, the nature of the amino acids beyond the P_1 position (toward the NH_2 terminus) may influence the substrate's properties.

The data obtained in the present study demonstrate that activation of ACE is markedly substrate dependent. It is improbable that multiple enzymes or separate active sites are responsible for hydrolyzing the three classes of peptides. Indeed, several results suggest that only a single active site is involved. (1) A class I substrate, Bz-Gly-Gly-Phe, inhibits hydrolysis of a class II substrate, with $K_i \simeq K_m$. (2) At pH 6, chloride concentrations greater than 50 mM inhibit hydrolysis of class I and II substrates similarly. (3) The effects of chemical modification by butanedione, tetranitromethane, and CMC do not depend on which substrate is employed for the assay (Table IV). These reagents are thought to modify essential arginine, tyrosine, and carboxyl residues of ACE, respectively (Bünning et al., 1978). (4) The acidic limb of the pH–activity profile is coincident for class I and II substrates (Figure 6). The alkaline limb for FA-Phe-Phe-Arg is shifted up by about 0.5 pH unit with respect to that for FA-Phe-Gly-Gly. However, this may be due to the differences in the K_A' values over this pH range with the two substrates: 0.3 M NaCl is decreasingly effective for activation of FA-Phe-Gly-Gly hydrolysis as the pH increases, while it is saturating for FA-Phe-Phe-Arg throughout.

Scheme III



The substrate-dependent properties of ACE should be accounted for by molecular events occurring within a single active site. Although the present work does not provide such an accounting, it allows some of the problems posed to be defined. The key problem, perhaps, is to explain how the measured activation constant (K_A) for chloride can vary with substrate. For class I and II peptides, the term K_A in the respective kinetic schemes (I and II) represents the same substrate-independent quantity: the dissociation constant for chloride and *free* enzyme. It is therefore paradoxical that the calculated K_A values for the two classes of substrates at pH 7.5 differ by 20-fold. Two possible resolutions of this paradox can be suggested.

First, the different K_A values could be accounted for by two separate chloride binding sites. Binding at a high-affinity site (K_A = 5 mM) could be sufficient for activation of ACE toward class II peptides, while binding at the low-affinity site (K_A = 100 mM) would be necessary for hydrolysis of class I substrates. With class III substrates, binding at the high-affinity site could afford partial activation, with binding at the low-affinity site resulting in additional activation.

Alternatively, the K_A values may not represent true binding constants. The diagnostic plots with class I and II substrates conform reasonably well with those predicted by Schemes I and II [see Figure 3 and Bünning & Riordan (1983)]. However, other mechanisms can produce similar or even identical plots and yet yield K_A values which do not represent the dissociation constant for anion and free enzyme. For example, consider a nonessential activator scheme in which anion binds after substrate (Scheme III).

Steady-state treatment produces an apparent activation constant [equal to $(k_{-3} + k_4)(k_{-1} + k_2)/(k_3k_4)$] which now varies with substrate. Lineweaver–Burk plots and plots of $K_m^0/\Delta K_m$ and $V_{max}^0/\Delta V_{max}$ vs. $[Cl^-]$ have the same shapes observed in Figures 2 and 3 with the class II substrate. In fact, this mechanism can also apply to class I substrates if the K_m component of the activation [equal to $(k_{-1} + k_2)/k_4$] is sufficiently large. Scheme III becomes inadequate, however, when the effect of chloride on inhibitor binding is studied (R. Shapiro, unpublished results) and hence is presented primarily as an illustration. It is possible, of course, that this mechanism

[4] The specificity of converting enzyme has been thought to exclude peptides having a glutamic acid at the ultimate position. This is based primarily on the work of Elisseeva et al. (1971), but Rohrbach et al. (1981) have reported a similar result. In contrast, we have found FA-Phe-Ala-Glu to be a substrate for ACE, in fact having a lower K_m than either of the more commonly employed synthetic substrates, Bz-Gly-His-Leu and Bz-Gly-Gly-Gly (Cushman & Cheung, 1971a; Dorer et al., 1976). Among the 11 FA-Phe-X-Y peptides we have examined, however, it does have the highest K_m, indicating that glutamic acid in this position is at least somewhat unfavorable. The ability of ACE to cleave peptides with P'_2 glutamic acid has also been reported by Krutzsch (1980).

could be modified by the introduction of additional terms in such a way as to account for the inhibition kinetics as well.

Finally, it should be noted that steady-state (rather than rapid equilibrium) treatment of Scheme II can under some conditions also allow substrate-dependent deviations of the measured activation constant from the true dissociation constant for $E + A \rightleftharpoons EA$. Such treatment may be particularly applicable to class III substrates. Since the steady-state rate equation contains $[A]^2$ terms in both numerator and denominator, it is possible for $K_m^0/\Delta K_m$ to bear a nonlinear relationship to $1/[Cl^-]$ of the same type observed in Figure 5.

In order to resolve these questions, we are at present pursuing several different approaches. (1) A detailed study of the substrate and chloride dependences of inhibition kinetics should distinguish among some of the possibilities suggested. (2) Stopped-flow, radiationless energy transfer measurements (Lobb & Auld, 1979) may allow visualization of intermediates in the hydrolytic reactions and reveal whether rapid equilibrium or steady-state assumptions are more appropriate for kinetic analysis. (3) We are exploring a variety of physical (i.e., nonkinetic) methods for the direct determination of the dissociation constant(s) for free enzyme and anion. The magnitude of this value would rule out some kinetic mechanisms and support others (e.g., a true K_A of 100 mM would be difficult to reconcile with Scheme III). This method might also determine whether there are one or two chloride binding sites.

As noted above, there is a discrepancy between some of our findings and those reported by previous researchers. Cheung et al. (1980) observed only a 3-fold activation by chloride with the bradykinin analogue Bz-Gly-Phe-Arg and found that hydrolysis of BPP_{5a} and its analogue Bz-Gly-Ala-Pro was *inhibited* rather than activated by chloride. In contrast, we observed at least a 24-fold activation by chloride for all of the 23 FA-blocked tripeptides tested here. In addition, we tested the two Bz-tripeptides in question and found activation patterns similar to those obtained with the corresponding FA-peptides.

We assessed the degree of chloride activation of hydrolysis of these substrates by performing assays with substrate concentrations well below K_m, so that k_{cat}/K_m would be measured. An activator may affect either or both k_{cat} and K_m (in fact, it is largely K_m which is altered with ACE), and, therefore, this is the only way for the full effect of the activator to be observed. Also, this condition is most relevant to in vivo activation of the enzyme where substrate concentrations are much lower than K_m at least for angiotensin I and bradykinin (Williams & Hollenberg, 1977; Hulthén & Holkfelt, 1978; Johnston et al., 1979). Cheung et al. (1980) determined the extent of chloride activation with their substrates by using very high substrate concentrations, i.e., under zero-order reaction conditions. With Bz-Gly-Phe-Arg, therefore, one would expect (from Scheme II) to observe only the effect of chloride on k_{cat}, which we find to be increased about 3-fold with class II substrates. Similar considerations apply to their assessment of the effect of chloride on Bz-Gly-Ala-Pro hydrolysis. However, we cannot explain why *inhibition* was observed with this substrate. Even using the same high concentration (5 mM), we found a 5-fold *increase* in velocity as the chloride concentration was raised from 0 to 0.3 M. Amino acid and elemental analysis both indicate a high degree of purity for our substrate, which we synthesized by two different routes to give products with identical properties.[5] These factors, combined with the fact that the corresponding FA-tripeptide behaves similarly to Bz-Gly-Ala-Pro (as do all class III peptides), provide strong evidence that the activation behavior observed here is characteristic for this peptide.

In addition to masking the effect of anions on K_m, using high substrate concentrations for studying activation introduces a further problem. Since the effective K_A varies inversely with [S], contamination by small amounts of extraneous anions becomes much more critical and could eventually account for a large degree of activation. Thus, as is apparent from Scheme I, if the substrate concentration is high enough, an anion concentration which merely equals that of the enzyme would be sufficient to provide maximal activation. (All monovalent anions tested, with the exception of borate, phosphate, and sulfonates such as Hepes, are to some extent activators.) As a consequence, the essential mechanism would appear to be a nonessential one. It is clear that caution is required when assessing the chloride activation of ACE. Failure to recognize the substrate dependence of activation, the striking effect of substrate concentration, and the possible presence of anionic contaminants can lead to serious errors in analysis. These factors must also be considered when evaluating whether "new" peptidases are in fact ACE, since chloride activation has been used as a major identifying characteristic of the enzyme and obviously, under certain conditions, it could appear to be minimal or nonexistent.

The present report demonstrates the existence of major differences in the manner by which anions activate ACE hydrolysis of various substrates. Aside from being of considerable interest in relation to the molecular mechanism of the enzyme, these differences may be relevant to the physiological actions of ACE. It might seem unlikely that anion activation would be involved in regulation of ACE activity in vivo. The only documented function of the enzyme is to act on angiotensin I and bradykinin as they circulate through the pulmonary vasculature. The concentration of chloride in the enzyme environment (i.e., about 100 mM in the blood) is high enough, based on in vitro studies, to fully activate the enzyme toward both of these substrates. This concentration is not believed to undergo marked fluctuations. However, several additional factors must be considered. First, the enzyme is present in many other tissues. In some of these, the basal chloride levels are lower than in the blood and may be subject to temporal variation. Neither the substrates nor the roles of ACE in these tissues are known. Second, activation of the enzyme has only been studied in solution, in vitro, and K_A values in vivo, in the plasma membrane where the enzyme is located (Lanzillo & Fanburg, 1974; Soffer et al., 1974; Ryan et al., 1975), may be considerably higher, allowing more control through this mechanism. Third, the degree of activation is influenced by a variety of factors which are not necessarily constant, including pH (with class I and III substrates, K_A' changes dramatically with pH precisely in the physiological range) and the presence of other anions which are less effective activators and may compete with chloride. Hydrolysis of angiotensin I may be particularly sensitive to pH, if this peptide behaves like its analogue FA-Phe-His-Leu. Variations in pH could alter the extent to which this substrate will act as class I (high K_A') or class II (low K_A'), due to ionization of the P'_1 histidine. Thus, the already rapid decrease in K_A' as pH is lowered will be further accelerated by the gain in class II character and loss of class I character. Clearly, the 50-fold range of K_A' values (Table I) observed with the three classes of substrates in this report presents at least the possibility that particular substrates can be selectively hydrolyzed under certain in vivo conditions and that others can be spared.

[5] The details of these analyses are given in the supplementary material.

Finally, we note that these substrate-dependent effects of anions may also pertain to the interaction of ACE with inhibitors. At present, a number of converting enzyme inhibitors are being used to treat hypertension in humans. Studies now in progress should help to delineate the effect of chloride and other anions on the binding of these inhibitors to ACE.

Acknowledgments

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Supplementary Material Available

A description of the synthesis of 14 FA- and Bz-tripeptides (6 pages). Ordering information is given on any current masthead page.

Registry No. ACE, 9015-82-1; FA-Phe-Gly-Gly, 64967-39-1; FA-Phe-Leu-Gly, 56186-51-7; FA-Phe-Ala-Glu, 86064-72-4; FA-Phe-(N^{ϵ}-*t*-Boc)Lys-Ala, 86064-73-5; FA-Leu-Ala-Gly, 71067-13-5; FA-Leu-Leu-Gly, 83803-10-5; FA-Ala-Leu-Ala, 83803-12-7; FA-Gly-Ala-Gly, 56186-46-0; FA-Gly-Leu-Phe, 56186-49-3; FA-Lys-Gly-Gly, 86064-74-6; FA-(N^{ϵ}-*t*-Boc)Lys-Gly-Gly, 86064-75-7; Bz-Gly-Gly-Phe, 24853-77-8; FA-Phe-Phe-Arg, 86064-76-8; FA-Phe-Ala-Lys, 86064-77-9; FA-Phe-Ala-Arg, 86064-78-0; FA-Phe-Lys-Ala, 86064-79-1; Bz-Gly-Phe-Arg, 73167-83-6; FA-Gly-Ala-Pro, 86064-80-4; FA-Phe-Ala-(N^{ϵ}-*t*-Boc)Lys, 86064-81-5; FA-Lys-Ala-Phe, 86064-82-6; FA-(N^{ϵ}-*t*-Boc)Lys-Ala-Phe, 86064-83-7; FA-Phe-Ala-Phe, 71067-11-3; FA-Phe-Ala-Gly, 71067-12-4; FA-Ala-Ala-Ala, 56186-47-1; Bz-Gly-Ala-Pro, 73167-84-7; FA-Phe-His-Leu, 71067-14-6; chloride, 16887-00-6; bromide, 24959-67-9; nitrate, 14797-55-8; iodide, 20461-54-5; acetate, 71-50-1; FA-(N^{ϵ}-*t*-Boc)Lys, 86064-91-7; FA-ONSu, 56186-54-0; (N^{ϵ}-*t*-Boc)Lys, 2418-95-3; FA-(N^{ϵ}-*t*-Boc)-Lys-Gly-Gly-OEt, 86064-84-8; FA-(N^{ϵ}-*t*-Boc)Lys-ONSu, 86064-85-9; Ala-Phe, 3061-90-3; FA-Phe-Ala-ONSu, 86064-86-0; FA-Phe-ONSu, 71067-15-7; FA-Phe-(N^{ϵ}-*t*-Boc)Lys-Ala-OMe, 86064-87-1; Glu, 56-86-0; FA-Phe-Phe-ONSu, 86064-88-2; Arg, 74-79-3; FA-Gly-ONSu, 56186-55-1; Bz-Gly-Ala-ONSu, 86064-89-3; Pro, 147-85-3; Bz-Bly-ONSu, 66134-74-5; Ala-Pro, 13485-59-1; Bz-Gly-Phe-ONSu, 86064-90-6.

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