Identification of Essential Tyrosine and Lysine Residues in Angiotensin Converting Enzyme: Evidence for a Single Active Site†

Ying-Nan P. Chen and James F. Riordan*
Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, Massachusetts 02115
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ABSTRACT: Inactivation of rabbit lung angiotensin converting enzyme (ACE) by 1-fluoro-2,4-dinitrobenzene (Dnp-F) has been shown to be due primarily to the modification of a tyrosine residue [Bünning, P., Kleeman, S. G., & Riordan, J. F. (1990) *Biochemistry* (preceding paper in this issue)]. Rabbit testicular ACE is also inactivated by Dnp-F. The specific site of modification has been identified by peptide mapping of tryptic digests of the Dnp-modified protein. Two principal 340-nm-absorbing peaks, not observed with protein modified in the presence of inhibitor, have been characterized. Amino acid and sequence analyses show that these peptides contain two distinct residues that have been selectively modified. The sequence of the major (>90% of the total) modified peptide is YVEFTNK with the Dnp group on tyrosine. The sequence of the second, minor peptide is KVQDLQR with the Dnp group on lysine. Identical peptides were obtained from Dnp-modified rabbit lung ACE. These modified amino acids correspond to residues 200 and 118, respectively, in testicular ACE (human enzyme numbering). Both peptides are present only in the carboxy-terminal half-domain of lung ACE, corresponding to residues 776 and 694, respectively. These results indicate that the Dnp-F sensitive, catalytically functional active site is located in the "testicular" half of lung ACE.

Angiotensin converting enzyme (EC 3.4.15.1) (ACE)[1] is a dipeptidyl carboxypeptidase that participates in the regulation of blood pressure (Piquilloud et al., 1970; Stevens et al., 1972; Soffer, 1976) by activating angiotensin I (Ng & Vane, 1967, 1968) and inactivating bradykinin (Yang et al., 1970). Its activity has been detected in a variety of tissues but especially lung and kidney; the molecular weight of the enzyme from these sources (referred to throughout as lung ACE) has been reported to range from 130 000 to 180 000 (Erdös, 1975; Soffer, 1976). A smaller protein of approximately 100 kDa has been found in mature testis (El-Dorry et al., 1982a,b; Lanzillo et al., 1985; Soffer et al., 1987). Its catalytic properties appear to be similar to those of lung ACE (Soffer et al., 1987) although its function is unknown. The complete amino acid sequence of the larger protein (from human endothelial cells as well as mouse kidney) has been determined from its cDNA (Soubrier et al., 1988; Bernstein et al., 1989). It contains 1277 (human) or 1278 (mouse) amino acids and consists of two homologous, repeated domains each of which contains a potential catalytic site. The amino acid sequence of ACE from human and rabbit testis has also been deduced from its cDNA (Ehlers et al., 1989; Kumar et al., 1989; Lattion et al., 1989), and this together with Northern analysis of mouse testis mRNA (Bernstein et al., 1989) indicates that testicular ACE shares the carboxy-terminal half-structure of its lung counterpart. This raises important questions such as the following: Why does lung ACE possess two possible active sites? Do each of the two bind zinc, and are they both catalytically active?

We have attempted to answer these questions by carrying out chemical modification of ACE. This approach has two potential benefits: it may identify amino acid residues in the primary structure essential for catalytic activity, and it may localize these critical residues to one of the two domains.

Previous studies have shown that 1-fluoro-2,4-dinitrobenzene (Dnp-F) inactivates rabbit lung ACE and the inactivation can be prevented by the presence of a competitive inhibitor (Bünning et al., 1990). Chemical analysis identified *O*-Dnp-Tyr as the major product of modification and ε-Dnp-Lys as a minor product. Since knowledge of the sequence surrounding these residues should allow them to be assigned in the primary structure of the protein, we have therefore isolated and characterized the tryptic peptides containing the modified tyrosine and lysine residues from both rabbit lung and rabbit testicular ACE. The results indicate that these residues occur only in the second half of the lung ACE, which is identical with almost the entire testicular enzyme, and thus this half would seem to contain the Dnp-F-sensitive, functional active site.

Materials and Methods

Dnp-F and ε-Dnp-Lys were purchased from Sigma. Phenylphosphoryl-L-Phe-L-Phe and Enalaprilat [(*S*)-1-[*N*-(1-carboxy-3-phenylpropyl)-L-Ala]-L-proline] were kindly provided by Dr. Barton Holmquist.

ACE was isolated both from frozen rabbit lung and mature rabbit testis (Pel-Freez Biologicals Inc., Rogers, AR) and purified to homogeneity by affinity chromatography (Pantoliano et al., 1984; Bünning & Escher, 1986). The final preparation was >95% pure as judged by SDS–PAGE. Concentrations of lung ACE were determined from measurements of the absorbance at 280 nm and converted to molar concentrations based on a molar absorptivity of 290 000 M^{-1} cm^{-1} (Bünning et al., 1990). Testicular ACE concentrations were based on amino acid analysis. Enzyme activity was measured with Fa-Phe-Gly-Gly as substrate as described (Holmquist et al., 1979).

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* Address correspondence to this author at the Center for Biochemical and Biophysical Sciences and Medicine, Seeley G. Mudd Building, 250 Longwood Ave., Boston, MA 02115.

[1] Abbreviations: ACE, angiotensin converting enzyme; Fa, 2-furanacryloyl; Dnp-F, 1-fluoro-2,4-dinitrobenzene; Dnp, dinitrophenyl; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

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Dnp-F Modification. The modification was initiated by adding an aliquot of Dnp-F stock solution (100 mM in ethanol) to 1–2 μM ACE in 0.1 M borate, pH 8.5, at room temperature (final Dnp-F concentration was 2 mM). The reaction was carried out in the dark at room temperature until >95% inactivation had occurred. It was then terminated by acidification, followed by dialysis against 0.1 M ammonium acetate, pH 5, and lyophilized.

Trypsin Digestion. The protein samples were dissolved in 1 mL of 8 M urea/10 mM EDTA/50 mM sodium phosphate, pH 5, for 3 h at 37 °C. The protein solution was dialyzed against 0.1 M ammonium bicarbonate and then digested with 2% (w/w) of TPCK-treated trypsin for 24 h at 37 °C. At the end of the digestion, 10% acetic acid was added to stop the reaction, and the digests were analyzed by reversed-phase HPLC.

Peptide Mapping and Peptide Isolation by HPLC. For peptide mapping, 1–2 nmol of protein digest was analyzed on an Ultrasphere octadecylsilane column (0.46 × 25 cm). Solvent A was 0.1 M perchloric acid/0.1% *o*-phosphoric acid, titrated to pH 2 with sodium hydroxide. Solvent B was 75% acetonitrile and 25% solvent A. The protein digest was eluted at a flow rate of 1 mL/min with linear gradients of 0% B to 15% B in 15 min and then 15% B to 75% B in 95 min followed by 75% B to 90% B in 5 min. Peptides of interest were rechromatographed on the same column by use of a 120-min linear gradient from 0.1% TFA in water to 75% acetonitrile containing 0.1% TFA, at a flow rate of 1 mL/min. Peptides were detected by their absorbance at 214 nm while, simultaneously, Dnp-peptides were detected by their absorbance at 340 nm.

Amino Acid and Sequence Analysis. Amino acid analysis was performed as previously described according to the PicoTag derivatization method (Bidlingmeyer et al., 1984). Automated Edman degradation was performed on a Beckman 890C sequencer with 0.1 M quadrol coupling buffer and an updated Beckman program as described (Strydom et al., 1986).

Results

Modification of Testicular ACE with Dnp-F. Incubation of rabbit testicular ACE with 2 mM Dnp-F at pH 8.5 results in loss of >95% of the initial activity in 60 min. The presence of the competitive inhibitor phenylphosphoryl-Phe-Phe, 0.5 mM, partially protects the enzyme from modification, suggesting that the modified amino acid residue is located at the active site of ACE. These data are consistent with earlier observations obtained with rabbit lung ACE (Bünning et al., 1990).

Tryptic Peptide Mapping. For peptide mapping, testicular ACE was treated with 2 mM Dnp-F in the absence and presence of a 0.2 mM sample of the much stronger competitive inhibitor Enalaprilat [$K_i = 5 \times 10^{-11}$ M, i.e., 10^4-fold tighter than phenylphosphoryl-Phe-Phe (Shapiro & Riordan, 1984)]. After removal of excess reagents, the modified proteins were denatured and digested with TPCK-treated trypsin, and the digests were subjected to reversed-phase HPLC. The peptide elution profiles for Dnp-modified testicular ACE modified in the absence and presence of inhibitor are shown in Figures 1 and 2, respectively. Two principal 340-nm-absorbing peptides, A and B, with retention times of 59 and 70 min, respectively, are observed in the digest of protein modified in the absence of inhibitor (Figure 1) but are not present when the inhibitor was present during modification (Figure 2). The 214-nm profiles indicate that peak B is only present in the digest from the minus inhibitor-modified ACE and is absent from that of

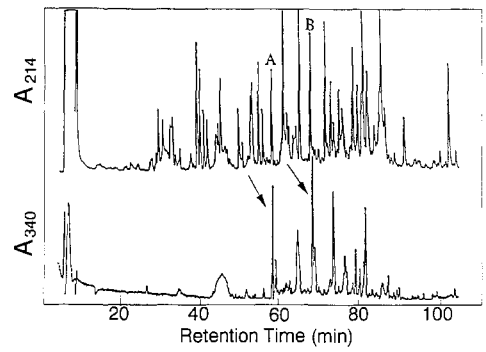

FIGURE 1: HPLC chromatogram of tryptic digest of ACE modified with 2 mM Dnp-F in the absence of inhibitor at room temperature for 1 h. Approximately 2–3 nmol of the tryptic digest was injected onto a C18 column and eluted with linear gradients as described under Materials and Methods. Peptides A and B (and arrows) are not present when modification is performed in the presence of inhibitor (see Figure 2).

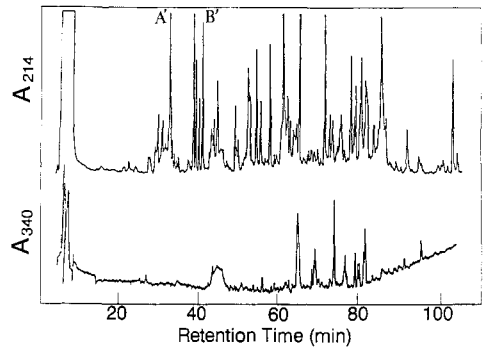

FIGURE 2: HPLC chromatogram of tryptic digest of ACE modified with Dnp-F as in Figure 1, but in the presence of 0.2 mM Enalaprilat. A′ and B′ are peptides not present when modification is performed in the absence of inhibitor (see Figure 1).

plus inhibitor-modified ACE. Peak A is found in the 214-nm profiles from both digests. Rechromatography of peak A reveals that it is composed of several peptides, only one of which is modified. Two peptides, A′ and B′, with retention times of 31 and 41 min, respectively, are present in the inhibitor control (Figure 2), and are correspondingly decreased in the chromatogram of the unprotected sample (Figure 1). A number of peptides that absorb at 340 nm are present in both samples, indicating nonspecific modification of several amino acids not essential for enzymatic activity. Peptide maps from separate preparations are highly reproducible.

Thiolysis of Dnp-ACE. Literature precedents have shown that *O*-Dnp-Tyr, Im-Dnp-His, and *S*-Dnp-Cys are susceptible to thiolysis, whereas α- and ε-amino-Dnp derivatives are stable to treatment with thiols (Shaltiel, 1967). Since tyrosine was thought to be the major site of modification (Bünning et al., 1990), attempts were made to restore native activity to lung Dnp-ACE by thiolysis, but these were unsuccessful owing to inhibition of ACE by the thiol reagent itself. We also performed thiolysis on a tryptic digest of testicular Dnp-ACE. The digest was treated overnight with 10 mM dithiothreitol, pH 8.5, at room temperature, and then analyzed by HPLC. Peptide B (which elutes at 70 min) was no longer present as indicated by the near-absence of both the 214- and 340-nm peaks (data not shown). In contrast, peptide A (which elutes at 59 min) as well as the nonspecifically modified peptides were unaffected by this treatment (data not shown). The data suggest that peptide B likely contains a modified tyrosine, whereas the remaining 340-nm peaks are due to Dnp-lysines.

O-Acetylation of ACE. The presence at the active site of ACE of an essential tyrosine residue that reacts with Dnp-F

Table I: Amino Acid Compositions[a] of the Tryptic Peptides of Dnp-Modified Testicular ACE[b]

amino acid	A (59 min)[c]	A′ (31 min)	B (70 min)	B′ (41 min)
Asp	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Glu	1.8 (2)	1.0 (2)	1.0 (1)	1.0 (1)
Ser	0.4		0.2	0.2
Gly	0.7		0.3	0.4
His	0.6		0.4	0.2
Arg	0.9 (1)	1.0 (1)		0.2
Thr	0.2	0.2	0.9 (1)	1.1 (1)
Ala	0.3	0.2	0.2	
Pro	0.3	0.4		0.2
Tyr	0.3		(1)[d]	0.7 (1)
Val	1.0 (1)	0.3 (1)	0.8 (1)	0.9 (1)
Met	0.2			
Ile				
Leu	1.0 (1)	0.6 (1)	0.2	
Phe	0.4	0.5	0.8 (1)	1.0 (1)
Lys	0.3 (1)[d]		1.0 (1)	1.0 (1)

[a] Residues per molecule determined on the basis of calculated average nanomoles per residues. The values in parentheses are the number of residues from sequence analysis. [b] Peptides A′ and B′ were non-Dnp-peptides isolated from plus inhibitor-modified ACE. Peptides A and B were Dnp-peptides from ACE modified in the absence of inhibitor. [c] Mixture of peptides as revealed by sequence analysis. [d] Number in parentheses includes the Dnp residue.

is further supported by the results of modification of the enzyme with *N*-acetylimidazole. It has been demonstrated that this reagent inactivates both lung and testicular ACE and that activity can be recovered in each case by addition of 0.2 M hydroxylamine (Bünning et al., 1978, 1989; J. W. Harper, R. Shapiro, and J. F. Riordan, unpublished results). Hydroxylamine has no effect on the activity of the native enzymes. Moreover, prior acetylation prevents subsequent irreversible inactivation of lung ACE by Dnp-F (Bünning et al., 1990). To test whether the tyrosine modified by Dnp-F is also the one that reacts with *N*-acetylimidazole, testicular ACE was first modified with 10 mM *N*-acetylimidazole and then with Dnp-F and then digested with trypsin. HPLC analysis indicated that the amount of peak B (70 min), the putative tyrosine-containing peptide, was greatly decreased. In a separate experiment, ACE was first treated with 10 mM *N*-acetylimidazole at pH 8.5 for 30 min and then with Dnp-F for 15 min. The excess reagents were removed by extraction with ether, and hydroxylamine was added to the aqueous phase to induce O-deacylation. The product was found to have 45% of the initial activity.[2] Collectively, these and earlier data indicate that Dnp-F and *N*-acetylimidazole modify the same tyrosine residue which is likely located at or near the active site of ACE.

Stoichiometry. Previous studies had shown that just over one Dnp group is incorporated per enzyme molecule during inactivation of lung ACE with [^{3}H]Dnp-F and that as much as 95% of the modification occurs at tyrosine (Bünning et al., 1990). We have confirmed the relative stoichiometry of the Dnp-F reaction with testicular ACE by estimating the absorptivities and peak areas of the principal modified peptides by dual-wavelength monitoring at 214 and 340 nm. The molar absorptivity for ε-Dnp-Lys at 360 nm is 17 400 M^{-1} cm^{-1}, about 6 times higher than that for *O*-Dnp-Tyr at 350 nm, 2900 M^{-1} cm^{-1} (Fraenkel-Conrat et al., 1954). Since the area of the 70-min peak (the putative tyrosine-containing peak) is greater than that of the 59-min peak, it accounts for >85% of the specific modification. Quantitative data from sequence analysis of both peptides (Tables II and III) give a similar ratio between the two modified peptides.

[2] When this experiment was performed at pH 7.5 rather than pH 8.5, more than 90% of the initial activity was recovered after deacylation with hydroxylamine (Bünning et al., 1990).

Table II: Edman Degradation of Peptides A and A′

	peptide A (59 min)		peptide A′ (31 min)	
cycle	PTH-aa	nmol	PTH-aa	nmol
1	blank[a]		Val	0.17
2	Val	0.13	Gln	0.06
3	Gln	0.07	Asp	0.15
4	Asp	0.08	Leu	0.04
5	Leu	0.07	Gln	0.05
6	Gln	0.05	Arg	0.05
7	Arg	0.03		

[a] No PTH-amino acid was observed at this cycle.

Table III: Edman Degradation of Peptides B and B′

	peptide B (70 min)		peptide B′ (41 min)	
cycle	PTH-aa	nmol	PTH-aa	nmol
1	Tyr	0.07[a]	Tyr	0.61
2	Val	0.39	Val	0.59
3	Glu	0.23	Glu	0.39
4	Phe	0.28	Phe	0.52
5	Thr	0.05[b]	Thr	0.04[b]
6	Asn	0.19	Asn	0.28
7	Lys	0.12	Lys	0.19

[a] Location of *O*-Dnp-Tyr. [b] This cycle contains a 313-nm peak indicative of Thr.

Isolation and Characterization of the Major Dnp-Modified Tryptic Peptides. The two Dnp-modified tryptic peptides, A and B, from the unprotected sample of testicular ACE, as well as the two unmodified peptides, A′ and B′, from the protected sample, were isolated and purified by rechromatography (chromatograms not shown). The four purified peptides were subjected to amino acid analysis and automated Edman degradation (Tables I–III). Amino acid analyses show that peptides A and A′ correspond to the same peptide, except for the presence of Dnp-lysine in peptide A. In addition, tyrosine is present in B′ whereas Dnp-tyrosine is present in B.

The sequence data for peptide A (59 min) are shown in Table II. PTH amino acid derivatives were identified for six of the seven cycles. The first cycle did not yield any of the usual PTH derivatives, but instead gave a peak that eluted at a later retention time, absorbed at 313 nm, and coeluted with the PTH derivative of ε-Dnp-Lys under the same HPLC conditions (data not shown). Repeated sequence analysis of peptide A yielded identical results. Sequence analysis of peptide A′ (31 min) shows that the first 6 residues are exactly the same as residues 2–7 of peptide A. Residue 1 of peptide A would not be expected to be present in peptide A′ since, in the absence of its modification by Dnp-F, it would be removed by tryptic cleavage. These results are consistent with modification of a lysine in unprotected ACE.

The amino acid sequence data for peptide B (70 min) are shown in Table III. In the first cycle, a small amount of unmodified PTH-Tyr was observed, which probably arises from tyrosine generated from *O*-Dnp-Tyr by the dithioerythritol used in the sequencer wash. Repeated sequence analysis of this peptide gave the same results. Sequence analysis of peptide B′ (41 min) gave identical results except for the much higher yield of PTH-Tyr in the first cycle. These results, together with amino acid analysis, indicate that in peptide B the residue modified is tyrosine.

Modification of Lung ACE with Dnp-F. In contrast to testicular ACE, lung ACE consists of two large, repeated sequences, the second of which corresponds to almost the entire

sequence of the testicular enzyme (Soubrier et al., 1988; Ehlers et al., 1989; Kumar et al., 1989; Lattion et al., 1989). Dnp-F treatment of both lung and testicular ACE has indicated that both tyrosine and lysine are chemically modified. To test whether these critical residues are present in just one or in both halves of the lung enzyme, we repeated the above experiments with rabbit lung ACE. The results obtained revealed two 340-nm-absorbing peptides which had virtually the same retention times as those from the testis enzyme and which were protected from dinitrophenylation by Enalaprilat. Composition and sequence analyses of the two peptides confirmed that their sequences were identical with those of the testis enzyme (data not shown). No additional specifically modified peptides were found.

Discussion

Studies by Bünning et al. (1990) revealed that pulmonary ACE contains a Dnp-F-reactive, catalytically essential tyrosine residue as well as a second critical residue, lysine, which is also modified by Dnp-F but to a much lesser extent. The present results confirm and extend these findings.

The inactivation of ACE by Dnp-F can be prevented by a competitive inhibitor, phenylphosphoryl-Phe-Phe or Enalaprilat, which implies that Dnp-F acts as an active-site-directed inhibitor. This reagent has been used with many proteins to study a variety of functional amino acid residues involved in catalysis, e.g., a lysine in ribonuclease A (Hirs et al., 1965), a histidine and a cysteine in L-lactate oxidase (Giegel et al., 1987), and N-terminal proline in the 30S subunit of *Escherichia coli* ribosomes (Olah et al., 1988), and a tyrosine and a lysine in D-amino acid oxidase (Swenson et al., 1982). This report demonstrates that lung and testis ACE contain reactive tyrosine and lysine residues at or near their active sites.

A critical tyrosyl residue has been thought to be involved in the catalytic activity of ACE based on the results of modification with several chemical reagents, i.e., *N*-acetylimidazole, tetranitromethane, and Dnp-F (Bünning et al., 1978, 1990). The peptide mapping, double modification, and sequence analyses presented here are all consistent with the finding that a tyrosine residue is essential for ACE activity. Although the detailed function of this critical tyrosine residue has not been specified, it likely plays a role in substrate binding analogous to that of Tyr-248 of bovine carboxypeptidase A (Ondetti & Cushman, 1982; Vallee et al., 1984).

ACE inactivation with Dnp-F also involves a critical lysine residue whose modification is prevented by Enalaprilat. A lysine residue has been suggested previously, both by acetylation with acetic anhydride and by reductive methylation, to be part of the anion activation site. It also is involved in substrate binding, perhaps by mediating the activation effect of chloride (Shapiro & Riordan, 1983). It is not yet known whether the critical lysine residue found in this study is identical with the one modified by reductive methylation.

The complete primary structures of human and mouse lung ACE and human and rabbit testicular ACE are now known (Soubrier et al., 1988; Bernstein et al., 1989; Lattion et al., 1989; Ehlers et al., 1989; Kumar et al., 1989). In addition, partial sequence information (about 150 residues) for rabbit lung ACE is available (Soffer et al., 1987; M. R. W. Ehlers and D. J. Strydom, personal communication) and indicates a sequence identity with the human enzyme of about 90%. Therefore, it should be possible to locate the essential lysine and tyrosine residues in the overall sequence of ACE. The sequence of the N^{ϵ}-Dnp-Lys heptapeptide from rabbit testicular ACE, K(-Dnp)VQDLQR (Figure 3), corresponds to residues 122–128 (Kumar et al., 1989), which is essentially the same as that of residues 118–124 of human testicular ACE (Ehlers et al., 1989; Lattion et al., 1989) and residues 694–700 of human lung ACE (Soubrier et al., 1988) and mouse kidney ACE (Bernstein et al., 1989). The sequence of the *O*-Dnp-Tyr heptapeptide obtained from rabbit testicular ACE, Y(-Dnp)VEFTNK (Figure 3), corresponds to residues 204–210, which is similar to the sequence of residues 200–206 of human testicular ACE and of residues 776–782 of human lung ACE and mouse kidney ACE. Together these results indicate that Dnp-F modifies Tyr-776 and Tyr-200 in the lung and testis forms of ACE, respectively (human ACE numbering), whereas the modified lysine is residue 694 in lung ACE and 118 in testis ACE. It is no doubt significant that the sequences around Tyr-776 and Lys-694 are conserved within the primary structures of ACE from different species.

Peptide A

ε-N-Dnp-Lys-Val-Gln-Asp-Leu-Gln-Arg	(Dnp-R_TACE)
^{122}Lys-Val-Gln-Asp-Leu-Gln-Arg	(R_TACE)
^{118}Lys-Val-Gln-Asp-Leu-Glu-Arg	(H_TACE)
^{694}Lys-Val-Gln-Asp-Leu-Glu-Arg	(H_LACE)
^{694}Lys-Leu-Gln-Asn-Leu-Asp-Arg	(M_KACE)

Peptide B

O-Dnp-Tyr-Val-Glu-Phe-Thr-Asn-Lys	(Dnp-R_TACE)
^{204}Tyr-Val-Gln-Phe-Thr-Asn-Lys	(R_TACE)
^{200}Tyr-Val-Glu-Leu-Ile-Asn-Gln-Ala-Ala-Arg	(H_TACE)
^{776}Tyr-Val-Glu-Leu-Ile-Asn-Gln-Ala-Ala-Arg	(H_LACE)
^{776}Tyr-Val-Glu-Phe-Ser-Asn-Lys	(M_KACE)

FIGURE 3: Sequence of the Dnp-labeled peptides from Figure 1. Comparison of the tryptic peptides of ACE from rabbit testis (R_TACE), human testis (H_TACE), human endothelial cell (H_LACE), and mouse kidney (M_KACE). The positions of the labels were determined from the cDNA-derived amino acid sequences (Kumar et al., 1989; Ehlers et al., 1989; Lattion et al., 1989; Soubrier et al., 1988; Bernstein et al., 1989).

The amino acid sequences of human and mouse lung ACE have also revealed that these larger forms of the protein contain two highly conserved segments (residues 361–365 and 959–963) each with the sequence HEMGH analogous to that found in other zinc metalloproteases (Jongeneel et al., 1989; Vallee & Auld, 1989) where it has been thought to constitute the metal binding site. By analogy to thermolysin, the two His residues in the HEMGH sequence would serve as zinc ligands. A third ligand, most often Glu, is typically found in zinc proteases, but this can be separated from the first two by from ~20 to ~120 residues (Vallee & Auld, 1989). Hence, it is not possible to predict the location of this third ligand. Previous studies had indicated that only a single zinc atom is bound per molecule of ACE (Das & Soffer, 1975; Bünning et al., 1983; Bünning & Riordan, 1985). Hence, it would seem that just one of the two HEMGH sites in lung ACE actually binds zinc and is catalytically active. In this regard, lung ACE has been found to be completely inhibited on binding a single molecule of inhibitor (Bull et al., 1985; Strittmatter & Synder, 1986) and, in fact, to bind only one inhibitor molecule as judged by equilibrium dialysis (Shapiro & Riordan, 1984; Cumin et al., 1989). It is most intriguing, therefore, that testis ACE has just a single domain with the same sequence as the carboxy-terminal domain of its larger counterpart and contains only one HEMGH site (Figure 4) (Ehlers et al., 1989; Lattion et al., 1989; Kumar et al., 1989). The lung and testis isozymes are catalytically very similar (Soffer et al., 1987), and chemical modification of the latter form has indicated the presence of the same "essential" residues as in lung ACE (J. W. Harper, R. Shapiro, and J. F. Riordan, unpublished results). Thus, the putative active site located

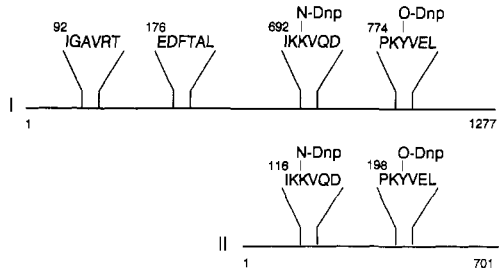

FIGURE 4: Schematic sequences of the peptides containing Dnp-modified lysine and tyrosine residues of human endothelial ACE (I) and human testis ACE (II). The positions of the labels were determined by sequence analysis of rabbit lung and testicular ACE and based on the cDNA-derived amino acid sequences of rabbit testicular ACE (Kumar et al., 1989) and the homologous human enzymes (Soubrier et al., 1988; Ehlers et al., 1989; Lattion et al., 1989). Numbering in each case corresponds to the sequence of the mature protein. Residues 94 and 178 in endothelial ACE correspond to residues 694 and 776 the Lys and Tyr modified by Dnp-F, respectively. The corresponding residues in testicular ACE are 118 and 200, respectively.

in the carboxy-terminal half of lung ACE and corresponding to the testicular one would seem to be catalytically functional.

It should be emphasized that the metal content of ACE noted above has been reported only for the rabbit pulmonary form of the enzyme and that the stoichiometry obtained was based on an apparent molecular weight of ~130000 (Das & Soffer, 1975; Bünning et al., 1983; Bünning & Riordan, 1985). Human lung ACE contains 1277 amino acids (Soubrier et al., 1988). If rabbit lung ACE also has essentially the same number of amino acids, and 26% carbohydrate (Das & Soffer, 1975), its molecular weight should be approximately 185000. If, in addition, it has an amino acid composition similar to that of the human lung enzyme, its reported molar absorptivity, 204000 M^{-1} cm^{-1} (Bünning et al., 1983), should be revised to 290000 M^{-1} cm^{-1} to correspond with the molecular weight of 185000 [see Bünning et al. (1990)]. As a consequence, its metal content would be increased to at least 1.3 mol/mol and perhaps as much as 1.8 mol/mol. Given that zinc binding to rabbit lung ACE is relatively weak compared with other zinc metalloenzymes (Kleemann et al., 1986), the possibility of two metal binding sites per molecule is not unreasonable. Additional metal analyses are required to verify this suggestion. Whether both sites are catalytically active remains to be established as well.

If there are two active sites in lung ACE, they likely do not have equal catalytic efficiency. Loss of activity on dinitrophenylation correlates with modification of just over one residue per molecule. Moreover, the present evidence indicates that the two "critical" residues modified by Dnp-F are located only in the second half and not in the first half of lung ACE. This is in contrast to the putative Zn binding sites which are present in both halves. It is interesting to note, in fact, that the amino-terminal domain of human lung ACE, despite substantial sequence identity to its carboxyl-terminal domain, lacks the tyrosine and lysine residues corresponding to Tyr-776 and Lys-694. Instead, residue 178 is Phe rather than Tyr, and residue 94 is Ala rather than Lys (Figure 4).

Significantly, Phe-178 is in one of the least conserved regions of the amino-terminal domain of lung ACE. There is an overall 68% sequence identity between residues 220–582 of the amino-terminal domain and residues 818–1180 of the carboxyl-terminal domain. However, there are segments where the percent identity is even greater: between residues 220–270 and 818–868, it is 80%, between residues 300–349 and 898–947, it is 86%, and between residues 360–390 and 958–988, it is 87%. In contrast, the percent identity between residues 164–189, which encompass Phe-178, and the corresponding residues 762–787, which encompass Tyr-776, is only 19%. Similarly, Lys-694 is in a region (residues 693–710) where just 5 of 18 residues, 28%, are identical with the corresponding region around Ala-94. Curiously, there are two other short regions, one immediately preceding and the other somewhat after the putative metal binding sites, where the degree of sequence identity drops to about 20%. This may also signal that one of these two sites is nonfunctional.

The absence of essential tyrosine and lysine residues in the first half of the ACE molecule may not be merely adventitious; it would provide an explanation for any inactivity or diminished activity of the putative active site located in the first half-structure. It implies that tyrosine and lysine play important roles in catalysis, i.e., through interactions either with the substrate directly or with other amino acids, helping to create the proper active-site conformation. The substitution of Phe and Ala (or Ser in mouse ACE) could preclude proper interaction with substrate. Further studies including site-directed mutagenesis, now in progress, are needed in order to establish possible structure–function relationships.

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Registry No. ACE, 9015-82-1; Tyr, 60-18-4; Lys, 56-87-1.

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Phosphonate Analogue Substrates for Enolase†

Vernon E. Anderson‡ and W. W. Cleland*
Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53705
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ABSTRACT: Phosphonate analogues in which the bridge between C-2 and phosphorus is a CH_2 group are slow substrates for yeast enolase. The pH variation of the kinetic parameters for the methylene analogue of 2-phosphoglycerate suggests that the substrate binds as a dianion and that Mg^{2+} can bind subsequently only if a metal ligand and the catalytic base are unprotonated. Primary deuterium isotope effects of 4–8 on V/K_{Mg}, but ones of only 1.15–1.32 on V for dehydration, show that proton removal to give the carbanion intermediate largely limits V/K_{Mg} and that a slow step follows which largely limits V (presumably carbanion breakdown). Since there is a D_2O solvent isotope effect on V for the reverse reaction of 5, but not an appreciable one on the forward reaction, it appears that the slow rates with phosphonate analogues result from the fact that the carbanion intermediate is more stable than that formed from the normal substrates, and its reaction in both directions limits V. Increased stability as a result of replacement of oxygen by carbon at C-2 of the carbanion is the expected chemical behavior.

When one is trying to determine the identity of enzymic acid–base catalysts by study of pH profiles, one obtains equivocal data if the substrates are "sticky", that is, if substrate release from the enzyme is slower than the rate of reaction and release of the first product. Consequently, it is desirable to use slow alternate substrates where the rate of the chemical reaction has become slower than both product and substrate dissociation rates. These conditions will normally hold if the maximum velocity is considerably smaller and the Michaelis constant is larger for the alternate substrate than for the normal one. This approach was fruitful in the study of serine as an alternate substrate for alanine dehydrogenase (Grimshaw et al., 1981).

Stubbe and Kenyon reported that MePEP[1] (the methylene-bridged analogue of phosphoenolpyruvate) was a slow alternate substrate for enolase. The maximum velocity was 1.6% of that with phosphoenolpyruvate, and K_{MePEP} was larger than K_{PEP}. We report in this paper the isolation of MePGA (the methylene analogue of 2-phosphoglycerate, and the product of the hydration of MePEP), the pH variation of the kinetic parameters, and the primary deuterium and D_2O solvent isotope effects with the methylene analogue substrates. We conclude that these phosphonate analogues are slow substrates because the intermediate carbanion is too stable, so that its decomposition is rate limiting in both directions.

Materials and Methods

Materials. Yeast enolase was obtained from Sigma as the lyophilized powder. MePEP was synthesized by the method of Stubbe and Kenyon (1972). The free acid, crystallized after removal of the 48% HBr hydrolysis solvent, was purified by dissolving 400 mg in 20 mL of water, titrating to pH 9.0 with KOH, loading this solution on a 2.5 × 25 cm column of Do-

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‡ Present address: Department of Chemistry, Brown University, Providence, RI 02912.

[1] Abbreviations: MePEP, the methylene-bridged analogue of phosphoenolpyruvate; MePGA, the methylene-bridged analogue of 2-phosphoglycerate.

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