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Critical Lysine Residue at the Chloride Binding Site of Angiotensin Converting Enzyme†

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ABSTRACT: Pulmonary angiotensin converting enzyme has been reductively methylated by using formaldehyde and sodium cyanoborohydride. This modification virtually eliminates enzyme activity toward some substrates (e.g., furanacryloyl-Phe-Gly-Gly) while less drastically affecting activity toward others (e.g., furanacryloyl-Phe-Phe-Arg). Affinity chromatography and analysis of radiolabeled reaction products reveal that this effect is due to methylation of a single critical lysine residue. Loss of activity primarily represents an increase in K_m values, indicating that the critical lysine plays a role in substrate binding. This lysine can be protected by a competitive inhibitor, suggesting that it is at or near the active site. Addition of chloride at pH 6.1 specifically protects against methylation of this lysine. These findings support the idea that the critical lysine is part of the binding site for chloride and other monovalent anions which are strong activators of the enzyme.

Angiotensin converting enzyme (dipeptidyl carboxypeptidase, EC 3.4.15.1) (ACE)[1] is a zinc metalloexopeptidase that releases dipeptides from the C-terminus of a wide variety of oligopeptide substrates (Soffer, 1976). Chemical modification studies from this laboratory (Bünning et al., 1978) and others (Fernley, 1977; Harris & Wilson, 1982, 1983) have suggested that the active site of ACE is closely related to that of carboxypeptidase A (Vallee et al., 1983; Lipscomb et al.,

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[1] Abbreviations: ACE, angiotensin converting enzyme; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; FA, *N*-furanacryloyl; *t*-Boc, *tert*-butyloxycarbonyl; PPPP, *N*-(phenylphosphoryl)-L-Phe-L-Phe; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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1970): in addition to the essential zinc atom, critical arginine, tyrosine, and glutamic residues appear to be involved in catalytic activity or substrate binding. However, unlike carboxypeptidase A, ACE is also inactivated by treatment with reagents which, in general, modify only lysine residues (Bünning et al., 1978; Weare, 1982). The function of a critical lysine residue in ACE might be associated with mechanistic characteristics of this enzyme not shared by carboxypeptidase A, and one such feature of ACE is its strong activation by chloride and other monovalent anions (Skeggs et al., 1954). Since the ϵ-amino group of lysine has the appropriate charge for interacting with anions, a plausible hypothesis would invoke an essential lysine as a component of the activator binding site. It has already been demonstrated that anion activation does not operate through the zinc atom (Bünning & Riordan, 1981).

The present study examines the effects of reductive methylation of lysines on ACE, using formaldehyde and $NaCNBH_3$ (Jentoft & Dearborn, 1979). Although a large number of lysine residues are modified by this procedure, their pK_a values are barely altered (Means & Feeney, 1968; Bradbury & Brown, 1973), and the steric effect of the added methyl groups is relatively small. The effects on enzyme function of such a "minimal" modification of a critical residue were expected to be especially informative concerning its precise role.

We have recently reported (Shapiro et al., 1983) that the anion activation properties of ACE vary markedly with the particular substrate being hydrolyzed, allowing an empirical division of the 26 substrates examined into three "classes". Hence, it was of particular interest to determine whether methylation differentially affects the activity of the enzyme toward various substrates. At pH 7.5, the anion appears to be essential for hydrolysis of class I substrates (Bünning & Riordan, 1983), while for class II and III substrates it is nonessential but still gives rate enhancements of 24–160-fold at optimal concentrations (Shapiro et al., 1983). The primary effect of the activator in all cases is to decrease K_m values, although with class II and III substrates there is also some increase in k_{cat} values. The activation constant for chloride is strongly substrate dependent, ranging from $\sim$3 mM with class II substrates to $\sim$100 mM with class I substrates. Thus, if a lysine is part of the anion binding site, the effects of methylation might also be substrate dependent.

We report here that methylation of a single lysine residue, probably at or near the active site, greatly reduces ACE activity. The extent of this decrease is indeed substrate dependent, being much larger for class I than for class II substrates. In addition, the activity reduction in large part represents an increase in K_m values, suggesting a role for this lysine in mediating substrate binding, and consistent with its participation in anion activation. Finally, addition of chloride can, under appropriate conditions, specifically protect against methylation of this lysine.

Materials and Methods

ACE was isolated from frozen rabbit lungs (Pel-Freez Biologicals Inc., Rogers, AR) as described by Bünning et al. (1983). The final preparation was >95% pure as judged by specific activity and SDS–polyacrylamide gel electrophoresis. ACE 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).

Activity Measurements. Enzyme activities were measured by using furanacryloyl (FA)-blocked tripeptides as described previously (Holmquist et al., 1979). The absorbance decrease accompanying hydrolysis was continuously monitored with a Varian Model 219 spectrophotometer. The standard assay conditions were 25 °C, 50 mM Hepes and 300 mM NaCl, pH 7.5. Initial velocities were measured during the first 10% of hydrolysis. All substrates were from previous studies, and their synthesis is described elsewhere (Blumberg & Vallee, 1975; Holmquist et al., 1979; Bünning et al., 1983; Shapiro et al., 1983).

The kinetic parameters K_m and k_{cat} were derived from Lineweaver–Burk plots. K_i values were determined from plots of $1/v_0$ vs. [I] at [S] $\ll K_m$, where the intersection with the [I] axis approximates $-K_i$.

Inhibitors. *N*-(Phenylphosphoryl)-L-Phe-L-Phe (PPPP) was kindly provided by Dr. Barton Holmquist (Holmquist & Vallee, 1979) and *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly by Dr. M. Pantoliano.

Reductive Methylation. The procedure developed by Jentoft & Dearborn (1979) was employed. ACE (0.5–4.0 μM) was incubated at 25 °C with 2 mM formaldehyde (Fisher) and 10 mM sodium cyanoborohydride (Alfa) in 50–100 mM Hepes, pH 7.5. The cyanoborohydride solution was prepared immediately prior to use. At various times, aliquots were removed and diluted (usually 1:10 and 1:20) into 50 mM Hepes, pH 7.5, on ice. Assays were performed within 30 min after dilution, using 10–60 μL of the diluted enzyme solution per mL of assay mixture. In some cases, the reaction mixture was dialyzed extensively against 50 mM Hepes, pH 7.5, prior to being assayed. Enzyme concentration was found to affect the rate of inactivation somewhat and, hence, was held constant among all incubation mixtures within each experiment.

[^{14}C]Formaldehyde (New England Nuclear) with a specific activity of 47–55 mCi/mmol was purified by passage through Dowex 1-X8 (acetate form) as suggested by Jentoft & Dearborn (1979). For determinations of the extent of ACE methylation, [^{14}C]formaldehyde was added to the incubation mixtures (giving 1500–3000 cpm/nmol). At the stated times, the reaction was terminated by addition of 2 M ammonium chloride, pH 7.5, to a final concentration of 0.1 M. After dialysis against 20 mM Hepes, pH 7.5, an ultraviolet absorption spectrum was recorded in order to quantitate the enzyme, and a portion was counted in Econofluor (New England Nuclear).

Identification of the Modified Amino Acids. ^{14}C-Methylated ACE was dialyzed against water and hydrolyzed in 5.7 N HCl for 18–22 h at 108 °C. After evaporation of HCl, the hydrolysate was dissolved in 0.01 N HCl, and N^{ϵ}-methyllysine and $N^{\epsilon},N^{\epsilon}$-dimethyllysine (Vega) were added as carriers. Chromatography was performed on a silica gel thin-layer sheet (E. Merck) in two dimensions: first with chloroform/methanol/ammonia (6:4.5:2) and then with 1-butanol/acetic acid/water (4:1:1). This system gives excellent separation of the methyllysines from each other and from the other amino acids. The positions of the two lysine derivatives were visualized with ninhydrin. The plate was then sprayed with EN^{3}HANCE spray (New England Nuclear) and autoradiography performed with X-Omat film (Eastman Kodak Co.) (exposure time 2 days).

Affinity Chromatography. Affinity column separations of modified ACE were performed by using immobilized *N*-[1-(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly (M. W. Pantoliano, B. Holmquist, and J. F. Riordan, unpublished results). Modified ACE (up to 0.6 mg) was dialyzed against 20 mM Mes, 0.2 M NaCl, and 0.1 mM zinc acetate, pH 6.0, and loaded onto a 0.9 × 2.2 cm column containing resin with a capacity of 20 mg of ACE/mL. The flow rate was 3.0 mL/h. The column was then washed with the same buffer until the absorbance at 280 nm returned to the base-line level. Bound

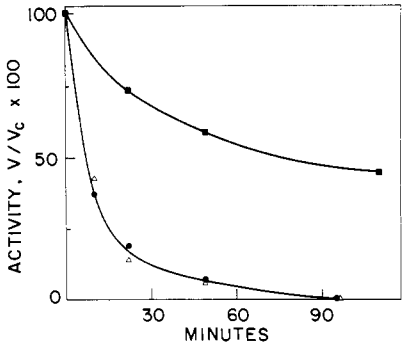


FIGURE 1: Effects of reductive methylation on ACE activity. All reactions were performed with 2 mM formaldehyde and 10 mM sodium cyanoborohydride in 100 mM Hepes, pH 7.5, at 25 °C. Samples were incubated with no additions (●), 0.5 M NaCl (▲), or 100 μM PPPP plus 0.3 M NaCl (■). Activity was measured with 50 μM FA-Phe-Gly-Gly as substrate in 50 mM Hepes/300 mM NaCl, pH 7.5, at 25 °C.

enzyme was then eluted with 50 mM Tris/0.5 M NaCl, pH 8.9.

Results

Inactivation of ACE by Reductive Methylation. The effect of reductive methylation of ACE was initially examined with FA-Phe-Gly-Gly as substrate, at [S] $\ll K_m$. Incubation of the enzyme with 2 mM formaldehyde and 10 mM $NaCNBH_3$ at pH 7.5 for 100 min at 25 °C decreases the activity by >99% (Figure 1). Dialysis of the reaction mixture does not restore activity. Neither formaldehyde nor cyanoborohydride alone significantly inactivates the enzyme.

Protection by Inhibitor and Chloride. A competitive inhibitor, *N*-(phenylphosphoryl)-L-Phe-L-Phe (PPPP), and chloride were tested for their effect on the rate of inactivation. In the presence of 100 μM PPPP and 0.3 M NaCl, the rate of inactivation is reduced markedly (Figure 1). This concentration of PPPP is 100× its K_i value under these conditions. In the absence of chloride,[2] 500 μM PPPP slows the inactivation rate about 2-fold (not shown).

Chloride by itself (0.5 M) has no significant effect on the reaction at pH 7.5 (Figure 1). However, as the pH is lowered from 7.5 to 6.0, the kinetically determined dissociation constant for chloride decreases 24-fold from 80 to 3.3 mM with FA-Phe-Gly-Gly as substrate (Bünning & Riordan, 1983). Thus, if the critical residue(s) is (are) at the activator binding site, a measurable degree of protection with chloride might occur at a lower pH where this site could be more fully saturated. Figure 2 shows that chloride indeed protects against inactivation at pH 6.1. This protection is specific for the critical residue since chloride does not affect the overall rate of methylation: the numbers of methyl groups incorporated in 30 min are 14.8 and 15.6 in the absence and presence of 1.0 M NaCl, respectively. (These data are based on the incorporation of ^{14}C from formaldehyde.) It is also apparent from Figure 2 that the inactivation rate is severalfold faster at pH 6.1 than at pH 7.5. This effect is again specific, since the overall rate of methylation actually *decreases* slightly with pH (16.6 methyl groups in 30 min at pH 7.5 vs. 14.8 at pH 6.1).

Activity of Modified ACE toward Various Substrates. The effect of reductive methylation on ACE activity depends strongly on the particular substrate employed in the assay (Figure 3). Thus, while <1% of the native activity toward

[2] The chloride concentration in the incubation mixture is estimated to be below 10 μM, based on measurements with a chloride-selective electrode (Graphic Controls, Buffalo, NY).

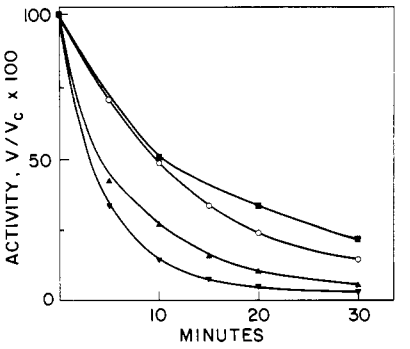


FIGURE 2: Effects of pH and chloride on reductive methylation of ACE. Enzyme was incubated with 2 mM formaldehyde and 10 mM sodium cyanoborohydride in 50 mM Hepes, pH 7.5 (■), or in 50 mM Mes/0.1 mM zinc acetate, pH 6.1, with 0 (▼), 0.3 (▲), or 1.0 (○) M NaCl. Activity was measured with 50 μM FA-Phe-Gly-Gly under standard conditions.

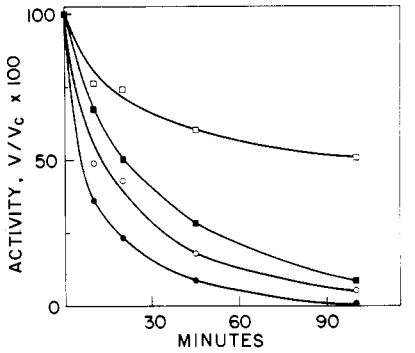


FIGURE 3: Effect of reductive methylation on ACE activity toward FA-Phe-Gly-Gly (●, ○) and FA-Phe-Phe-Arg (■, □). FA-Phe-Gly-Gly concentrations were 0.05 (●) and 5 mM (○). FA-Phe-Phe-Arg concentrations were 3.2 (■) and 300 μM (□). Assays were performed in 50 mM Hepes/300 mM NaCl, pH 7.5, at 25 °C. The methylation conditions were as described in the legend to Figure 1.

FA-Phe-Gly-Gly remains after 100 min of reaction, there is 8.5% activity toward FA-Phe-Phe-Arg (with both substrates at concentrations well below K_m). When high substrate concentrations ([S] $\gg K_m$ for native ACE) are employed, this difference becomes more striking: 6.2% activity with FA-Phe-Gly-Gly and 48% with FA-Phe-Phe-Arg. If the methylation reaction is allowed to proceed for 6 h, activity toward FA-Phe-Gly-Gly (at [S] $\gg K_m$) decreases to <1%, while activity toward FA-Phe-Phe-Arg (at all [S] values) undergoes no significant further change. Addition of fresh formaldehyde and cyanoborohydride (after dialysis of the reaction mixture) also has little effect on the activity toward FA-Phe-Phe-Arg, measured after treatment for another 2 h.

FA-Phe-Gly-Gly and FA-Phe-Phe-Arg are class I and class II substrates, respectively (Shapiro et al., 1983). In order to determine whether the difference in the activities of modified ACE toward these two substrates is related to this classification, which is based on anion activation properties, ten additional substrates were employed. Activities toward high concentrations ($12–20K_m$) of these substrates were measured with native ACE and with enzyme which had been methylated for 2 h. The data in Table I show that there is good correlation between the degree of inactivation and the substrate class. Thus, modified ACE hydrolyzes all four of the class I substrates poorly, while three of the four class II substrates are hydrolyzed at almost half the native enzyme rate. Activity toward class III substrates is intermediate. The single exception to the pattern is the class II peptide FA-Phe-Lys-Ala. In this case, the activity, while much greater than that measured with the class I substrates, is closer to the class III than

Table I: Activities of Methylated ACE toward Various Substrates[a]

	K_m (μM)	[S]/K_m	(v/v_c) × 100
Class I Substrates			
FA-Phe-Gly-Gly	300	17	5.9
FA-Leu-Ala-Gly	410	12	3.5
FA-Phe-Leu-Gly	250	12	3.8
FA-Phe-(N^{ϵ}-*t*-Boc)Lys-Ala	160	20	7.2
Class II Substrates			
FA-Phe-Phe-Arg	16	19	48
FA-Phe-Ala-Lys	23	20	40
FA-Phe-Ala-Arg	6.5	20	49
FA-Phe-Lys-Ala	68	20	22
Class III Substrates			
FA-Phe-Ala-Phe	120	20	22
FA-Phe-Ala-(N^{ϵ}-*t*-Boc)Lys	97	20	25
FA-Phe-Ala-Gly	500	20	14
FA-Lys-Ala-Phe	200	18	14

[a] K_m values are for native ACE and are from Bünning et al. (1983) and Shapiro et al. (1983). ACE was methylated for 2 h under standard conditions and then dialyzed. All assays were performed in 50 mM Hepes/0.3 M NaCl, pH 7.5, at 25 °C with [S]/K_m as indicated. v/v_c values are determined with high (12–20K_m) substrate concentrations. v_c values are for unmodified ACE.

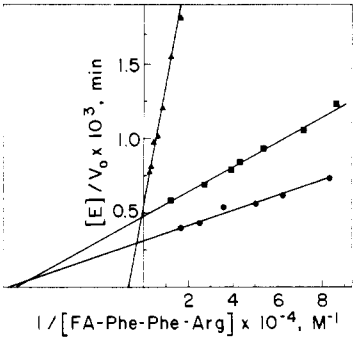


FIGURE 4: Lineweaver–Burk plots for the hydrolysis of FA-Phe-Phe-Arg by native ACE (●) and by ACE methylated for 3 h in the presence (■) and absence (▲) of 500 μM PPPP and 300 mM NaCl. Methylation was performed as described in the legend for Figure 1. Methylated samples were dialyzed extensively against 50 mM Hepes, pH 7.5, prior to assay. Assays were in 50 mM Hepes/300 mM NaCl, pH 7.5 at 25 °C.

to the class II values. This peptide differs from the other class II substrates studied in that it contains a positively charged side chain at the penultimate rather than the ultimate position.

Characterization of Methylated ACE. The data in Figure 3 indicate qualitatively that a major effect of methylation is to weaken substrate binding to the enzyme. Lineweaver–Burk plots for native and modified ACE with FA-Phe-Phe-Arg as substrate (Figure 4) quantitate the changes in K_m and k_{cat} values accompanying methylation. The modified enzyme has a 9-fold higher K_m (143 vs. 16 μM) and a 1.9-fold lower k_{cat} (1760 vs. 3260 min^{-1}). Figure 4 also plots the activities of ACE modified in the presence of PPPP and chloride. This enzyme exhibits 54% of the native activity toward this substrate under standard assay conditions. Almost all of the activity loss is due to a 1.6-fold change in k_{cat}. Thus, the difference between the activities of ACE modified in the presence and absence of inhibitor is largely attributable to an altered K_m value. No attempt was made to obtain K_m and k_{cat} values for FA-Phe-Gly-Gly with the methylated enzyme since the activity toward this substrate was too low. However, it is clear from Figure 3 that the K_m value for FA-Phe-Gly-Gly has also been increased substantially.

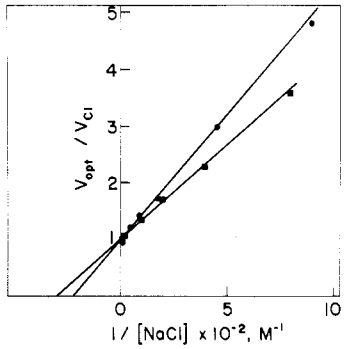


FIGURE 5: Effect of chloride concentration on the activity of native (■) and methylated (●) ACE toward FA-Phe-PheArg. The latter was the same sample used in the experiment shown in Figure 4 (methylated in the absence of inhibitor). Substrate concentrations were 3.2 μM for native and 20 μM for methylated ACE (i.e., well below K_m in both cases). Standard assay conditions were employed. V_{opt} is the optimum activity that is achieved at high chloride concentration, and V_{Cl} is the activity observed at lower chloride concentrations.

Methylation of the enzyme also affects the apparent K_i value for PPPP. Measured with FA-Phe-Phe-Arg as substrate, the apparent K_i value increases from 5 μM for unmodified ACE to 160 μM for methylated ACE.[3] The chloride dependence of PPPP binding resembles that of class I substrates (R. Shapiro, unpublished results).

Activation of the methylated enzyme by chloride is similar to that of the native enzyme. ACE methylated for 2 h has no measurable activity toward FA-Phe-Gly-Gly (at [S] $\gg$ K_m for unmodified enzyme) in the absence of chloride. Activity toward FA-Phe-Phe-Arg (at [S] $\gg$ K_m) is decreased at least 30-fold when chloride is omitted from the assay mixture. The apparent activation constant for chloride with FA-Phe-Phe-Arg as substrate (Figure 5) is 4.4 mM for the modified enzyme and 3.4 mM for the native enzyme.

Stoichiometry of Inactivation. Any attempt to ascertain the number of residues whose methylation is responsible for inactivation of the enzyme is complicated by the lack of specificity in the reaction. Thus, for example, during 2 h of incubation, an average of 33 methyl groups are incorporated into each enzyme molecule, as judged by ^{14}C incorporation from [^{14}C]formaldehyde (see Materials and Methods). [Rabbit lung ACE contains 35 lysines and a free N-terminus (Das & Soffer, 1975; R. Shapiro, unpublished results). All of its amino sugars are reported to be acetylated (Das & Soffer, 1975).] This incorporation rate is too rapid to allow detection of any difference between samples methylated in the presence and absence of inhibitor. The specificity of the reaction can be increased by first modifying the enzyme extensively in the presence of inhibitor with unlabeled formaldehyde and then treating it with [^{14}C]formaldehyde. If the second modification is performed in the presence and absence of inhibitor, a difference in [^{14}C]methyl incorporation can be compared with a change in activity.

The initial modification had to be carried out for 6 h in order to limit sufficiently the amount of additional methylation that would occur during the second reaction. During these 6 h, about 75% of the ACE is inactivated even in the presence of inhibitor and chloride, thus precluding direct stoichiometry

[3] This K_i value for native enzyme (5 μM) is 5 times higher than the value given earlier, which was obtained with FA-Phe-Gly-Gly as substrate (Holmquist & Vallee, 1979). With several inhibitors thus far examined, we have found apparent K_i values that vary with the substrate employed (R. Shapiro, unpublished results).

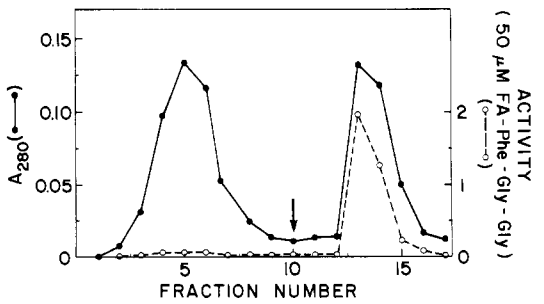


FIGURE 6: Affinity chromatography of ACE methylated for 6 h in the presence of 60 μM *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly and 300 mM NaCl. The reaction (2 mM formaldehyde/10 mM cyanoborohydride in 70 mM Hepes, pH 7.5, 25 °C) was terminated by the addition of ammonium chloride to 30 mM. The enzyme was dialyzed against 50 mM Tris-HCl/500 mM NaCl, pH 8.9, then against 50 mM Hepes, pH 7.5, and finally against 20 mM Mes, 200 mM NaCl, and 0.1 mM zinc acetate, pH 6.0. It was then applied to a column of immobilized *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly equilibrated with the Mes buffer. At the time indicated by the arrow, the buffer was changed to 50 mM Tris-HCl/500 mM NaCl, pH 8.9. One-milliliter fractions were collected. The absorbance at 280 nm (●) and the activity toward 50 μM FA-Phe-Gly-Gly (○) (under standard assay conditions) were measured.

determinations. This problem was largely alleviated through the use of an affinity chromatographic resin which removed most of the inactive ACE.

ACE was methylated with unlabeled formaldehyde for 6 h in the presence of 0.3 M NaCl and 60 μM *N*-[1(*S*)-carboxy-5-aminopentyl]-L-Phe-Gly, a very good competitive inhibitor with an apparent K_i value of 0.13 μM under these conditions (M. W. Pantoliano and R. Shapiro, unpublished results). As judged by a similar experiment using [^{14}C]-formaldehyde, 53 methyl groups were incorporated. The enzyme was then dialyzed extensively and chromatographed on the affinity resin (Figure 6). The bound fraction contained 93% of the activity and less than 40% of the absorbance at 280 nm. (All activities here refer to hydrolysis of FA-Phe-Gly-Gly at [S] $\ll K_m$ unless stated otherwise.) Fractions 13–15 (Figure 6) were pooled, concentrated, and dialyzed. The specific activity of this pool was almost 3 times higher than that of the material applied to the column (71% native vs. 25%). The enzyme was then methylated by using [^{14}C]formaldehyde plus cyanoborohydride in the presence and absence of 200 μM PPPP and 0.3 M NaCl. After 65 min, the reactions were terminated by addition of ammonium chloride. Activities and specific radioactivities of the products were determined after dialysis.

The protected enzyme retained 89% of its initial activity and had incorporated 3.64 methyl groups per ACE molecule, while the sample without inhibitor was only 5.4% active and had incorporated 4.40 methyl groups. Thus, by difference, an activity change of 84% accompanied the addition of 0.76 methyl group. However, as already stated, the enzyme used for the ^{14}C reductive methylation was not fully active (i.e., it only had 71% native activity). Hence, calculation of the precise inactivation stoichiometry requires knowledge of what the "missing" 29% activity represents. The two most likely possibilities are (i) enzyme methylated at the critical lysine(s) despite the presence of inhibitor or (ii) ACE not methylated at the critical, protectable residue(s) but having a lower k_{cat} value due to modification of many other lysines.

In order to distinguish between these possibilities, it was necessary to determine whether or not unprotected, methylated ACE would bind to the affinity resin. Although virtually inactive toward FA-Phe-Gly-Gly, such enzyme has considerable activity toward FA-Phe-Phe-Arg when assayed at high [S] (see Figure 3). About 65% of this activity was found to bind to the resin, suggesting that the 29% loss in activity is accounted for by enzyme that has been modified at the critical residue. Consistent with this, the 71% active enzyme exhibits nearly 100% activity when assayed at high concentrations of FA-Phe-Phe-Arg. Thus, incorporation of 0.76 methyl group results in an activity change of 59% (71% of 84%) which extrapolates to complete inactivation on incorporation of 1.3 methyl groups. These data indicate that methylation of a single protectable residue is primarily responsible for the activity changes.

Identification of Lysine as the Critical Amino Acid. It is generally presumed that the effects of reductive methylation are due to lysine modification. With both *N*-acetyl amino acids and a variety of proteins (Jentoft & Dearborn, 1979), formaldehyde/cyanoborohydride have been demonstrated to form stable adducts only with amino groups. However, in any specific case, the possibility of reaction with other functional groups, particularly at an enzyme active site, cannot be ruled out a priori. In addition, even if methylation of an amine is responsible for the loss of activity, this amine could be either on a lysine or at the N-terminus (or even, perhaps, on a posttranslationally modified amino acid). Hence, the identity of the critical residue which is modified in ACE had to be established.

ACE was methylated with unlabeled formaldehyde for 6 h in the presence of 100 μM PPPP plus 0.3 M chloride, dialyzed, and then incubated for 75 min with [^{14}C]formaldehyde/cyanoborohydride. The enzyme was then acid hydrolyzed and chromatographed in two dimensions on a thin-layer plate. An excess of unlabeled N^{ϵ}-methyllysine and $N^{\epsilon},N^{\epsilon}$-dimethyllysine was added in order to assure unambiguous determination of the positions of these amino acids. Autoradiography demonstrated that >99% of the radioactivity comigrated with the methyllysines. There was no significant loss of radioactivity during hydrolysis and the subsequent evaporations. These results indicate that the critical residue is indeed a lysine.

Discussion

Previous chemical modification studies (Bünning et al., 1978; Weare, 1982) have suggested that ACE contains one or more essential lysine residues. Its activity is decreased by treatment with pyridoxal phosphate, trinitrobenzenesulfonate, and imido esters, all of which are considered to react exclusively with amino groups. ACE is also inactivated by acetic anhydride, diethyl pyrocarbonate, and diketene. In these cases, the failure of hydroxylamine to restore activity has been interpreted to indicate that the effect is probably due to lysine modification. The present work was designed to obtain more direct evidence on this question and to investigate the function of any such lysines.

Reductive methylation was chosen as a potentially informative modification for two reasons. First, it does not significantly change the charge of the residues modified, and, hence, the function of a critical lysine might be only partially impaired by methylation. Some understanding of the role of the residue might be gained by examining the precise nature of the activity changes induced. Second, in contrast with many of the reagents employed previously, reductive methylation should not cause extensive steric perturbations of the immediate surroundings of the modified residues. Thus, the observed consequences of modification would be specifically attributable to alteration of the groups methylated.

Reductive methylation indeed causes a marked loss of ACE activity, suggesting direct involvement of a lysine residue(s)

in the enzyme mechanism. With the aid of an affinity chromatographic resin for ACE, we have determined that methylation of a single, protectable residue is responsible for this effect. Moreover, analysis of the (radiolabeled) products of the reaction confirms that the critical residue is, as expected, a lysine.[4]

The protection against inactivation provided by high concentrations of inhibitor in the absence of activating anions implies further that the essential lysine is located at or near the active site. Protection increases substantially when chloride is added together with the inhibitor, likely reflecting the marked synergism between chloride and inhibitor binding (R. Shapiro and J. F. Riordan, unpublished results). Thus, just as chloride increases inhibitor binding, inhibitor increases chloride binding. As a result, in the presence of 100 μM PPPP, 0.3 M chloride could be equivalent (in terms of binding to the activator site) to as much as 30 M chloride in the absence of inhibitor. While 0.5 M chloride (at pH 7.5) does not protect the enzyme, it is possible that 30 M would, if the critical residue were the anion binding site (see below).

Some inactivation of the enzyme occurs even in the presence of chloride and high inhibitor concentrations (Figure 1). In part, this appears to reflect incomplete protection of the critical residue with the rest probably due to nonspecific inactivation accompanying extensive methylation. (In 6 h, an average of about 1.5 methyl groups are incorporated per lysine.) Jentoft & Dearborn (1979) have reported that even with enzymes not thought to contain essential lysines, reductive methylation can cause up to 47% inactivation. However, the possibility that a second essential lysine undergoes methylation very slowly and is not protectable cannot be excluded.

Three lines of evidence support the idea that the critical lysine is a component of the binding site for activating anions. First, both anion activation and methylation primarily affect K_m. With the native enzyme, optimal chloride concentrations decrease the K_m value for FA-Phe-Phe-Arg 15-fold compared with the value measured in the absence of activators (Shapiro et al., 1983). Methylation of the essential lysine increases the K_m value 9-fold, while barely changing k_{cat}. For both anion activation and methylation, the K_m changes should reflect substrate binding since K_i values are also altered by both. The effect of anion activation on substrate binding has been seen directly in a radiationless energy-transfer study (Bünning & Riordan, 1981).

There are, of course, other ways in which a lysine could play a role in substrate (or inhibitor) binding, e.g., through hydrogen-bonding or ionic interactions either with amino acids, helping to create the proper active-site geometry, or with the substrate directly. Nevertheless, the drastic (in the case of class I substrates) decrease in activity accompanying the mere addition of a methyl group is especially easily understood if the function of the lysine is to bind the activator. The effects of substituting other anions for chloride (Bünning & Riordan, 1983; Shapiro et al., 1983) demonstrate that there are stringent size requirements associated with activation. For example, although bromide (Bünning & Riordan, 1983), nitrate, iodide, and acetate all appear to bind to the enzyme at least as well as chloride [by kinetic criteria (R. Shapiro, unpublished results)], they are only 52%, 14%, 10%, and 2% as effective as chloride, respectively, at activating the enzyme (with class I substrates). Thus, it would not be surprising for methylation of the activator binding site to produce a pronounced effect on activity.

Second, both the anion activation properties of ACE and the effects of methylation are substrate dependent. In contrast, the consequences of modification of arginyl, tyrosyl, and carboxyl residues are independent of the substrate employed (Shapiro et al., 1983). There are, in particular, some striking parallels in the effects of activation and methylation. Substrates whose hydrolysis is decreased the most by methylation (i.e., class I) are the same substrates whose hydrolysis is activated the most by anions. Hydrolysis of these substrates is also the most sensitive to substitution of other anions for chloride. [For example, compare the values in the previous paragraph with percentages of 72, 46, and 40 for bromide, nitrate, and iodide, respectively, with class II substrates (Shapiro et al., 1983).] The differences in the effects of anion substitution may indicate that the precise structural relationships at the activator binding site are more critical for class I than for class II substrates. If so, then the consequences of placing an additional methyl group in this site would be more pronounced for class I than for class II substrates.

Alternative interpretations, however, must be considered. The mechanistic basis for the substrate dependence of anion activation is not understood. It is possible that any factor which influences substrate binding will be observed kinetically to affect class I more than class II substrate hydrolysis, whether or not this factor bears any relationship to the phenomenon of activation.

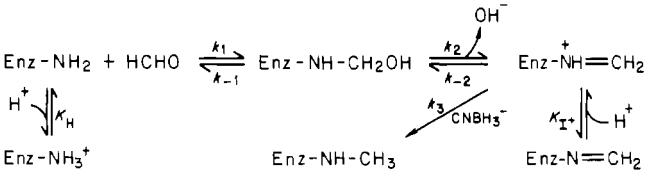
Third, addition of chloride at pH 6.1 markedly slows the rate of inactivation due to reductive methylation (Figure 2). It should be noted that this protection is specific for the critical lysine, since the overall rate of methylation is unaffected by chloride. While this finding further strengthens the idea that the critical lysine is located within the anion binding site, it is striking that protection is only observed at very high chloride concentrations. Thus, at pH 7.5, there is no protection by 0.5 M chloride (Figure 1), a concentration about 6-fold higher than the chloride dissociation constant measured kinetically with FA-Phe-Gly-Gly as substrate. Even at pH 6.1, where there is considerable protection, chloride concentrations 100–300-fold higher than the (kinetic) dissociation constant are required to see this effect (Figure 2), and the degree of protection is still not as great as with chloride plus inhibitor at pH 7.5 (Figure 1).

There are three conditions under which such high amounts of chloride would be required for protection of a chloride binding site: (i) if chloride does not significantly decrease the concentration of the reactive free amine; (ii) if the true binding constant for chloride is much greater than those determined kinetically (3–100 mM); or (iii) if the concentration of free amine is not kinetically significant. The first condition might apply if the amino group were already involved in an ionic interaction of comparable strength in the chloride-free enzyme (e.g., with a neighboring carboxylate). Concerning the second condition, one must in general use caution when equating kinetic and thermodynamic binding constants. This may be particularly relevant to ACE, since the kinetically determined chloride binding constants vary greatly with the substrate being hydrolyzed.

The third condition must be considered in terms of the chemical mechanism for inactivation [see Cordes & Jencks (1963) and Jencks (1969) for a discussion of the mechanism

[4] Gidley & Sanders (1982) have reported that reductive methylation of protein amino groups with formaldehyde and sodium cyanoborohydride can give up to 25% yield of a *N*-cyanomethyl derivative which is rapidly hydrolyzed back to the starting amine at low pH. In the present case, the recovery of all radioactivity after acid hydrolysis of labeled ACE as either methyl- or dimethyllysine indicates that little or no cyanomethylation had occurred.

of imine formation]. A reasonable scheme would be



Any protection by chloride would most likely be due to the equilibrium

$$\text{Enz–NH}_3^+ + \text{Cl}^- \overset{K_{Cl^-}}{\rightleftharpoons} \text{Enz–NH}_3^+\text{Cl}^-$$

which would decrease the concentration of Enz–NH_2 available for reaction with formaldehyde. The rate expression for inactivation is[5]

$$k_{obsd} = \frac{k_1 k_2 k_3 [\text{HCHO}][\text{CNBH}_3^-]}{X + Y}$$

where

$$X = \left(1 + \frac{[\text{H}^+]}{K_\text{H}} + \frac{[\text{H}^+][\text{Cl}^-]}{K_\text{H} K_{\text{Cl}^-}}\right) \times (10^{-14} k_{-1} k_{-2}/[\text{H}^+] + k_2 k_3 [\text{CNBH}_3^-] + k_{-1} k_3 [\text{CNBH}_3^-])$$

and

$$Y = k_1[\text{HCHO}] \times \{10^{-14} k_{-2}/[\text{H}^+] + k_3[\text{CNBH}_3^-] + k_2(1 + K_{\text{I}^+}/[\text{H}^+])\}$$

It is apparent that if X is small compared to Y (or if $[\text{H}^+]/K_\text{H} \ll 1$, which we consider unlikely),[6] no protection by chloride would be observed.

Such a predominance of Y over X at pH 7.5 is in fact suggested by the severalfold increase in the rate of inactivation as the pH is lowered from 7.5 to 6.1 (Figure 2): the terms contained in X contribute either toward a decrease or toward no change in k_{obsd} as the pH decreases, while only the terms in Y can contribute toward an increase in k_{obsd}. (This assumes that $[\text{H}^+]/K_\text{H} \gg 1$. See footnote 6.)

The fact that protection by chloride *is* observed at pH 6.1 (Figure 2) lends support to these kinetic considerations. By increasing both $[\text{H}^+]/K_\text{H}$ and $[\text{Cl}^-]/K_\text{Cl}$, the pH change enlarges X compared with Y and thus allows the chloride concentration to become kinetically relevant.

In conclusion, our studies have revealed that a lysine residue, probably at the active site, is essential for ACE activity. The function of this residue is clearly related to substrate binding. An association between this lysine and binding of activating anions is suggested by the parallelism between the effects of methylation and anion activation. Further, the specific protection of the critical lysine afforded by chloride demonstrates minimally that this residue has an unusual affinity for chloride. Nuclear magnetic resonance studies using [^{13}C]formaldehyde should be able to establish more definitively the relationship of this lysine to anion binding and possibly shed light on the physical basis of the activation phenomenon.

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Registry No. ACE, 9015-82-1; FA-Phe-Gly-Gly, 64967-39-1; FA-Leu-Ala-Gly, 71067-13-5; FA-Phe-Leu-Gly, 56186-51-7; FA-Phe-(N^ϵ-*t*-Boc)Lys-Ala, 86064-73-5; 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; FA-Phe-Ala-Phe, 71067-11-3; FA-Phe-Ala-(N^ϵ-*t*-Boc)Lys, 86064-81-5; FA-Phe-Ala-Gly, 71067-12-4; FA-Lys-Ala-Phe, 86064-82-6; PPPP, 72772-32-8; chloride, 16887-00-6; Lys, 56-87-1.

[5] The assumptions made in deriving this expression are that (i) steps in which protons or chloride is bound are in rapid equilibria, (ii) the carbinolamine intermediate and (protonated) imine are at steady state, and (iii) the activity measurements reflect all forms of the enzyme except Enz–NH–CH_3. An alternative mechanism involving acid-catalyzed conversion of the carbinolamine to the protonated imine by loss of water may also pertain. In this mechanism, the rate expression is similar to that presented except that k_2 should be replaced by $k'_2[\text{H}^+]$ and $10^{-14}k_{-2}/[\text{H}^+]$ by k'_{-2} where k'_2 and k'_{-2} are rate constants for the alternative second step. The same kinetic arguments can be made for both mechanisms.

[6] In fact, the opposite condition, i.e., $[\text{H}^+]/K_\text{H} \gg 1$, probably applies here. The reaction is occurring at about 3 pH units below the normal pK_a for a lysine ϵ-amino group. If the critical lysine group had an abnormally low pK_a value, it would probably be methylated much more rapidly than other lysines. The ^{14}C incorporation data, however, indicate that this is not the case.

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