An Experimental Study of the Effect of Zinc on the Activity of Angiotensin Converting Enzyme in Serum

Philip G. Reeves and Boyd L. O'Dell

The activity in serum of zinc-dependent angiotensin converting enzyme (ACE), is measured to aid in diagnosis and monitor treatment of certain diseases. This report shows the effect of dietary zinc deprivation on ACE activity in the serum of rats. The mean (and SE) of the zinc concentration (μmol/L) in serum was 3.5 (0.3) in rats deprived of dietary zinc for four days, 16.3 (0.2) in control rats, and 19.8 (0.9) in rats deprived of zinc for four days, then replented with zinc for 12 h. The respective mean (and SE) of ACE activities (nmol/min per ml) in serum were 390 (15), 543 (13), and 545 (20). Serum ACE activity was restored also by adding zinc to the assay mixture in vitro. The V max for ACE was 1.4 times greater when serum was diluted 40-fold as compared to twofold dilution. There was a small effect on the K m for the substrate, but the K m for zinc was decreased by 22-fold when serum was diluted 40-fold. The V max under these conditions was decreased by only 9%.

Additional Keyphrases: rats · therapy with steroids

High activity of angiotensin converting enzyme (ACE; dipeptidyl carboxypeptidase I, or peptidyl-dipeptide hydrolase, EC 3.4.15.1) is found in lung and serum. ACE activity in serum may be measured clinically to assist in diagnosing sarcoidosis and monitoring therapy with steroids (7, 2), with antihypertensive drugs that inhibit the enzyme (3), and in other clinical and research uses as well (4, 5).

The enzyme is activated by zinc (6), and White et al. (7) reported recently that the activity of the enzyme in plasma of rats fed zinc-deficient diets for only four days was decreased by half, the activity being directly dependent on the concentration of zinc in the plasma. If these results are substantiated, they considerably affect the use of the assay in assessment of disease states that have a secondary hypozincemia associated with them. We have extended these studies and also report some kinetic parameters for the enzyme.

Materials and Methods

Animal protocol: We divided female Wistar rats, weighing between 120 and 150 g, into three groups of five rats each. All rats were fed a semipurified zinc-deficient diet, 25 mg of Zn per kilogram of diet (8), during a four-day adaptation period. Two groups were then fed a similar diet, but without added zinc. The third group continued to receive the zinc-deficient diet. These regimens were continued for another three days, at which time one of the zinc-deprived groups was fed the zinc-deficient diet for an additional 12 h. On the morning of the fourth day all the rats were anesthetized, and blood was sampled.

Serum samples: Blood was collected from the abdominal aorta of anesthetized rats (50 mg of pentobarbital sodium per kilogram body weight, i.p.), allowed to clot at room temperature for 20 min, and centrifuged (1500 × g, 20 min). Serum was stored at −20 °C until used.

Reagents: Hippurylglycylglycine (HGG), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), NaCl, Na 2 SO 4, glycylglycine, sodium tungstate, "Chelating Resin," and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma Chemical Co., St. Louis, MO. These and other chemicals were analytical grade.

Procedures

Preparation of chelating resin: Rinse 50 g of chelating resin four times with four 500-mL portions of de-ionized water, to remove fines. Then treat the resin twice with four bed volumes of sodium acetate (0.5 mol/L, pH 6.3) and wash it five times with four successive bed volumes of de-ionized water. Aspirate the resin free of excess water, and store it in a tightly closed bottle at 4 °C.

Preparation of substrate solution: Place 476 mg of HEPES, 702 mg of NaCl, 2.272 g of Na 2 SO 4, 358 mg of HGG, 15 mL of de-ionized water, and 50 μL of saturated NaOH in a 50-mL polypropylene test tube. Dissolve the ingredients completely by adjusting the pH to 6.3 with an all-glass combination electrode (Fisher Scientific, St. Louis, MO). Then pass the solution through a column (1.0 cm, i.d.) made with 3 g of the prepared chelating resin. After the solution has passed through, wash the resin with 3 mL of de-ionized water and add the wash to the substrate solution. Re-adjust the pH to 7.4 with HCl and bring the final volume to 20 mL. Store the solution in 1.0-mL aliquots at −20 °C.

Enzyme assay: Assay for ACE activity by modifying the procedure of Neels et al. (9), as follows. To tubes immersed in an ice bath, add 50 μL of either whole serum or serum diluted 20-fold with assay buffer, and 50 μL of substrate solution (final substrate concentration, 30 mmol/L). Begin the reaction by placing the rack of tubes into a shaking water bath at 37 °C. Stop the reaction after 10 min for the undiluted serum and after 30 min for the 20-fold diluted serum by placing the rack of tubes back in the ice bath and adding to each tube 200 μL of equilibrium mixture of sodium tungstate (100 g/L solution) and dilute H 2 SO 4 (0.33 mol/L solution). Add 1 mL of water to each tube. Prepare serum blanks by adding the sodium tungstate-sulfuric acid mixture before incubation.

Vortex-mix the contents of the tubes, then centrifuge them at 1500 × g for 20 min. Transfer appropriate aliquots of the supernatant fluid (0.1 mL when undiluted serum is used, 0.75 mL when the 20-fold dilution is used) to glass tubes and bring the final volume to 0.75 mL. To each tube, add 1.0 mL of borate buffer (Na 2 B 4 O 7 · 10H 2 O, 100 mmol/L, pH 9.6) and 50 μL of TNBS (60 mmol/L). Vortex-mix, and allow the tubes to stand at room temperature for 40 min until color development has stabilized. Measure the absorbance at 420 nm, using 1.0-mL disposable cuvettes (1.0-cm lightpath).
Preparation of standards: Prepare glycylglycine standards and treat them in the same manner as the sample blanks. The concentrations of standard used in the initial incubation volume should be such that 0.75 mL of the tungstate–acid supernate contains 0 to 100 nmol of glycylglycine. Add the sodium tungstate–sulfuric acid solution to these tubes before incubation.

Serum zinc determinations: Determine the concentration of zinc in serum by flame atomic absorption procedures with an instrument equipped with automatic background correction capabilities (we used a Perkin-Elmer Model 2380). Dilute the serum threefold with de-ionized water and aspirate the sample directly. Prepare zinc standards with a concentration range of 0 to 1 mg/L in a solution of glycerin so that the viscosity will be the same as that of the diluted serum.

Results

Experiments on Rats

Our first attempt to repeat the experiments of White et al. (7) showed no effect of zinc deprivation on ACE activity in serum of rats. We soon discovered, however, that the substrate preparation was contaminated with zinc (5 μmol/L). When we treated the substrate with chelating resin to remove the zinc and repeated the assay we obtained the results shown in Table 1. The short-term zinc deficiency (treatment 1) decreased zinc in serum by almost fivefold as compared with serum of rats fed diets with adequate zinc (treatment 2). This low concentration in these samples evidently led to the decrease in ACE activity, a surmise substantiated when we added zinc to the assay preparation of zinc-deficient serum at concentrations equal to that found in the assay of zinc-adequate serum and found no difference in ACE activities (treatment 4). In addition, when zinc-deprived rats were fed a zinc-adequate diet (25 mg/kg) for 12 h before sampling, zinc concentrations in serum returned to normal and ACE activity was restored (treatment 3).

Kinetic Characteristics of the Serum Enzyme

We studied the kinetics of the enzyme under various conditions. In the assay described above we used 30 mmol/L of substrate and a final twofold dilution of serum. Other investigators (9, 10) used much greater dilutions of serum in similar types of assays. Figure 1 shows the effect of dilution of serum on enzyme activity expressed as nanomoles of glycylglycine released per milliliter of serum per minute, in graded substrate concentrations. Activity was measured in the presence of 20 μmol of zinc per liter. A Lineweaver–Burk plot of these data (Figure 2) shows that a 40-fold dilution of serum increased the Vₘₐₓ by 43% but decreased

| Table 1. Effect of Short-Term Zn Deficiency and Resupplementation on Zn Concentration and ACE Activity in Rat Serum |
|-----------------|-----------------|-----------------|
| Serum* | In Assay | In Medium |
| Treatment * | Concent of Zn, μmol/L, mean (and SE) | ACE Acty, mmol mL per min* |
| 1 | 3.5 (0.3) | 1.8 | 390 (15) |
| 2 | 16.3 (0.2) | 8.2 | 542 (13) |
| 3 | 19.8 (0.9) | 9.9 | 545 (20) |
| 4 | 3.5 (0.3) | 8.8 | 534 (19) |

* Each treatment is described in the text. Each value is the mean of five replicates. * The mean concentration of zinc in the serum of rats in treatment 1 differs significantly (p < 0.001) from those in experiments 2 and 3. * The activity of ACE in the serum of rats in experiment 1 differs significantly (p < 0.01) from those in experiments 2, 3, and 4.

Fig. 1. Effect of various concentrations of substrate (hippurylglycylglycine, HGG) on the activity of angiotensin converting enzyme in serum.

The assay was run at 37°C, pH 7.4, in the presence of 20 pmol of Zn per liter. Two dilutions of sera were used: twofold (+) and 40-fold (+).

Fig. 2. Reciprocal plot of the data in Figure 1.

The Vₘₐₓ for the enzyme in serum diluted twofold (*) was 610 mmol/mL per min; the Kₘ was 5 mmol/L. When serum was diluted 40-fold (+) the Vₘₐₓ was 870, the Kₘ 4.3

the Kₘ by only 14% as compared with the twofold dilution. These results suggest that serum contains a natural inhibitor of the enzyme. We have not used substrate concentrations higher than 30 mmol/L, but these data (Figure 1), as well as others from our laboratory (not shown), suggest that concentrations exceeding 30 mmol/L would inhibit the enzyme.

Figure 3 shows the effects of serum dilution on the activation of the enzyme by zinc. Serum samples from five zinc-deprived rats were pooled for this assay. Activity was measured in the presence of 30 mmol of substrate per liter. The results show that the enzyme was fully activated at approximately 2.5 μmol of zinc per liter when serum was diluted 40-fold. However, when serum was diluted by only one-half, the concentration of zinc required for full activation was 10 μmol/L. The normal zinc concentration in the serum of a healthy human is approximately 15 μmol/L. If this serum were diluted 10- to 20-fold, as in some ACE assay methods (9, 10) used today, the zinc concentration in the reaction vessel would be 1.5 and 0.75 μmol/L, respectively. Clearly, if the medium were otherwise free of zinc the observed ACE activity would be below the maximum.

A Lineweaver–Burk plot (Figure 4) of the data in Figure 3...
enzyme in serum may be decreased. The activity of the enzyme, and the enzyme characteristics, were studied by Falchuk et al. (12) and Vaz Parente et al. (14). For example, in lung and other tissues as a metallo-enzyme, containing one gram atom of tightly bound zinc per mole. Whether or not zinc is tightly bound to the enzyme in serum remains to be determined, but the characteristics of the enzyme in tissue and serum appear to be somewhat different. For example, some studies (17, 6) show that lung ACE requires a relatively high concentration (0.1 mmol/L) of EDTA and extensive dialysis to inactivate the enzyme, whereas, as shown in the present study, a short-term (four day) decrease in zinc in serum by dietary means causes a large decrease in the activity of the enzyme. We also have observed that as little as 10 μmol of EDTA per liter will completely de-activate serum ACE, and that activity can be restored by adding zinc (data not shown). These observations suggest that at least part of the zinc is more loosely bound to the enzyme in serum than it is to EDTA. Its mechanism of activation by zinc may be different from that of the tissue enzyme.

In this discussion we have stressed the importance of knowing the concentration of zinc in serum and in the reaction mixture if one is to adequately assess the maximal value for serum ACE activity. It is highly probable that, under the assay conditions prescribed for most current methods (9, 10), the zinc concentration in the reaction mixture would be adequate, because the substrate and other assay components might be contaminated with it during the manufacturing process—but one should not rely on contamination as a means of controlling the quality of a clinical assay.

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References