Essential Residues in Angiotensin Converting Enzyme: Modification with 1-Fluoro-2,4-dinitrobenzene†

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ABSTRACT: The peptidase and esterase activities of rabbit pulmonary angiotensin converting enzyme (ACE) are rapidly abolished on reaction with 1-fluoro-2,4-dinitrobenzene (Dnp-F). Inactivation follows first-order kinetics with respect to the reagent and is accompanied by stoichiometric incorporation of 3,5-[^{3}H]Dnp, indicating that the effect is due to a specific modification of the enzyme. Thin-layer chromatography of an acid hydrolysate of the modified enzyme indicates that most of the radioactive label is present as *O*-Dnp-tyrosine (65 to >95%) and the rest as N^{ϵ}-Dnp-lysine. The pH dependence of the reaction is consistent with modification of either tyrosine or lysine. The presence of a competitive inhibitor effectively protects the enzyme against inactivation by Dnp-F. Acetylation of ACE with *N*-acetylimidazole also protects the enzyme against modification with Dnp-F. The results indicate the presence of catalytically essential tyrosine and lysine residues at the active site of ACE.

Angiotensin converting enzyme (EC 3.4.15.1) (ACE)[1] is a chloride-activated dipeptidyl carboxypeptidase that catalyzes the hydrolytic release of dipeptides from the C-termini of oligopeptide substrates (Soffer, 1976). Although the enzyme exhibits broad substrate specificity, its best known physiological functions are to activate angiotensin I (Skeggs et al., 1954, 1956) and inactivate bradykinin (Yang et al., 1970).

To establish a molecular basis for the catalytic activity of ACE, we have investigated the essential components of its active site. The enzyme contains zinc which is essential for activity (Das & Soffer, 1975; Bünning & Riordan, 1981, 1985; Kleemann et al., 1986). In addition, chemical modification studies from this (Bünning et al., 1978; Keung et al., 1980) and other laboratories (Fernley, 1977; Harris & Wilson, 1982, 1983) have provided evidence for the participation of tyrosine, arginine, and glutamic acid residues in the enzymatic process. In this regard, the active site of ACE seems to be closely similar to that of carboxypeptidase A (Vallee et al., 1983). However, ACE differs in that it is also inactivated by treatment with reagents that selectively modify the ϵ-amino group of lysine (Bünning et al., 1978; Weare, 1982; Shapiro & Riordan, 1983). This loss of activity has been shown to be related to the chloride activation characteristic of ACE (Shapiro & Riordan, 1983). Reductive methylation of one critical lysine markedly reduces substrate binding and, under appropriate conditions, chloride can protect against this modification. Since methylation with formaldehyde/cyanoborohydride is nonspecific, the stoichiometry of inactivation by lysine modification had to be determined by differential labeling of ACE in the presence and absence of an inhibitor (Shapiro & Riordan, 1983). ACE contains more than 50 lysines, and during a typical modification, almost as many methyl groups are incorporated per enzyme molecule. We therefore looked for other reagents that might exhibit greater selectivity for the critical lysine residue. The present study describes our findings with 1-fluoro-2,4-dinitrobenzene (Dnp-F), which proves to be very selective. Surprisingly, however, it preferentially modifies an active-site tyrosine and is seemingly much less reactive toward the critical lysine.

Experimental Procedures

Materials. Dnp-F and Dnp-amino acids were purchased from Sigma Chemical Co. (St. Louis, MO) or synthesized from *N*-acetyl-Cys and *N*-acetyl-His by dinitrophenylation and hydrolysis by hydrochloric acid. 3,5-[^{3}H]Dnp-F, 14.2 Ci mmol^{-1}, in benzene was obtained from New England Nuclear (Boston, MA). Phenylphosphoryl-L-phenylalanyl-L-phenylalanine (Holmquist & Vallee, 1979) was kindly provided by Dr. Barton Holmquist.

Preparation of ACE. ACE isolated from frozen rabbit lungs (Pel-Freez Biologicals Inc., Rogers, AR) was purified to homogeneity by a previously described procedure (Pantoliano et al., 1984; Bünning & Escher, 1986). Its specific activity was 72 units/mg. ACE concentrations were determined from the absorbance at 280 nm and expressed in molar concentrations by using a molar absorptivity[2] of 290 000 M^{-1} cm^{-1}.

Enzyme Assays. Peptidase activity was determined with Fa-Phe-Gly-Gly as substrate (Holmquist et al., 1979), and esterase activity was measured with Fa-Phe-OGly-Gly (Keung et al., 1980). Assays were carried out at 25 °C with 50 μM substrate in 0.05 M Hepes, pH 7.5, containing 0.3 M NaCl and 5 μM $ZnCl_2$.

Reaction of Dnp-F with ACE. Reactions were generally initiated by adding an aliquot of a 30 mM or 100 mM Dnp-F stock solution in ethanol to a solution of ACE, 0.01 mg/mL (0.05 μM), in 0.1 M borate, pH 8.5 at 25 °C. The final ethanol concentration in the reaction mixture did not exceed

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[1] Abbreviations: ACE, angiotensin converting enzyme; AcIm, *N*-acetylimidazole; ATPase, adenosinetriphosphatase; Dnp, dinitrophenyl; Dnp-F, 1-fluoro-2,4-dinitrobenzene; Fa, 2-furanacryloyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; RNase, ribonuclease; TLC, thin-layer chromatography.

[2] A molar absorptivity of 290 000 M^{-1} cm^{-1} for rabbit lung ACE was estimated from the amino acid composition of human lung ACE (Soubrier et al., 1988). Although the complete sequence of the rabbit lung enzyme is not yet available, it seems reasonable to assume that it will be comparable to that of the human lung enzyme based on the sequence similarities of rabbit testicular ACE (Kumar et al., 1989) and human testicular ACE (Ehlers et al., 1989; Lattion et al., 1989).

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10% (v/v) and had no effect on ACE activity over the time course of the reaction. The reaction mixtures were kept in the dark. The pH dependence of the modification reaction was determined with 1 mM Dnp-F and 0.1 M Hepes (pH 7.0, 7.5, and 8.0), 0.1 M sodium borate (pH 8.5, 9.0, 9.5, and 10.0), and 0.1 M sodium carbonate (pH 10.5, 11.0, and 11.5) as buffers. Aliquots were withdrawn from the reaction mixture at set time intervals and diluted 50-fold into substrate solution for measurement of activity (peptidase activity unless otherwise indicated). First-order rate constants for the modification reaction, k_{obs}, were calculated from half-lives or determined by plotting log $[V_c/(V_c - V)]$ versus time, where V_c is the activity of the unmodified enzyme and V the activity of the modified enzyme at time t.

The incorporation of [^{3}H]Dnp-F into ACE was followed by reacting the enzyme, 0.84 mg/mL (4.5 μM), with 0.5 mM Dnp-F containing 170 μCi/mL [^{3}H]Dnp-F (0.24 Ci/mmol) in 0.1 M borate, pH 8.5 at 25 °C in the dark. At intervals, samples were withdrawn from the reaction mixture to measure enzyme activity, and 200-μL aliquots taken at the same time were chromatographed on Sephadex G-15 (0.5 × 30 cm) equilibrated with 0.2 M ammonium acetate, pH 5.0. The eluate from the column was collected in 180-μL fractions and analyzed for radioactivity. Fractions containing the protein were combined, dialyzed exhaustively against three changes of 0.2 M ammonium acetate, pH 5.0, and again analyzed for radioactivity. The measurements were made with a Beckman LS-355 multichannel β spectrometer using Biofluor (New England Nuclear) as scintillation liquid. Counting efficiency was determined to be 30.8%, and quenching by buffer was 14.4%.

Identification of the Modified Amino Acid Residues. ACE (2.6 μM) was treated with 0.5 mM [^{3}H]Dnp-F (0.4 Ci/mmol) at pH 8.5 in 0.1 M borate until residual enzymatic activity decreased to about 40%. After the reaction was terminated and excess reagent removed by chromatography on Sephadex G-15 (1 × 30 cm) as described above, the [^{3}H]Dnp-enzyme was dialyzed against three changes of 1 L of water, lyophilized in the dark, and hydrolyzed with 6 M HCl at 110 °C for 12 h. The hydrolysate was evaporated, dissolved in 2 mL of 1 M HCl, and extracted 5 times with 2 mL of diethyl ether. The aqueous phase retained 90% of the radioactivity. It was evaporated to dryness and taken up in 50 μL of water, and 2 μL was analyzed by two-dimensional thin-layer chromatography on a silica gel 60–F254 TLC plate that had been prerun with the first-dimension solvent (E. Merck, Darmstadt, West Germany). The first-dimension solvent was 1-butanol/acetic acid/water (4:1:1), and the second-dimension solvent was 0.05 M sodium phthalate, pH 6.0, saturated with *tert*-amyl alcohol. The [^{3}H]Dnp-amino acids were located by autoradiography. The TLC plate was sprayed lightly with three coats of EN^{3}HANCE (New England Nuclear) at 10-min intervals, and the film (Kodak X-omat AR) was exposed at −80 °C for 3 days prior to development.

All samples were protected from light as far as possible to prevent decomposition of the Dnp label.

Protection Experiment. ACE was successively modified with (1) *N*-acetylimidazole to O-acetylate tyrosine residues, then with (2) Dnp-F, and finally with (3) hydroxylamine to remove the *O*-acetyl groups (Riordan et al., 1965; Bünning et al., 1978). In this procedure, 2 μL (0.54 μmol) of a freshly prepared solution of 29.5 mg of *N*-acetylimidazole in 1 mL of dry benzene was evaporated under a stream of dry nitrogen. Then 100 μL of ACE (0.30 μM) in 0.05 M Hepes, pH 7.5, containing 5 μM $ZnCl_2$ was added, and enzymatic activity was monitored versus time with Fa-Phe-Gly-Gly as the substrate. After 25 min, one aliquot was removed and diluted with an equal volume of 2 M NH_2OH, pH 7.5, and enzymatic activity was followed for 10 min. The remaining enzyme was combined with enough Dnp-F to produce a final concentration of 3.5 mM. After 10-min incubation, the excess Dnp-F was removed completely by extracting 3 times with 1 mL of diethyl ether. The aqueous phase was diluted 1:1 with 2 M NH_2OH, pH 7.5, and enzymatic activity was again followed.

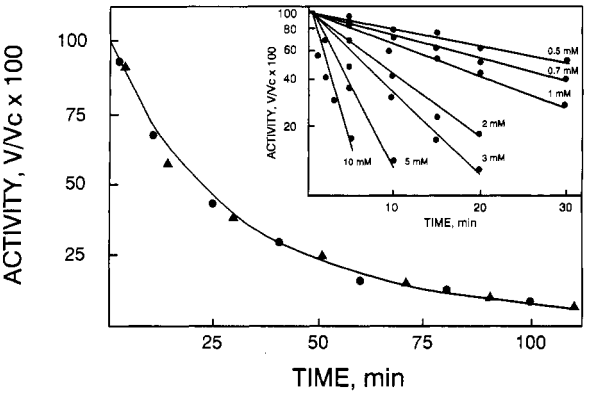

FIGURE 1: Changes in activity on modification of ACE with 1.0 mM Dnp-F in 0.1 M borate, pH 8.5. The time course of the reaction was followed by monitoring both peptidase (●) and esterase (▲) activity. Activities are expressed as the ratio of that of the modified enzyme, V, and the unmodified control, V_c, times 100. Inset: Effect of the Dnp-F concentration on the rate of ACE inactivation.

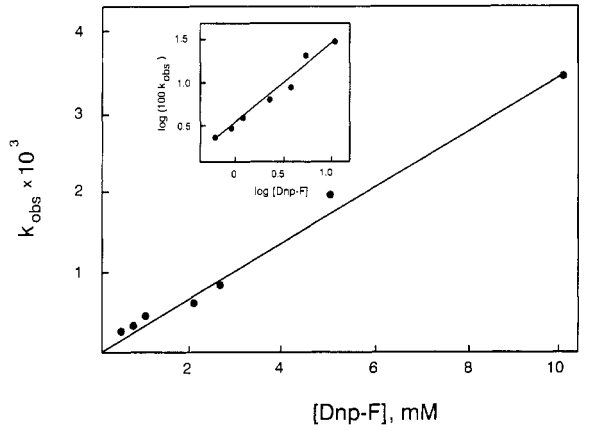

FIGURE 2: Variation of the rate of inactivation of ACE as a function of the Dnp-F concentration (data from Figure 1, inset). Inset: Determination of the order of the reaction between ACE and Dnp-F with respect to reagent. The data from Figure 2 are replotted in the form of log k_{obs} vs log [Dnp-F]. The slope of the plot gives n, the order of the reaction, as 0.9.

Results

Inactivation of ACE by Dnp-F. The time course of the inactivation of ACE by 1.0 mM Dnp-F is shown in Figure 1. Treatment of the enzyme in 0.1 M borate, pH 8.5, progressively abolishes both peptidase and esterase activities. Inactivation with concentrations of Dnp-F ranging from 0.5 to 10 mM (Figure 1, inset) follows pseudo-first-order kinetics with respect to enzyme concentration for at least two half-lives. Prolonged incubation with Dnp-F abolishes activity completely, and there is no restoration of activity on extensive dialysis (data not shown).

The pseudo-first-order rate constant is linearly dependent on reagent concentration (Figure 2), an indication that rapid reversible binding of the reagent to the enzyme prior to reaction does not occur. If such binding does occur, the dissociation constant of the resultant enzyme–Dnp-F complex would

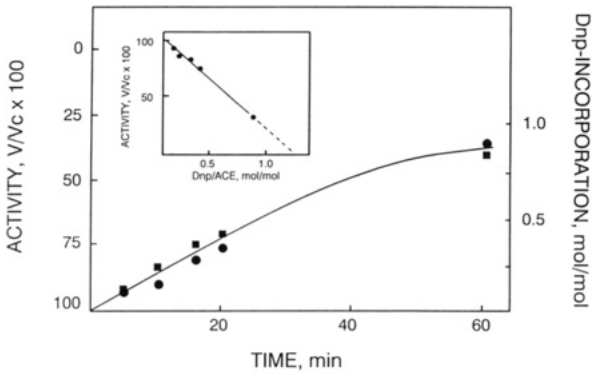

FIGURE 3: Time course of the inactivation of ACE (●) by [^{3}H]Dnp-F and the incorporation of radioactivity into protein (■). Activities are expressed as the ratio of that of the modified enzyme, V, and the unmodified control, V_c, times 100. Experimental details are described under Experimental Procedures. Inset: Plot of the ratio of the activities vs the incorporation of radioactive label.

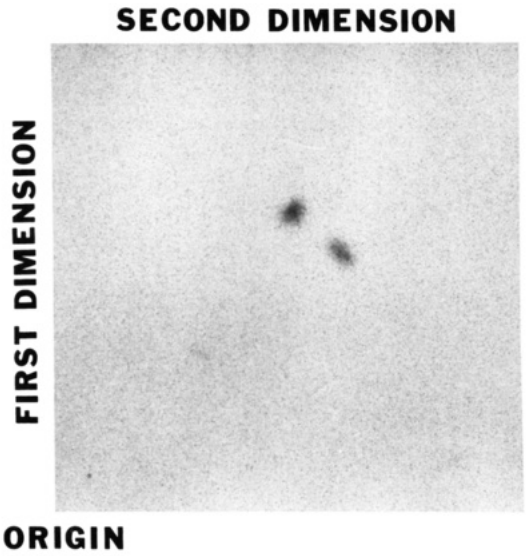

FIGURE 4: Two-dimensional thin-layer chromatography of an acid hydrolysate of [^{3}H]Dnp-F-modified ACE and detection of [^{3}H]-Dnp-amino acids by autoradiography. Experimental details are described under Experimental Procedures.

have to be high for the plot to appear linear over the range tested. The second-order rate constant for inactivation is 39 M^{-1} min^{-1} at pH 8.5. A plot of log k_{obs} versus the log of the Dnp-F concentration has a slope of 0.9 (Figure 2, inset), which indicates that the reaction is also first order with respect to reagent over the range of concentrations studied.

[^{3}H]Dnp-F Incorporation. The stoichiometry of the inactivation reaction was examined using [^{3}H]Dnp-F by monitoring both the time course of the decrease in enzyme activity and the accompanying incorporation of radioactivity into the protein. The results, summarized in Figure 3, indicate that the rate of ^{3}H incorporation parallels the disappearance of enzymatic activity (Figure 3). A plot of the ratio of [^{3}H]Dnp label per molecule of ACE versus activity (Figure 3, inset) gives a straight line (for the region of specific reaction) with a slope equal to the number of residues modified per molecule of ACE, and indicates that inactivation correlates with modification of just over one residue per molecule of ACE.

Identification of the Modified Residues. To identify which kind of amino acid residues were modified, ACE was treated with [^{3}H]Dnp-F to give a residual activity of about 40%. An acid hydrolysate of the modified enzyme was dissolved in dilute hydrochloric acid and extracted with diethyl ether. More than 90% of the radioactivity was retained in the aqueous phase

Table I: Two-Dimensional Thin-Layer Chromatography of a Dnp-ACE Hydrolysate and Dnp-amino Acids[a]

sample	R_f values: first dimension[b]	R_f values: second dimension[c]
Dnp-ACE, spot 1 (65–95%)	0.58	0.48
Dnp-ACE, spot 2 (35–5%)	0.52	0.58
O-Dnp-Tyr	0.58	0.48
ε-Dnp-Lys	0.52	0.58
Im-Dnp-His	0.32	0.43
S-Dnp-Cys	0.58	0.64
N-Dnp-Thr	0.71	0.81

[a] Standard Dnp-amino acids were detected by fluorescence quenching. The Dnp-ACE spots were detected by autoradiography and quantitated by densitometry. When *O*-Dnp-Tyr and ε-Dnp-Tyr and ε-Dnp-Lys were added to the hydrolysate of [^{3}H]Dnp-ACE prior to thin-layer chromatography, most of the radioactivity (65–95%) was subsequently found in the spot corresponding to *O*-Dnp-Tyr. The rest comigrated with ε-Dnp-Lys. [b] 1-Butanol/acetic acid/H_2O (4:1:1). [c] 0.05 M sodium phthalate, pH 6.0, saturated with *tert*-amyl alcohol.

Table II: pH Dependence of Rate Constants for Dnp-F Modification[a]

pH	k_{obs} (min^{-1})	k_{St} (min^{-1})	k_r (min^{-1})
7.0[b]	7.1×10^{-3}		7.1×10^{-3}
7.5[b]	1.5×10^{-2}		1.5×10^{-2}
8.0[b]	3.0×10^{-2}		3.0×10^{-2}
8.5[c]	4.1×10^{-2}		$4.1 \times 1-^{-2}$
9.0[c]	$7.6 \times 10-^{-2}$		7.6×10^{-2}
9.5[c]	0.18		0.18
10.0[c]	0.35	5.9×10^{-3}	0.34
10.5[d]	0.74	3.9×10^{-2}	0.70
11.0[d]	1.9	0.10	1.8
11.5[d]	2.8	0.65	2.2

[a] k_{obs} = inhibition rate constant; k_{St} = stability rate constant, i.e., that part of inhibition due to diminished stability of the enzyme at that pH. $k_I = k_{obs} - k_{St}$ gives the corrected reaction rate constant. The high-pH data are not corrected for spontaneous hydrolysis of Dnp-F. [b] In 100 mM Hepes, with 1 mM Dnp-F. [c] In 100 mM borate, with 1 mM Dnp-F. [d] In 100 mM sodium carbonate, with 1 mM Dnp-F.

which was then analyzed by two-dimensional thin-layer chromatography and subsequent autoradiography (Figure 4). An amount of 65 to >95% of the applied radioactivity was found in a spot whose mobility coincided with that of the reference standard *O*-Dnp-Tyr run at the same time (Table I). A second spot containing the remainder of the applied radioactive material corresponds to ε-Dnp-Lys (Table I). No other Dnp-amino acids were detected. In particular, Im-Dnp-His and *S*-Dnp-Cys, also known to be potential products of the Dnp-F reaction, were absent. The degree of inactivation was approximately the same irrespective of the ratio of tyrosine to lysine modification. Therefore, it would seem that both residues are essential to the catalytic activity of ACE.

Effect of pH. The pH dependence of the Dnp-F reaction with ACE was determined between pH 7 and 11.5. Above pH 10, the reaction rate was corrected for spontaneous loss of activity due to the reduced stability of the enzyme (Table II); valid measurements could not be obtained above pH 11.5. Between pH 7 and 11.5, the rate of ACE inactivation by Dnp-F increases as the pH is raised. The pH dependence curve of the reaction gives an apparent pK for the modified residue(s) of 10.7, which is consistent with the modification of either tyrosine or lysine.

Protection of ACE against Inactivation by Dnp-F. The rate of inactivation of ACE by Dnp-F is markedly reduced by the presence of the competitive inhibitor phenylphosphoryl-Phe-Phe (Figure 5). A semilogarithmic plot of the time course of the reaction indicates that the rate of inactivation decreases by 45 and 75% in the presence of 10^{-4} and 5×10^{-4} M in-

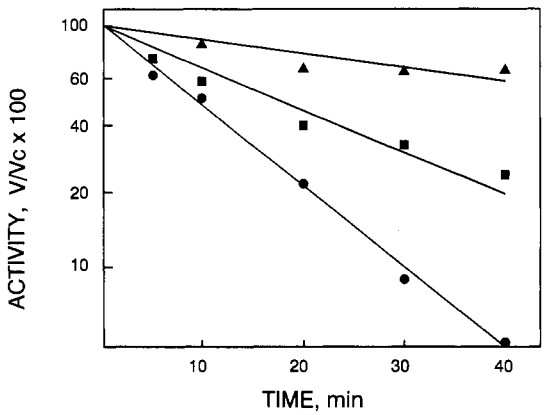

FIGURE 5: Effect of phenylphosphoryl-Phe-Phe on ACE inactivation by Dnp-F. The reaction was carried out with 1.0 mM reagent in 0.1 M borate, pH 8.5, and the following phenylphosphoryl-Phe-Phe concentrations: (●) none; (■) 0.1 mM; (▲) 0.5 mM. Activities are expressed as the ratio of that of the modified enzyme, V, and the unmodified control, V_c, times 100.

Table III: Modification of ACE with *N*-Acetylimidazole and Dnp-F[a]

reaction sequence	activity, $V/V_c \times 100$[b]
5 mM AcIm	9
5 mM AcIm; 1 M NH_2OH	97
5 mM AcIm; 3.5 mM Dnp-F	3
5 mM AcIm; 3.5 mM Dnp-F; 1 M NH_2OH[c]	91
3.5 mM Dnp-F	35
3.5 mM Dnp-F; 1 M NH_2OH[c]	35

[a] Reactions were in 0.05 M Hepes, pH 7.5, containing 5 μM $ZnCl_2$. The reaction time for acetylation was 25 min, for hydroxylamine treatment, 10 min, and for Dnp-F modification, 10 min. [b] Activities are expressed as the ratio of that of the modified enzyme at the end of the reaction sequence, V, and the unmodified control, V_c, times 100. [c] Prior to the addition of hydroxylamine, excess Dnp-F was removed by three extractions with diethyl ether.

hibitor, respectively. This protective effect of phenylphosphoryl-Phe-Phe against inactivation by Dnp-F suggests that the modified residues are located at the active site of ACE.

The presence of an essential tyrosine residue in the active site of ACE had previously been demonstrated by the inactivation of the enzyme on acetylation with *N*-acetylimidazole and the subsequent restoration of activity on addition of hydroxylamine (Riordan et al., 1965; Bünning et al., 1978). To test whether the tyrosine residue(s) acetylated with *N*-acetylimidazole is (are) the same as that modified by Dnp-F, a sequential modification experiment was carried out. The enzyme was first O-acetylated with 5 mM acetylimidazole at pH 7.5, and its activity decreased to 9% (Table III). Treatment of an aliquot of the modified enzyme with hydroxylamine restored essentially all activity, an indication that loss of activity was due to tyrosine acetylation (Riordan et al., 1965). The acetylated enzyme was next reacted with 3.5 mM Dnp-F for 10 min, and the activity decreased a bit more to 3%. When the doubly modified enzyme was finally incubated with 1 M hydroxylamine, more than 90% of the original activity was restored. Hydroxylamine treatment had no effect on the activity of ACE modified with Dnp-F alone. These results indicate that O-acetylation protects the tyrosine against Dnp-F modification and that the two reagents react with the same site of the enzyme.

Discussion

N-Terminal α-amino groups as well as cysteine, lysine, histidine, and tyrosine side chains of proteins are all potentially reactive toward Dnp-F. At pH 8.5, Dnp-F reacts primarily with a single functional residue at the active site of ACE. The specificity is apparent from a combination of radioactivity and kinetic data for Dnp incorporation. Determination of the residue modified was made by autoradiography of a two-dimensional TLC of the acid hydrolysate of Dnp-F-modified ACE. The predominant Dnp product was *O*-Dnp-Tyr, but some ε-Dnp-Lys was always present. Quantitative determination by counting each of the two TLC spots indicated that *O*-Dnp-Tyr ranged from 65 to >95% of the radioactivity incorporated. Im-Dnp-His and *S*-Dnp-Cys were never detected, the latter consistent with the fact that ACE is not known to have free thiol groups. Although some losses occurred during the workup of the hydrolysate, close to 90% of the radioactivity remained in the aqueous phase after extraction with diethyl ether. This method can be used to separate ε-Dnp-Lys, *O*-Dnp-Tyr, Im-Dnp-His, *S*-Dnp-Cys, and α-Dnp-Arg from all other Dnp-amino acids (Fraenkel-Conrat et al., 1955). Thus, the product of modification of the N-terminal amino acid residue of ACE, α-Dnp-Thr, would be extracted into the ether phase. Since the ether phase accounts for less than 10% of the incorporated radioactivity, modification of the N-terminal Thr of ACE can occur only to a very small extent. Extensive destruction of Dnp-amino acids during hydrolysis, reported to occur sometimes when carbohydrate is present in the protein (Fraenkel-Conrat & Porter, 1952), was not observed with ACE, though it is known to contain a high amount of carbohydrate. Special care was taken to prevent destruction of the light sensitive Dnp label by excluding light from all operations.

The variable yield of Dnp-Tyr and Dnp-Lys may likely reflect small differences in the reaction conditions for each preparation. Results presented in the following paper demonstrate that a specific tyrosine and a specific lysine are both protected from dinitrophenylation by the competitive inhibitor Enalaprilat (Chen & Riordan, 1990). Thus, it would appear that both a tyrosine and a lysine are components of the active site of ACE although complete inactivation correlates with modification of just over one residue per molecule. It is possible that modification of these two residues may be mutually exclusive, but additional studies would be required to clarify this point.

Double modification experiments with *N*-acetylimidazole and Dnp-F, based on the ability of hydroxylamine to deacetylate *O*-acetyltyrosine (Riordan et al., 1965), indicate that Dnp-F reacts with a specific tyrosine residue in ACE. Modification of ACE with *N*-acetylimidazole prior to treatment with Dnp-F at pH 7.5 essentially abolishes enzymatic activity. Subsequent addition of hydroxylamine restores it, indicating that O-acetylation of a tyrosine residue prevents it from reacting with Dnp-F. These experiments were done at pH 7.5 in order to minimize hydrolysis of *O*-acetyltyrosine which can occur spontaneously at the higher pH. At pH 7.5, dinitrophenylation of lysine is minimal (Y.-N. P. Chen, unpublished observations).

Protection studies to prevent Dnp-F modification of ACE were done at pH 8.5 in the presence of various amounts of the competitive inhibitor phenylphosphoryl-Phe-Phe. This inhibitor, in sufficient concentration, was able to afford up to 75% protection against modification by Dnp-F, indicating that the susceptible residue(s), i.e., tyrosine/lysine, is (are) at or near the active site and/or that its (their) reactivity is altered as a result of inhibitor binding.

The highly selective reaction of Dnp-F with ACE is rather unusual in that modification of tyrosine takes place in preference to lysine. Moreover, although there may be more than 50 tyrosine residues in rabbit lung ACE [extrapolated from

Table IV: Second-Order Rate Constants for the Reaction of Various Groups with Dnp-F at pH 8.0

group	k (M^{-1} min^{-1})
ϵ-amino of Lys_{avg} in immunoglobulins[a]	0.05–0.5
ϵ-amino of Lys_{avg} in concanavalin A[a]	0.68
α-amino in RNase[b]	0.35
ϵ-amino of Lys-41, RNase[b]	2.7
α-amino of Gly-His[a]	~0.4
ϵ-amino of Gly-Lys[a]	~0.05
O-Tyr in ACE[c]	30
O-Tyr in F_1 ATPase[d]	110
O-Tyr_{avg} in immunoglobulins[a]	0.10–0.23
O-Tyr_{avg} in concanavalin A_a	0.52
N-acetyl-Tyr-NH_2[a]	~0.03

[a] Calculated from pH-independent second-order rate constants for pH 8.0, 20 °C (Kaplan et al., 1980; Jackson & Young, 1986). [b] Reaction in water at pH 8.0, 15 °C (Murdock et al., 1966). [c] Reaction in 0.1 M borate, pH 8.0, 25 °C. [d] Reaction in 50 mM Hepes, 25 mM NaCl, 2 mM ethylenediaminetetraacetic acid, and 25% glycerol at pH 8.0, 24.5 °C (Ting & Wang, 1980).

Das and Soffer (1975) by using a revised molecular weight based on that for human lung ACE (Soubrier et al., 1988)], up to 60% inactivation occurs with a reaction involving only 1 of them. Obviously this residue is endowed with a unique reactivity. This is particularly apparent from the comparison of second-order rate constants for the reactions of various groups with Dnp-F, at pH 8.0, presented in Table IV. The second-order rate constant for the critical residue (predominantly tyrosine) in ACE at pH 8.0 is 30 M^{-1} min^{-1}, much greater than that of the average tyrosines or lysines in the immunoglobulins or concanavalin A, or any of the model compounds listed in Table IV. Moreover, while it is only one-fourth as reactive as the hyperreactive tyrosine in F_1 ATPase (Ting & Wang, 1980), it is 10 times more so than the relatively reactive Lys-41 in RNase (Murdock et al., 1966), albeit under somewhat different conditions.

An abnormally low apparent pK_a is often cited as an explanation for hyperreactivity of functional residues in proteins, but the pH dependence of Dnp-F inactivation of ACE (Table II) suggests an apparent pK_a of 10.7 which is within or somewhat higher than the normal range for the phenolic hydroxyl group (Jackson & Young, 1986). Thus, the modification of the critical side chain in ACE is an unusually fast reaction, especially since, under "normal" conditions, the order of reactivity with Dnp-F is SH > NH_2 > OH (Hirs, 1967).

Both ACE and carboxypeptidase A are metalloexopeptidases with active-site tyrosine, arginine, and glutamic acid residues. Further, ACE has been shown to contain an essential lysine residue and is also strongly activated by anions. Modification of the tyrosine residue in carboxypeptidase A typically results in loss of peptidase activity, though not necessarily in a parallel loss of esterase activity. Modification of the tyrosine residue in ACE, on the other hand, results in simultaneous loss of both peptidase and esterase activities (Keung et al., 1980). Results of the modification of ACE by Dnp-F confirm this. This difference between carboxypeptidase A and ACE implies a difference in function or in the stereochemical requirements of the essential tyrosine residues between the two enzymes, or both. Further investigations are needed to show if this observation is generally applicable.

Registry No. ACE, 9015-82-1; Dnp-F, 70-34-8; Tyr, 60-18-4; Lys, 56-87-1; phenylphosphoryl-Phe-Phe, 129492-17-7.

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