Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 310-316

A DEEPLY RECESSED ACTIVE SITE IN ANGIOTENSIN-CONVERTING ENZYME IS INDICATED FROM THE BINDING CHARACTERISTICS OF BIOTIN-SPACER-INHIBITOR REAGENTS

Kenneth E. Bernstein, Stephen L. Welsh and John K. Inman*

Department of Pathology, Emory University, Atlanta, GA 30322

*Bioorganic Chemistry Section, Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, MD 20892

Received December 12, 1989

SUMMARY - Two biotinylated derivatives of the angiotensin-converting enzyme (ACE) inhibitor, lisinopril, were synthesized. Compounds BL11 (ε-biotinamidocaproyl-lisinopril) and BL19 (ε-biotinamidocaproyl-β-alanyl-β-alanyl-lisinopril) have, respectively, 11 and 19 atoms of spacing structure between the biotin and the inhibitor moieties. Both compounds were found to be potent inhibitors of mouse kidney ACE, but they lost this ability in the presence of streptavidin in free solution. However, BL19 (but not BL11), when complexed to ACE, retained enough residual binding strength for streptavidin to allow the complex to be specifically removed from solution by streptavidin-agarose beads. It was thus possible to employ BL19 for the affinity isolation of ACE from crude mixtures. These results indicate that the bound determinant of lisinopril must lie at least 11 Å below the outer surface of the ACE molecule.

Angiotensin-converting enzyme (ACE) is a major factor determining systemic blood pressure through its action to release the potent vasoconstrictor, angiotensin II (1,2,3). The physiologic role of ACE is demonstrated by the clinical utility of converting enzyme inhibitors, now widely used to treat systemic hypertension, heart failure and the nephrotoxic effects of diabetes (4,5). Investigation of substrate specificity has shown that ACE is a rather nonspecific peptidase capable of degrading many small peptides including the vasodilator, bradykinin (6,7). ACE has been identified on the luminal surfaces of vascular epithelium and in areas of the gut, brain and testes (3).

Until the recent cloning of cDNA encoding ACE, little was known about its structural biochemistry. What is suggested from these results is that the ACE active site may lie within a deep cleft that precludes entry to all but small peptides (8). To test this idea, we constructed two biotinylated derivatives of the ACE competitive inhibitor, lisinopril. These compounds differ in the length of the spacing structure separating a biotinyl group (Fig. 1, part E) from the binding determinant portion of the lisinopril molecule (Fig. 1, part A); they are designated BL11 and BL19 and possess 11- and 19-atom spacer arms, respectively, between these two ligands. From a series of binding and inhibition experiments described in this report, it was determined that simultaneous binding of these heterobifunctional molecules to streptavidin and

0006-291X/90 $1.50

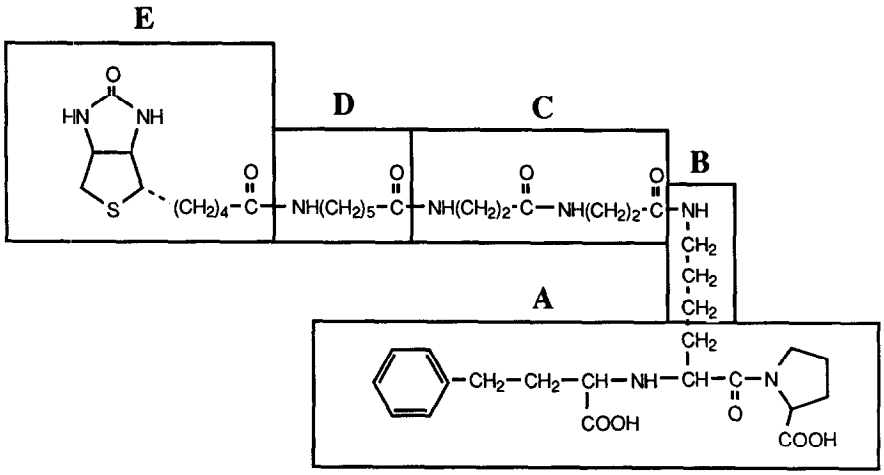


Fig. 1. Structures of the bifunctional probes. Compound BL11 (ε-biotinamidocaproyl-lisinopril) is comprised of parts A,B,D and E with the 11-atom spacer arm, BD. Compound BL19 (ε-biotinamidocaproyl-β-alanyl-β-alanyl-lisinopril) consists of all parts shown, ABCDE, and has the 19-atom spacer arm, BCD. The biotinyl group is E. Lisinopril is AB.

ACE occurred only in the case of BL19 and where streptavidin was bound to an agarose matrix. The length of spacing in this molecule (minus the depth of the avidin site) supports the notion that the lisinopril determinant is bound very deeply within the interior of the ACE molecule at a depth probably exceeding 11 Å below the outer surface contours. A molecule such as BL19, or one with a longer spacer arm, could serve as a versatile affinity probe for the separation of ACE or the visualization of its binding sites in a variety of experimental systems.

MATERIALS AND METHODS

Linsinopril was kindly provided by Dr. Herbert G. Bull, Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey. Diethylaminoethyl (DE-52) cellulose adsorbent was obtained from Whatman. ε-Caproylamidobiotin-N-hydroxysuccinimide ester (CAB-NHS) and streptavidin-agarose were purchased from Bethesda Research Laboratories, Bethesda, MD. Boc-β-alanyl-β-alanine was prepared by one of us (JKI) using conventional, solution-phase peptide synthesis methods. Disuccinimidyl carbonate (DSC) was obtained from Polysciences, Inc., Warrington, PA. Thin layer chromatographic (TLC) glass plates coated with silica gel containing fluorescent indicator were distributed by EM Laboratories, Inc. (made by E. Merck, Darmstadt): Preparative layers were 20 x 20 cm x 0.5 mm; analytical layers were 5 x 10 cm x 0.25 mm. Unless otherwise indicated, all other materials were purchased from Sigma Chemical Co. or were standard reagent grade chemicals and solvents.

Compound BL11 was prepared by reacting lisinopril with CAB-NHS (9) in absolute methanol (0.025 M each) overnight at room temperature. Boc-βAla-βAla-lisinopril (BL19 intermediate) was synthesized by coupling Boc-β-alanyl-β-alanine to the primary amino group of lisinopril by means of its N-hydroxysuccinimide ester, prepared with the reagent, DSC, according to published procedure (10). The coupling reaction was carried out overnight in methanol after the mixture was neutralized with small amounts of triethylamine. Methanol was removed, and the product was extracted into 1-butanol over 0.1 M $KHSO_4$. Compound BL19 was prepared from the above intermediate by removing the Boc protecting group with trifluoroacetic acid (Pierce Chemical Co., Sequanal grade), removing the TFA, dissolving the residue in methanol, alkalinizing the solution with diethylethanolamine (Aldrich), and adding an

equimolar amount of CAB-NHS. The reaction was allowed to proceed overnight at room temperature. BL11, BL19 intermediate, and BL19 were each purified by preparative thin layer chromatography on silica layers using a mixture of 1-butanol, acetic acid, water (4:1:1 by volume) as the developing solvent. Product zones were removed and extracted with methanol. Chromatography was repeated if necessary until materials were essentially pure as assessed by analytical TLC. Methanol was removed, and yields were determined as dry weights. Stock solutions of BL11 and BL19 were prepared in methanol. BL11 and BL19 possessed both ligand activities.

The mouse kidney ACE used to establish the binding characteristics of BL11 and BL19 was purified as previously described (11). Inhibition assays were performed as follows: 5 µl of inhibitor in buffer A (50 mM Hepes, 0.3 M NaCl, 0.4 M Na_2SO_4, pH 8.15) were added to 50 µl of buffered substrate solution (30 mM hippuryl-glycylglycine in buffer A) (12). This solution was warmed to 37°, and 25 ng of purified ACE in 5 µl of buffer A were added with rapid mixing. The reaction was carried out for 30 min at 37° and then terminated by the addition of H_2SO_4. Enzyme activity was quantified by the reaction of liberated glycylglycine with 2,4,6-trinitrobenzenesulfonic acid (12). A control assay contained no inhibitor. Enzyme units were calculated as described by Neels (12).

Two protocols were used to measure the effects of complexing BL11 or BL19 with soluble streptavidin. In one protocol, inhibitor, at various concentrations, was incubated with 25 ng of ACE for 10 min at 37° in buffer A. Streptavidin was then added in excess to a final concentration of 5 µM, and the mixture was assayed for ACE activity. In the second protocol, inhibitor was combined with 7.5 µM streptavidin for 30 min at 37°. ACE (25 ng) was added, and enzyme activity was determined as described above.

Affinity purification of mouse kidney ACE was attempted with BL11 and BL19. Mouse kidney was homogenized in the presence of Triton X-100 and then bound to and eluted from a DEAE-cellulose (DE-52) column as previously described (11). The desired fractions were pooled and dialyzed against Buffer B (20 mM Mes, 0.5 M NaCl, 0.1 mM $ZnCl_2$, 0.1 % Triton X-100, pH 6.0). The dialyzate was then divided into 3 equal portions. One portion was made 1.0 µM in BL11. A second portion was made 1.0 µM in BL19. These solutions were incubated 15 min at room temperature. Streptavidin-agarose beads were washed in Buffer B and added to the dialyzate-inhibitor mixture (0.5 ml of beads to 19 ml of dialyzate). This suspension was incubated with gentle mixing overnight at 4°. The beads were poured into mini-columns and washed with Buffer B, then with Buffer B without detergent until the absorbance at 280 nm fell below 0.01. Enzyme was eluted with 50 mM sodium borate, pH 8.9. A third portion of the dialyzate was incubated overnight with 0.5 ml of a previously described ACE-specific affinity adsorbent consisting of lisinopril covalently bound to Affi-Gel 15 (Bio-Rad Laboratories) (11). This material was treated identically to that of the streptavidin-agarose. Eluate fractions were pooled and concentrated (Minicon-B15, Amicon). Proteins were analyzed using 8 % SDS-polyacrylamide gels as described by Laemmli (13). Silver staining (Bio-Rad) of the SDS gels was done by the method of Merril et al. (14).

RESULTS

The inhibition of ACE by BL11 and BL19 is shown by standard inhibitory curves, constructed for these two, new compounds and the parent compound, lisinopril (Fig. 2). The IC_{50} of BL19 is 8.8 nM, 6 times the measured IC_{50} of lisinopril (1.5 nM). The IC_{50} of BL11 is 3.1 nM, intermediate between that of BL19 and lisinopril. Thus, in spite of binding less strongly than lisinopril, BL11 and BL19 remain very good inhibitors of ACE.

To determine if BL11 and BL19 retained the ability to bind streptavidin, 1×10^{-5} M lisinopril, BL11, or BL19 was first absorbed with increasing amounts of streptavidin-agarose for 60 min at room temperature. Supernatant fractions were assessed for residual inhibitor as

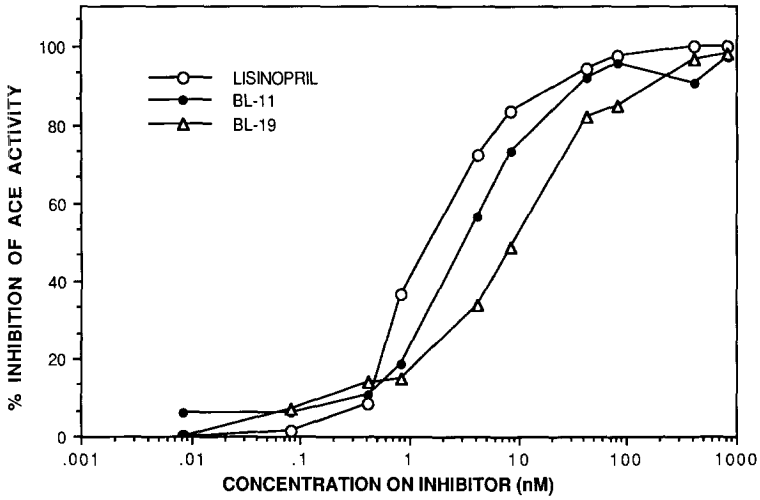
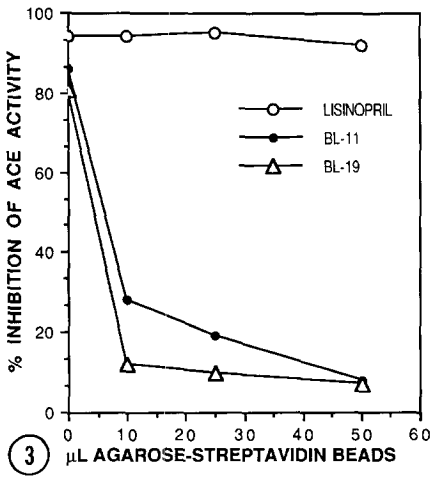


Fig. 2. Inhibition of ACE by lisinopril, BL11, and BL19. The method of Neels (12) was used to measure the activity of ACE in the presence of various concentrations of each inhibitor (see text).

described above. As shown in Fig. 3, streptavidin-agarose is able to bind BL11 and BL19 and unable to bind lisinopril as expected. An experiment in which BL11 was exhaustively absorbed with streptavidin-agarose demonstrated that approximately 1 % of the inhibitory capacity of this preparation is due to nonabsorbable material, presumably nonbiotinylated lisinopril.



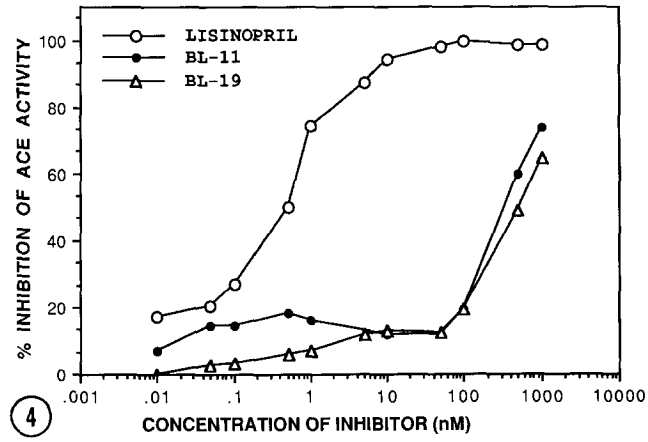


Fig. 3. Binding of lisinopril, BL11, and BL19 to streptavidin-agarose. Increasing quantities of streptavidin-agarose were incubated with each inhibitor for 60 min at room temperature, and the supernatants were assayed for the inhibition of ACE.

Fig. 4. Effect of soluble streptavidin on the inhibition of ACE by lisinopril, BL11, and BL19. Increasing concentrations of inhibitor were incubated with purified ACE for 10 min at 37°. Excess streptavidin was then added, and ACE activity was determined. Experiments in which inhibitor was first bound to streptavidin before addition to ACE gave identical results.

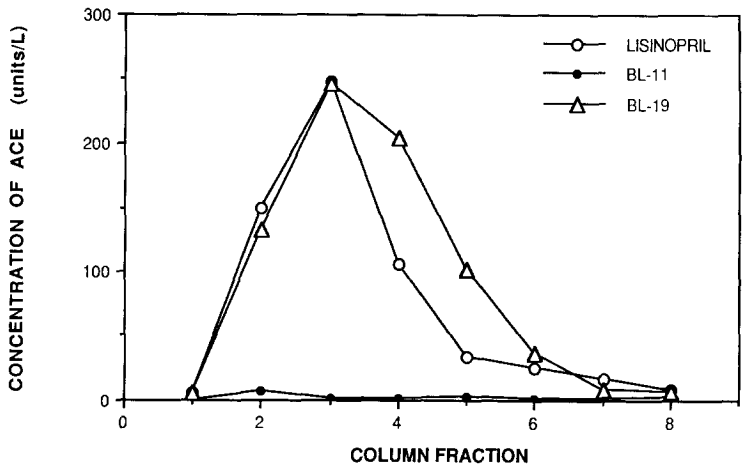


Fig. 5. Affinity isolation of ACE using BL11, BL19, and Affi-Gel 15-lisinopril. A detergent extract of mouse kidney was preliminarily purified as described in Materials and Methods. Portions of this crude material were treated with BL11 or BL19 and subsequently with streptavidin-agarose beads. Another portion was treated directly with Affi-Gel 15-lisinopril. All three loaded adsorbents were washed (Mes buffer, pH 6) and eluted with 50 mM sodium borate, pH 8.9; ACE activity was measured on eluates.

We next examined whether BL11 and BL19 could inhibit ACE in the presence of an excess of soluble streptavidin. In one protocol, inhibitor was bound to enzyme before the addition of streptavidin; in a second protocol, inhibitor was first bound to streptavidin. Both experiments used streptavidin in excess of that calculated as necessary to bind all biotinyl groups. Both protocols gave identical results. Comparison of the results shown in Fig. 4 with those of Fig. 2 reveals that lisinopril was nct much affected by the presence of soluble streptavidin, whereas the inhibitory activities of BL11 and BL19 were essentially abolished. The rapid rise in percent inhibition by these two preparations at approximately two orders of magnitude higher concentrations can be ascribed to the presence of roughly 1 % nonbiotinylated species (for BL11 and presumably for BL19, also).

We investigated whether BL11 or BL19 is capable of binding ACE in a manner sufficiently stable for the inhibitor-enzyme complex to be purified with streptavidin-agarose. To a crude preparation of mouse kidney ACE, we added BL11 or BL19 and then streptavidin-agarose beads. After an overnight incubation, the beads were washed, and protein was eluted at pH 8.9 with a low salt buffer. Another portion of the crude preparation was treated overnight directly with lisinopril that had been bound to the spacer arms of Affi-Gel 15 (11). This gel was washed, and protein was eluted in identical fashion. Fig. 5 shows the ACE activity of the elution fractions from the affinity matrices. In the case of BL11, no elution of enzyme activity was observed, suggesting no retention had occurred. However, BL19-streptavidin-agarose was equally as effective as the more conventional approach with the lisinopril-bound adsorbent in retaining and yielding enzyme activity upon elution. Eluted material from the two methods showed single bands in 8 % SDS-PAGE electrophoresis gels after silver staining. Both bands indicated the same molecular size (about 170 kDa).

DISCUSSION

Recent analyses of human and mouse cDNA showed that ACE is composed of a single polypeptide chain possessing two homologous domains (15,16). Each domain has amino acid sequence suggesting a putative catalytic site. Despite this fact, the ACE molecule is thought to accomodate only one Zn molecule and to have one catalytically active site. Pantoliano et al. (8) investigated a series of ACE-specific affinity adsorbents in which the ligands were attached to an agarose matrix with spacers of 10, 14, 22 and 28 Å length. Only those adsorbents with 28-Å spacer arms showed a high capacity to bind ACE. They concluded that ACE might be similar to alcohol dehydrogenase in containing a Zn atom within a deeply recessed active site.

In the present study, the nature of the ACE binding site was examined using soluble bifunctional reagents consisting of biotin linked with spacer arms to the ACE ligand, lisinopril. Two compounds, BL11 (Fig. 1, ABDE) with a 11-atom spacer between the biotin carboxyl carbonyl and the lisinopril determinant and BL19 (Fig. 1,ABCDE) with a 19-atom spacer, were synthesized in order to explore the minimally required spacing needed for simultaneous binding of streptavidin (or avidin) and mouse kidney ACE. BL11 and BL19 were strong inhibitors of proteolysis of hippuryl-glycylglycine substrate by ACE but had 2- and 6-fold larger IC_{50} values, respectively, than lisinopril itself (Fig. 2). This diminution of affinity could be ascribed to conformational and dynamical constraints that the additional structure places upon the lisinopril moiety. The binding of soluble streptavidin to inhibitor or inhibitor-ACE complexes virtually abolished inhibitor activity, signifying that the 19-atom (20 Å) spacing is inadequate for preventing steric hindrance by bound streptavidin to binding of the inhibitor ligand in the ACE site. Avidin was shown by Green et al. (17) to have a deeply recessed site where the bound biotin carboxyl may be as much as 9 Å beneath the outer surface of the molecule. The BL19 spacer arm (Fig. 1, BCD) has an extended length of 20 Å, thus indicating that the lisinopril determinant (Fig. 1, A) lies at least 11 Å below the surface of ACE. These studies show, however, that agarose-based adsorbents such as Affi-Gel l5-lisinopril, with 15 atoms of spacing plus the 4-atom spacer arm of lisinopril (11), and the agarose-streptavidin-BL19 (but not BL11) complex both allow enough binding to ACE to permit effective affinity purification from a preparation of crude material (Fig. 5).

In conclusion, a probe such as BL11 provides too little spacing to allow any significant, simultaneous binding of lisinopril and streptavidin. BL19 permits sufficient bifunctional binding for its use as an affinity purification linker; however, in systems where full binding affinity to both streptavidin and ACE is required, a spacer arm longer than 19 atoms (20 Å extended length) would be needed, consistent with the findings of Pantoliano et al. (8).

REFERENCES

1. Erdos, E. G. (1975) Circ. Res. 36, 247-255.
2. Skeggs, L. T., Dorer, F. E., Kahn, J. R., Lentz, K. E., and Levine, M. (1976) Am. J. Med. 60, 737-747.

3. Patchett, A. A., and Cordes, E. H. (1985) In Advances in Enzymology (A. Meister, Ed.), Vol. 57, pp. 1-84. John Wiley, New York.
4. Hollenberg, N. K. (1985) Am. J. Med. 79, Suppl. 3c, 1-2.
5. Anderson, S. (1987) In Pharmacotherapy of Renal Disease and Hypertension (W. M. Bennet, D. A. McCarron, B. M. Brenner and J. H. Stein, Eds.), pp. 277-297. Churchill Livingstone, New York.
6. Erdos, E. G., and Sloane, E. M. (1962) Biochem. Pharmacol. 11, 585-592.
7. Yang, H. Y. T., Erdos, E. G., and Levin, Y. (1970) Biochim. Biophys. Acta 214, 374-376.
8. Pantoliano, M. W., Holmquist, B., and Riordan, J. F. (1984) Biochemistry 23, 1037-1042.
9. Costello, S. M., Felix, R. T., and Giese, R. W. (1979) Clin. Chem. 25, 1572-1580.
10. Ogura, H., Kobayashi, T., Shimizu, K., Kawabe, K., and Takeda, K. (1979) Tetrahedron Letters No. 49, 4745-4746.
11. Bernstein, K. E., Martin, B. M., Striker, L., and Striker, G. (1988) Kidney Int. 33, 652-655.
12. Neels, H. M., van Sande, M. E., and Sharpe, S. L. (1983) Clin. Chem. 29, 1399-1402.
13. Laemmli, U. K. (1970) Nature (London) 227, 680-685.
14. Merril, C. R., Goldman, D., Sedman, S., and Ebert, M. H. (1981) Science 211, 1437-1438.
15. 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.
16. Bernstein, K. E., Martin, B. M., Edwards, A. S., and Bernstein, E. A. (1989) J. Biol. Chem. 264, 11945-11951.
17. Green, N. M., Konieczny, L., Toms, E. J., and Valentine, R. C. (1971) Biochem. J. 125, 781-791.