Vol. 184, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1992 Pages 306-309

The Functional Role of Tyrosine-200 in Human Testis Angiotensin-Converting Enzyme

Ying-Nan P. Chen, Mario R. W. Ehlers, and James F. Riordan*

Center for Biochemical and Biophysical Sciences and Medicine,
Harvard Medical School, 250 Longwood Avenue, Boston, MA 02115

Received March 2, 1992

SUMMARY: The active site of angiotensin-converting enzyme (ACE) has been shown by chemical modification to contain a critical tyrosine residue, identified as Tyr-200 in human testis ACE (hTACE). We have expressed a mutant hTACE containing a Tyr-200 to Phe mutation. The mutant exhibits a marked decrease in k_{cat}: 15-fold and 7-fold for the hydrolysis of furanacryloyl-Phe-Gly-Gly and angiotensin I, respectively, whereas its K_m increases by only 1.6- and 2.2-fold, respectively. We conclude that Tyr-200 is not required for substrate binding. Instead, the effect on k_{cat} together with a 100-fold decrease in affinity for the ACE inhibitor lisinopril indicates that Tyr-200 may participate in catalysis by stabilizing the transition state complex. Thus, Tyr-200 in hTACE has a role analogous to that of Tyr-198 in carboxypeptidase A.

Angiotensin-converting enzyme (ACE; EC 3.4.15.1) is a membrane-bound metallopeptidase that plays a critical role in blood pressure regulation by converting the inactive peptide angiotensin I to the vasoactive peptide angiotensin II (1). The enzyme contains essential tyrosine, arginine, lysine, and glutamic acid residues, as determined by chemical modification studies (2-5). There are two isozymes: a 160-170kDa somatic enzyme found in most mammalian tissues, and a 100-110kDa testis enzyme found only in developing sperm (1). Somatic ACE consists of two homologous, tandem domains each of which contains a putative metal-binding site (6,7), whereas testis ACE consists of only a single domain that corresponds to the C-terminal half of the somatic enzyme (8-10).

We have demonstrated that 1-fluoro-2,4-dinitrobenzene inactivates somatic ACE by selectively modifying a single tyrosine located in the C-terminal domain (5), the domain that is identical to testis ACE and that likely contains the principal angiotensin-converting site common to both isozymes (11). To define further the role of this tyrosine, we have constructed, by site-directed mutagenesis, a mutant human testis ACE (hTACE) in which the reactive tyrosine (Tyr-

*To whom correspondence should be addressed.

Abbreviations: ACE, angiotensin-converting enzyme; hTACE, recombinant wild-type human testis ACE; CHO, Chinese hamster ovary; fcs, fetal calf serum.

0006-291X/92 $1.50

200) is replaced by phenylalanine. The results demonstrate that Tyr-200 is primarily a catalytic residue and does not participate in substrate binding.

MATERIALS AND METHODS

Construction of expression vector. The plasmid pLEN-ACE-Y200F, which encodes human testis ACE in which Tyr-200 is replaced by Phe (hereafter referred to as the Y200F mutant), is based on pLEN-ACE6/5 (12,13) and was constructed by site-directed mutagenesis using the polymerase chain reaction according to standard protocols (14). Oligonucleotide primers spanning the region of interest were synthesized such that the codon TAC (nucleotides 713-715) that encodes Tyr-200 [numbering of nucleotides and residues as described for the full-length human testis ACE cDNA (8)] was changed to TTC encoding a Phe. After amplification the recombinant DNA was inserted into pLEN-ACE6/5 using suitable restriction sites, to give pLEN-ACE-Y200F. In common with pLEN-ACE6/5, pLEN-ACEΔ36N also lacks the 3′ end of the testis ACE cDNA that encodes the transmembrane and cytoplasmic domains. This results in direct secretion of the recombinant protein but does not alter its activity or stability (12,13). In the ensuing discussion, "wild-type" hTACE will refer to the anchor-minus recombinant protein encoded by pLEN-ACE6/5.

Expression of pLEN-ACEΔ36N. The mutant protein was expressed in Chinese hamster ovary (CHO) cells stably transfected with pLEN-ACE-Y200F and purified from the conditioned media by methods described previously (13), except for the following modifications. First, to eliminate the danger of copurifying contaminant bovine ACE present in the fetal calf serum (fcs) that is a constituent of the CHO cell media, the fcs was subjected to standard affinity chromatography (8) to extract the bovine ACE. Further, the fcs eluted from the affinity column was additionally treated with 1 mM chlorambucil (Sigma) at pH 7.0 for 1 h at room temp., conditions that have been shown to irreversibly inhibit bovine ACE (15). Following this treatment, no ACE activity was detectable in the fcs, and the growth of the CHO cells was not impaired. Second, since the Y200F mutant bound weakly to the affinity resin during purification, the steps of sample application, washing, and elution were performed rapidly (1-2 h versus ~24 h that is usual for native ACE) to optimize recovery.

RESULTS AND DISCUSSION

Expression of hTACE-Y200F in CHO cells. As expected for an active-site mutant, culture media of CHO cells stably transfected with pLEN-ACE-Y200F contained <5% of the ACE activity typically present in the media of cells expressing wild-type hTACE. Moreover, the mutant protein was recovered in low yield following standard affinity chromatography: 5% vs. 85% for wild-type hTACE (13). Only by rapidly washing and eluting the affinity column after application of conditioned media could the Y200F mutant be obtained in ~30% yield.

Physicochemical properties of hTACE-Y200F. By SDS-polyacrylamide gel electrophoresis the Y200F mutant migrates with the same M_r as wild-type hTACE (~100 kDa), and on Western blotting both proteins are similarly immunoreactive to a polyclonal anti-kidney ACE antiserum (not shown; see ref. 13). The amino acid composition of Y200F is essentially identical to that of wild-type hTACE (13); in a 650-residue protein with 26 tyrosines and 26 phenylalanines the conversion of one Tyr to a Phe cannot be distinguished. N-terminal sequence analysis revealed that the Y200F mutant has a blocked N terminus, as do wild-type hTACE and native human testis ACE (8,13). Fluorescence spectra of Y200F and wild-type hTACE are virtually

Table 1. Kinetic parameters for wild-type and Y200F recombinant testis ACE

	Fa-FGG hydrolysis		AI hydrolysis		Chloride	Lisinopril
	K_m	k_{cat}	K_m	k_{cat}	activation K_a'	inhibition K_i'
Enzyme	(x 10^{-4} M)	(min^{-1})	(x 10^{-5} M)	(min^{-1})	(M)	(x 10^{-10} M)
hTACE	2.9	22,000	3.6	2,050	0.25	1.9
Y200F	4.5	1,500	7.9	300	0.42	200

Determined as described previously (Ehlers et al., 1991b); K_i values were measured at [E] = 3 x 10^{-10} M and 1-3 x 10^{-9} M for hTACE and hTACE-Y200F, respectively. Fa-FGG, 2-furanacryloyl-L-Phe-Gly-Gly; AI, angiotensin I.

superimposable with maxima at 338 nm (not shown), indicating that the polypeptide conformation around the 20 tryptophans in the mutant protein has not changed significantly with respect to the wild-type protein. Taken together, these data indicate that in hTACE-Y200F the introduced mutation has not produced a gross physicochemical change in the protein.

Catalytic properties. hTACE-Y200F shows interesting differences from the wild-type enzyme with respect to several kinetic criteria (Table 1). Most notable is a marked decrease in the k_{cat} for substrate hydrolysis: 15-fold for the N-blocked tripeptide furanacryloyl-Phe-Gly-Gly, and 7-fold for the decapeptide angiotensin I. In contrast, the K_ms for these substrates are only slightly elevated by 1.6- and 2.2-fold, respectively. The effect on chloride activation is also small: the K_a' for the Y200F mutant is 1.7-fold greater than that for hTACE. A much larger change was observed for inhibitor binding: the Y200F mutant binds lisinopril 100-fold less tightly than wild-type hTACE [Table 1; this change may be even greater since lisinopril binds the wild-type enzyme so tightly that the true K_i is difficult to determine (13)]. This fall in inhibitor-binding affinity accounts for the poor binding of hTACE-Y200F to the lisinopril affinity resin during purification.

These data establish that Tyr-200 is an active site residue, as was indicated previously by chemical modification (5). The Y200F mutation selectively affects k_{cat} values for substrate hydrolysis with only minor changes in K_m. Thus, Tyr-200 is not significantly involved in substrate binding. This inference is corroborated by the minor effect of the Tyr-200-to-Phe substitution on chloride activation. Chloride activates ACE primarily through enhanced substrate binding and not by increasing the catalytic rate (16). Moreover, chloride binding is thought to be mediated by a critical lysine residue (3).

Whereas the Y200F mutation profoundly affects k_{cat}, it does not abolish enzymatic activity and thus the Tyr-200 hydroxyl is apparently not crucial in catalysis. This rules out a role for the phenolic hydroxyl in, for instance, general acid catalysis. Instead, the Tyr-200 hydroxyl may increase the k_{cat} by assisting in the stabilization of the transition state complex. Based on the changes in k_{cat}/K_m values, its contribution to binding energy in the rate-

determining transition state is 1.6 and 1.9 kcal/mol for angiotensin I and Fa-FGG, respectively. Further evidence for this is provided by the drastic (100-fold) decrease in binding affinity for the competitive inhibitor lisinopril, where the hydroxyl group contributes 2.8 kcal/mol to binding energy. Lisinopril and related slow- and tight-binding ACE inhibitors are considered to act, at least in part, as transition state analogs (17). In this regard Tyr-200 in testis ACE appears to be analogous to Tyr-198 in carboxypeptidase A, substitution of which similarly selectively affects k_{cat}, and has also been interpreted to be involved in transition state stabilization (18). In addition to Tyr-198, carboxypeptidase A contains a second active site tyrosine, Tyr-248, which plays a significant role in substrate binding. As yet, such a substrate-binding tyrosine has not been identified in ACE.

ACKNOWLEDGMENTS. We are indebted to Dr. Daniel J. Strydom, Wynford Brome, and Rebecca Ettling for the amino acid and sequence analyses.

REFERENCES

1. Ehlers, M. R. W., and Riordan, J. F. (1989) Biochemistry 28, 5311-5318.
2. Bünning, P., Holmquist, B., and Riordan, J. F. (1978) Biochem. Biophys. Res. Commun. 83, 1442-1449.
3. Shapiro, R., and Riordan, J. F. (1983) Biochemistry 22, 5315-5321.
4. Bünning, P., Kleemann, S. G., and Riordan, J. F. (1990) Biochemistry 29, 10488-10492.
5. Chen, Y.-N. P., and Riordan, J. F. (1990) Biochemistry 29, 10493-10498.
6. Soubrier, F., Alhenc-Gelas, F., Hubert, C., Allegrini, J., John, M., Tregear, G., and Corvol, P. (1988) Proc. Natl. Acad. Sci. USA 85, 9386-9390.
7. Bernstein, K. E., Martin, B. M., Edwards, A. S., and Bernstein, E. A. (1989) J. Biol. Chem. 264, 11945-11951.
8. Ehlers, M. R. W., Fox, E. A., Strydom, D. J., and Riordan, J. F. (1989) Proc. Natl. Acad. Sci. USA 86, 7741-7745.
9. Kumar, R. S., Kusari, J., Roy, S. N., Soffer, R. L., and Sen, G. C. (1989) J. Biol. Chem. 264, 16754-16758.
10. Lattion, A.-L., Soubrier, F., Allegrini, J., Hubert, C., Corvol, P., and Alhenc-Gelas, F. (1989) FEBS Lett. 252, 99-104.
11. Ehlers, M. R. W., and Riordan, J. F. (1991) Biochemistry 30, 7118-7126.
12. Ehlers, M. R. W., Chen, Y.-N. P., and Riordan, J. F. (1991) Proc. Natl. Acad. Sci. USA 88, 1009-1013.
13. Ehlers, M. R. W., Chen, Y.-N. P., and Riordan, J. F. (1991) Prot. Express. Purif. 2, 1-9.
14. Higuchi, R. (1989) In PCR Technology. Principles and Applications for DNA Amplification (H. A. Erlich, Ed.) pp. 61-70, Stockton Press, New York.
15. Harris, R. B., and Wilson, I. B. (1982) J. Biol. Chem. 257, 811-815.
16. Shapiro, R., and Holmquist, B., and Riordan, J. F. (1983) Biochemistry 22, 3850-3857.
17. Patchett, A. A., and Cordes, E. H. (1985) Adv. Enzymol. 57, 1-84.
18. Gardell, S. J., Hilvert, D., Barnett, J., Kaiser, E. T., and Rutter, W. J. (1987) J. Biol. Chem. 262, 576-582.