DEFINITIVE EVIDENCE FOR SIMILARITY IN THE ACTIVE SITE OF RENIN AND ACIDIC PROTEASE*

T. Inagami, K. Misono, A. M. Michelakis**

Departments of Biochemistry, Medicine** and Pharmacology**

Vanderbilt University, School of Medicine

Nashville, Tennessee 37232

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SUMMARY: The pressor enzyme renin from the mouse submaxillary gland was inactivated rapidly by diazoacetyl-D, L-norleucine methyl ester in the presence of cupric ion. The ion was essential to this reaction. The complete inactivation was obtained by the stoichiometric reaction of the reagent as indicated by the stoichiometric incorporation of norleucine into the renin molecule. The incorporated norleucine could be removed by treatment with 0.2 M hydroxylamine in 8 M urea indicating that a carboxyl group presumably essential for catalysis was esterified by the aliphatic diazo reagent. Renin from the hog kidney was also inactivated completely by a similar reaction. Since the requirement of cupric ion in the inactivation by aliphatic diazo compound is the most notable specific feature of acidic proteases, it is proposed that renin have a catalytic site similar to those of acidic proteases and that renin belongs to the family of acidic proteases, though its specificity determining site may be somewhat different.

Renin (EC. 3.4.4.15) produces angiotensin I from plasma renin substrate or synthetic peptide substrates by the limited proteolysis of one unique leucylleucine peptide bond of the substrates (1,2). The mechanism underlying such a highly selective catalysis is not known. Experiments designed to characterize renin action using specific inactivators of proteases such as diisopropyl phosphorofluoridate (3,4), p-hydroxymercuribenzoate (4) or EDTA (3) have indicated that renin does not belong to a known class of proteases such

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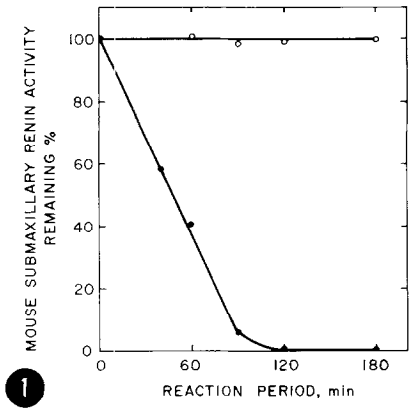
as serine-, cysteine-, or metallo protease. Not a single functional group essential for catalysis has been identified.

Recently the N-acylated pentapeptide pepstatin, a potent inhibitor of pepsin and other acidic proteases (5), has been found to inhibit renin competitively suggesting that renin may be an acidic peptidase (5-8). A notable and characteristic feature commonly found among acidic proteases is their sensitivity to cupric ion-catalyzed inactivation by aliphatic diazo compounds (9-13). We have applied diazoacetyl-D, L-norleucine methyl ester to the characterization of mouse submaxillary renin and hog renal renin, and obtained definitive evidence that these enzymes share characteristic features of acidic proteases.

Mouse submaxillary renin (peak A fraction) was prepared in a crystalline purity by the method described previously (2). Partially purified hog renal renin was prepared from a crude commercial product (Pentex) by a method developed in our laboratory using an affinity column of a pepstatin-bound polyacrylamide gel (8). It had a specific activity of 1.2 μmoles angiotensin I formed per hour per mg of enzyme protein. Diazoacetyl-D, L-norleucine methyl ester was prepared by the method of Rajagopalan et al. (10).

The submaxillary renin was modified with an 80-fold molar excess of diazoacetyl-D, L-norleucine methyl ester in the presence of a 40-fold molar excess of cupric acetate at 14° and pH 5.4 as described in detail in the legend to Fig. 1. The molar concentration of cupric ion had to be maintained lower than that of diazoacetyl compounds to avoid the precipitation of the enzyme. Aliquots of the reaction mixture extracted at intervals were treated with 10 mM EDTA to stop the cupric ion catalyzed reaction, exhaustively dialyzed against 0.05 M pyrophosphate buffer pH 5.5, hydrolyzed in 6 N HCl then subjected to amino acid analysis to determine the incorporated norleucine



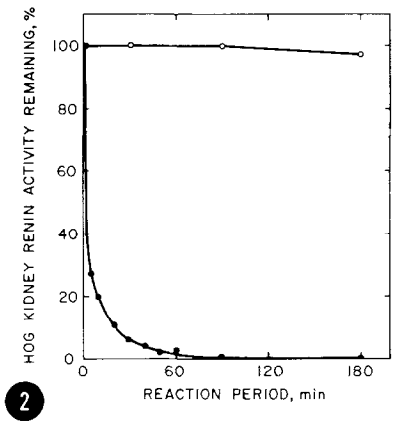


Fig. 1. Inactivation of mouse submaxillary renin by diazoacetyl-D, L-norleucine methyl ester. The reaction mixture consisted of 0.09% mouse submaxillary renin A, 2 mM diazoacetyl-D, L-norleucine methyl ester and 20 mM Na-acetate buffer, pH 5.4. After 10 min. of preincubation at 14° reaction was started by the addition of cupric acetate to 1 mM (●). Control experiments were performed in the absence of the cupric ion (O). Aliquots were withdrawn at intervals, diluted to appropriate concentration and assayed for renin activity immediately by the method of Reinharz and Roth using their fluorogenic octapeptide renin substrate (15).

Fig. 2. Inactivation of partially purified hog kidney renin by diazoacetyl-D, L-norleucine methyl ester in the presence of cupric ion. The reaction was carried out with a mixture containing the partially purified hog kidney renin at 0.07% (8), 4 mM diazoacetyl-D, L-norleucine methyl ester, 2 mM cupric acetate 0.01 M 2-(N-morpholino)ethane sulfonate-Cl buffer, pH 6.2 at 14°. The reaction of extracted aliquots (10 µl) was stopped by the addition of 10 µl of 10 mM EDTA. After a 1000-fold dilution with 0.1% bovine plasma albumine the renin activity was determined using 10 µl (3,5 ng protein) of the diluted solution by the radioimmunoassay method of Haber (17). Results were obtained in the presence of the cupric ion (●) and in its absence (O).

(14). The renin activity was assayed immediately after appropriate dilution without dialysis according to the method of Reinharz and Roth (15) using their fluorogenic octapeptide substrate (Bachem, Marina Del Ray, California). Details are given in Fig. 1. The pyrophosphate buffer used in the assay mixture chelates cupric ion and stops the cupric ion-catalyzed inactivation reaction. The partially purified hog renal renin was treated in

a similar manner at pH 6.2. Its activity was determined by the radio-immunoassay of Haber et al. (16) using sheep plasma substrate without prior dialysis of the modified enzyme. Details are given in the legend to Fig. 2.

A rapid inactivation of submaxillary renin took place resulting in complete inactivation in 2 hours at pH 5.4 as indicated by filled circles in Fig. 1. Diazoacetyl-D, L-norleucine methyl ester alone in the absence of the cupric ion did not affect the renin activity (open circles), nor did the cupric ion alone have any appreciable permanent effect at the concentration employed in this experiment.

The amino acid analysis of a completely inactivated enzyme preparation showed an incorporation of 1.2 residues of norleucine per enzyme molecule. This finding seems to indicate that the functional group modified by the cupric ion catalyzed reaction of diazoacetylnorleucine methyl ester plays an essential role in renin catalysis.

Rajagopalan et al. (10) were able to inactivate pepsin completely with diazoacetyl-D, L-norleucine methyl ester with incorporation of a stoichiometric amount of a norleucine derivative into the enzyme molecule. Furthermore, the incorporated norleucine derivative could be removed by a treatment with hydroxylamine. When submaxillary renin completely inactivated by incorporation of a stoichiometric amount of the norleucine derivative was treated with 0.2 M hydroxylamine at pH 9 in the presence of 8 M urea for 24 hours at room temperature, exhaustively dialyzed, hydrolyzed in 6 N HCl and subjected to amino acid analysis (14), the norleucine peak is reduced to less than 1% of the untreated protein. This observation indicates that the linkage between the norleucine derivative and submaxillary renin was susceptible to hydroxylaminolysis, strongly suggest-

ing involvement of a carboxyl group of renin in an esteric linkage with the hydroxyl group of glycolyl-D, L-norleucine methyl ester derived from diazoacetyl-D, L-norleucine methyl ester as suggested by Rajagopalan et al. (10). The presence of such an esteric linkage has been demonstrated by Baylis et al. (12) in pepsin inactivated by diazoacetyl-L-phenylalanine methyl ester.

Renal renin was also found to be inactivated completely by diazoacetyl-D, L-norleucine methyl ester. A partially purified renal renin preparation was treated in a manner similar to the inactivation of submaxillary enzyme and the loss in renin activity was followed by the specific radioimmunoassay of renin activity (Fig. 2). Again the cupric ion was an obligatory requirement for the inactivation, as the experiment without the cupric ion did not bring about the inactivation as indicated by open circles, whereas in the presence of 40 mM cupric ion a rapid inactivation resulted as shown by filled circles. Since the purity of the hog renal renin preparation has not been established, the stoichiometry of the modification reaction was not studied.

The requirement of the cupric ion for the inactivation of both submaxillary and renal renin by the aliphatic diazo compound, the stoichiometric relationship between the extent of the modification and the loss of renin activity, and the susceptibility of the incorporated modification reagent to hydroxylamine treatment are all in close analogy with reported characteristics of pepsin and other acidic proteases (9-13). Thus these observations can be taken as a strong evidence that the active site structure and most probably the catalytic mechanism of renins are closely related to those of acidic proteases and renins belong to the family of the acidic protease.

In the past it was noted that pepsin (17) and cathepsin D (18) also hydrolyzes the unique leucylleucine peptide bond of natural or synthetic renin substrates. Pepsin, cathepsin D and renin are all strongly inhibited

by pepstatin (5) though to somewhat different extents. These observations, when considered separately, may not present a very strong case for the similarity of renin with other acidic proteases. However, when taken in context with the finding of more definitive studies presented herewith, these findings all seem to fit in and support the proposal that the active site structure and catalytic mechanism of renins are closely related to those of acidic proteases.

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REFERENCES

1. Skeggs, L. T., Kahn, J. R., Lentz, K. E. and Sumway, N. P. (1957) J. Exp. Med., 106, 439-453.

2. Cohen, S., Taylor, J., Murakami, K. Michelakis, A. M., and Inagami T. (1972) Biochemistry 11, 4286-4293.

3. Pickens, P. T., Bumpus, F. M., Lloyd, A. M., Smeby, R. R. and Page, J. H. (1965) Circulat. Res. 17, 438-448.

4. Reinharz, A., Roth, M., Haefeli, L. and Schaetelin, G. (1971) Enzyme 12, 212-218.

5. Aoyagi, T., Morishima, H., Nishizawa, R., Kunimoto, S., Takeuchi, T., and Umezawa, H. (1972) J. Antibiotics 25, 689-694.

6. Gross, R., Lazar, J. and Orth, H. (1972) Science 175, 656.

7. Corvol, P., Devaux, C. and Menard, J. (1973) FEBS Letters, 34, 189-192.

8. Murakami, K., Inagami, T., Michelakis, A. M. and Cohen, S. (1973) Biochem. Biophys. Res. Commun. 54, 482-487.

9. Delpierre, G. R., and Fruton, J. S. (1965) Proc. Nat. Acad. Sci. U.S.A. 54, 1161-1167.

10. Rajagopalan, R. G., Stein, W. H. and Moore, S. (1966) J. Biol. Chem. 241, 4295-4297.

11. Keilova, H. (1970) FEBS Letters 6, 312-314.

12. Bayliss, R. S., Knowles, J. R., Wybrandt, G. B. (1969) Biochem. J. 113, 377-386.

13. Smith, G. D., Murray, M. A., Nichol, L. W. and Trikojus, V. M. (1969) Biochim. Biophys. Acta 171, 288-298.

14. Spackman, D. H. (1963) Fed. Proc. 22, 244.

15. Reinharz, A. and Roth, M. (1969) Eur. J. Biochem. 7, 334-339.

16. Haber, E., Koerner, T., Page, L. B., Kilman, B. M. and Purnode, A. (1969) Endocr. and Metab. 29, 1349-1355.

17. Fernandez, M. T. F., Paladini, A. C. and Delius, A. E. (1965) Biochem. J. 97, 540-546.

18. Reinharz, A. and Roth, M. (1971) Enzyme 12, 458-466.