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Minimal functional size of porcine lung and testicular angiotensin-converting enzymes deduced from radiation inactivation analysis

Interaction of two highly homologous domains in somatic isoenzyme

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Domain structures of porcine lung and testicular angiotensin-converting enzymes (ACE) were studied by radiation inactivation to test the hypothesis that lung ACE has two catalytic sites localized to discrete, structurally independent domains (the N- and C-domains) of approximately equal size. The minimum functional sizes of lung and testicular ACE, calculated from the inactivation curves obtained, were 140 and 74 kDa, respectively. Since testicular ACE has been demonstrated to contain only the C domain, this result indicates that the two domains in lung ACE are not independent but are, in fact, structurally tightly linked.

Angiotensin-converting enzyme; Radiation inactivation; Functional molecular size; Porcine lung; Porcine testis

1. INTRODUCTION

Angiotensin-converting enzyme (ACE) is a metallopeptidase localized mainly on the surface of vascular endothelial cells and involved in the maintenance of the circulatory homeostasis by catalyzing the formation of angiotensin II from angiotensin I and by inactivating bradykinin. In addition to this somatic ACE, testicular ACE has been demonstrated, which is much smaller than somatic ACE. The presence of these two types of ACE has been established by molecular cloning [1–4]. They have also been shown to be generated from the same single gene by use of two different transcription initiation sites [5,6]. As shown schematically in Fig. 1, somatic ACE consists of two homologous N and C domains, each containing a putative metal-binding motif (HEMGH) identified by sequence comparison with the active sites of other metallopeptidases; testicular ACE has a single domain that is identical to the C-terminal half of somatic ACE. The catalytic role of the C domain is therefore evident. However, the functional role of the N domain present only in the somatic enzyme is not clear; although it does exhibit a converting enzyme activity with different substrate specificity, as demonstrated by the expression of the corresponding cDNA [7], its activity is very weak, almost negligible, as compared to that of the C domain; therefore, biochemical analyses using competitive ACE inhibitors [8] and chemical modification [9] have failed to detect the enzymic activity of the N domain. Structurally, the N and C domains in somatic ACE are generally considered to form two independent, relatively stable domains connected loosely by a continuous polypeptide chain.

In the present study, we investigated the degree of the structural interaction between the N and C domains by radiation inactivation, which we previously employed for the analysis of atrial natriuretic peptide (ANP) activation of particulate guanylate cyclase [10,11]. Radiation inactivation is a useful technique for estimating the minimum functional size of biological macromolecules and their subunit or domain interactions. According to classic target theory [12], on which this technique relies, it is expected that if the N and C domains of somatic ACE are structurally and functionally independent of each other, almost identical inactivation curves will be obtained for both somatic and testicular ACE. Contrary to this expectation, distinct inactivation curves were obtained, indicating that the two domains are tightly linked.

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2. MATERIALS AND METHODS

2.1. *Materials*

Fresh porcine lung and testes were obtained from the Shibaura abattoir sanitation inspection station, Tokyo, Japan. Bz-Gly-His-Leu/H_2O and His-Leu were from Peptide Institute Inc., Osaka, Japan; *o*-phthaldialdehyde was from Kanto Chemical Inc., Tokyo, Japan; yeast alcohol dehydrogenase (ADH) and β-NAD were from Sigma, St. Louis, MO, USA.

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2.2. *Radiation inactivation*

Porcine lung and testicular membranes were prepared as described previously [10,11]; tissue membranes were suspended in an equal volume of PBS (20 mM phosphate, pH 7.5) containing 5 μg/ml of leupeptin and pepstatin, and 0.5 mM PMSF. To this membrane preparation was added ADH at a concentration of 50 U/ml as an internal standard, which has a functional size of 140 kDa. The membrane suspensions (0.5 ml) were placed into 5-ml ampules, rapidly frozen, lyophilized and sealed under vacuum.

Irradiation was performed at 25°C with ^{60}Co at the Japan Atomic Energy Research Institute, Takasaki, Japan, at a dose rate of 10 kGy/h [13]. After irradiation, ampules were opened and the samples were resuspended in 1 ml of PBS. The reconstituted suspension was centrifuged at 15,000 × g for 10 min; the supernatant was assayed for ADH activity [14]; the membranes were dispersed in 1 ml of PBS and assayed for ACE activity. Functional molecular size (kDa) of the enzyme was calculated from the following equation according to target theory [15]:

$$\text{mol.wt. (kDa)} = 6{,}400/D_{37}$$

where D_{37} is the radiation dose in kGy which reduced enzyme activity to 37% of its initial activity.

2.3. *Assay of angiotensin-converting enzyme activity*

ACE activity was measured by a modification of the fluorimetric method [15]; irradiated membrane preparations of porcine lung and testes were incubated at 37°C with 1.5 ml of reaction mixture containing 5 mM Bz-Gly-His-Leu (synthetic substrate), 150 mM NaCl and 50 mM Tris-HCl, pH 7.9, in the presence and the absence of 1 μM captopril (ACE inhibitor). At a given time, 100 μl of sample was removed from the reaction mixture and added to 1.4 ml of 0.5 M NaOH to stop the reaction. The product, His-Leu, was detected by adding 100 μl of 10 mg/ml *o*-phthaldialdehyde in ethanol, incubating at 25°C for 5 min and adding 250 μl of 6 N HCl. The fluorescence of the mixture was measured with emission at 495 nm and with excitation at 365 nm. The standard curve was obtained using known concentration of His-Leu. Enzyme activity was expressed as nanomoles of substrate hydrolyzed per hour per mg protein.

3. RESULTS AND DISCUSSION

Figure 2 shows the effects of radiation on porcine lung and testicular ACE activities; lung membranes were used as a typical source of somatic ACE. The loss of lung ACE activity with increasing radiation dosage was more rapid than the decay of the testicular enzyme. From the slopes of the linear inactivation curves, the functional sizes were determined to be 140 and 74 kDa for lung and testicular ACE, respectively. The fact that the target size of lung ACE is twice as large as that of testicular ACE indicates that the N and C domains are interacting quite strongly in the somatic enzyme. This view appears to be consistent with the earlier observation that the extracellular domain of somatic ACE could be solubilized from human lung membranes with trypsin (1 mg/500 mg protein) without further cleaving it into smaller fragments [16]. This structural information will be helpful for elucidating the role of the N domain.

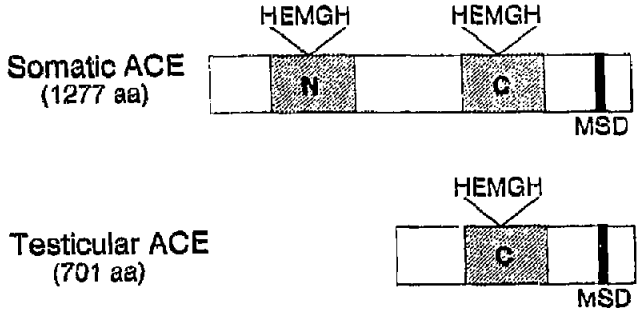

Fig. 1. Schematic structure of somatic and testicular ACE. The primary structure of ACE was deduced from cDNAs encoding human somatic (1,277 amino acids) [1,6] and testicular (701 amino acid) [4] ACE. The highly homologous domains, each containing the proposed metal-binding motif (HEMGH), are indicated by hatched boxes. The membrane-spanning domain (MSD) is represented by a black box. The highly homologous domains near the N- and C terminus are termed the N- and C domain, respectively, in this paper.

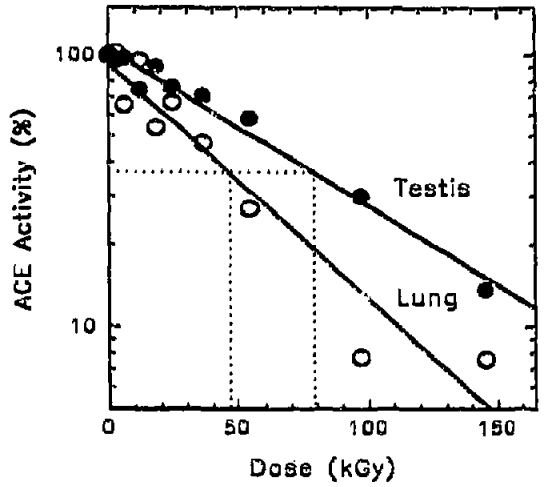

Fig. 2. Radiation inactivation of porcine lung and testicular ACE. Basal ACE activities in the non-irradiated membranes of lung and testis were 400 and 305 nmol/h/mg protein, respectively. ○, lung; ●, testis. Values are means of three determinations. Sample preparation and measurement of ACE activity were described in section 2.

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