Cleavage of Active Peptides by a Lung Enzyme[1]

Plasma[2] and organs such as lung[3-6] and kidney[6-8] contain the enzyme dipeptide hydrolase (DH) that cleaves dipeptides from the C-terminal end of angiotensin (Ang) I and bradykinin (Bk). The enzyme[3,4] can liberate the hypertensive Ang II from Ang I or inactivate the hypotensive peptide Bk. Thus it acts as an Ang I converting enzyme[9] and as a kininase, identical with kininase II[2,10]. We have purified the enzyme previously[2,4] from plasma and concentrated it from homogenates of kidney cortex[4,7] and lung[4]. Because substantial amounts of circulating Bk and Ang I are metabolized in the pulmonary circulation[11], we purified and studied the lung DH further to see how far is it involved in these processes. This report summarizes our studies on the inhibition of DH, on the development of a sensitive chemical assay for the enzyme and on its action in the perfused lung in situ[12,13].

Materials and methods. Hog lungs were homogenized in 0.25 *M* sucrose and the particulate fractions were sedimented by differential centrifugation. The final 100,000 *g* supernatant was the source of DH. The enzyme was purified by the sequential use of gel filtration on a Sephadex G-200 column, Amicon membrane filtration, DEAE-Sephadex column chromatography and filtration on a Biogel P 300 column. After a disc gel electrophoresis the protein band containing the enzyme was eluted. Repeating the electrophoresis yielded a homogenous DH preparation, as shown by the single protein band on the gel. For control studies, DH was concentrated from guinea-pig plasma by gel filtration on a Sephadex G-200 column[2,4]. (Of all animals tested, the guinea-pig has the highest DH activity in blood[4].)

Similar to the plasma DH[3,4] the purified lung enzyme showed relatively little substrate specificity in that it cleaved C-terminal dipeptides from several short and long substrates. Its peptidase action was measured also in the UV spectrophotometer with Hip-Gly-Gly[3], *t*-BOC-Phe (NO_2)-Phe-Gly[3], or Hip-His-Leu[4] used as substrates. The hydrolysis of other peptides in vitro was determined in thin-layer chromatography[3,4] or in bioassay[3,4]. The action of DH in situ was investigated in the perfused blood-free rat lung employing radioactive peptides as substrates. We used inhibitors of DH in the experiments to show that the various actions studied can be attributed to a single enzyme. The most active specific inhibitors were two recently synthesized peptides, that were first discovered in snake venoms[14,15], the undecapeptide 'peptide C', and the nonapeptide SQ 20881 (M. Ondetti, Squibb Institute).

Table I. I_{50} values of inhibitors of DH of hog lung (L) and guinea-pig plasma (P) obtained in the UV-spectrophotometer

Inhibitor	Substrates			
	HGG		HHL	BPPG
	L	P	P	L
Glutathione	2×10^{-6}	3×10^{-6}	2×10^{-6}	
Peptide C	3×10^{-8}	3×10^{-7}		3×10^{-7}
SQ 20881	3×10^{-8}	3×10^{-9}		1×10^{-8}

I_{50} = *M* concentration of inhibitor that inhibits 50% Substrates: HGG, Hip-Gly-Gly[3]; HHL, Hip-His-Leu[4]; BPPG, *t*-BOC-Phe (NO_2)-Phe-Gly[3]. Glutathione was tested in the presence of dithioerythritol. Peptide C, Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro, SQ 20881, Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro.

Results and discussion. The cleaving of optically active substrates was inhibited by 3 peptide inhibitors. Peptide C and SQ 20881 were the most potent with I_{50} values in the orders of 10^{-7} and 10^{-9} *M* (Table I). Glutathione had an I_{50} of 2.10^{-6} *M* in the presence of dithioerythritol. Low concentrations of all 3 inhibitors were approximately equally effective against DH prepared from either hog lung or guinea-pig plasma. This further suggests that the same enzyme is present both in blood and in lung.

Since insulin inhibited DH in vitro[4], it was also tested as a substrate of DH. The B chain of insulin was cleaved by DH when incubated in vitro from 0.5 to 24 h. Thin-layer chromatography indicated that DH removed the dipeptide Lys-Ala from the C-terminal end of the B chain. Thin-layer chromatography also proved that DH cleaved Phe-Arg from Ac-Ser-Pro-Phe-Arg representing the protected C-terminal tetrapeptide in Bk. The cleavage of Lys-Ala from the B chain of insulin was completely inhibited by 1×10^{-4} *M* SQ 20881.

Because DH cleaves a variety of peptidyl dipeptide bonds[3,4], we prepared a radioactive, fluorescent substrate to detect small quantities of the enzyme. The N-terminal end of triglycine was reacted with ^{14}C labeled dansyl (1-dimethylaminonaphthalene-5-sulfonyl; DNS) chloride[16]. The resulting ^{14}C-DNS-Gly-Gly-Gly (0.6 nmole) was cleaved by DH to ^{14}C-DNS-Gly and to Gly-Gly. DH did not release ^{14}C-DNS-Gly-Gly. The products were separated in thin-layer chromatography on silica coated glass microfiber sheets in chloroform: benzyl alcohol: acetic acid (50:15:2.5), and were localized under the UV-light. The Rf values of dansylated glycine derivatives on the chromatography sheets used were: 0.17 for DNS-Gly-Gly-Gly, 0.47 for DNS-Gly-Gly and 0.83 for DNS-Gly. The fluorescent spots were punched out from the chromatography sheets, placed in vials containing a liquid scintillator and the decrease in substrate concentration and the increase in labeled product (^{14}C-DNS-Gly) after

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hydrolysis by DH were measured in a liquid scintillation counter. ^{14}C-DNS-Gly-Gly-Gly was not hydrolyzed by DH in the presence of the inhibitor SQ 20881 (10^{-4} *M*).

The hog lung enzyme preparation inactivated Bk and converted Ang I to Ang II in vitro as determined by bio-assay of the peptides on the isolated rat uterus or on the isolated rat colon[4]. These two actions of DH were inhibited similarly by the inhibitors SQ 20881 (3×10^{-6} *M*), peptide C (1×10^{-6} *M*), insulin (1×10^{-6} *M*) and by the B chain of insulin (2×10^{-6} *M*) (Table II). Glutathione (1×10^{-4} *M*) also prevented the conversion of Ang I.

The action of DH was also studied in the rat lung perfused in situ with Krebs solution. Radioactive (Asp^1-Ile^5-^{14}C-Leu^{10})-Ang I was added to the perfusion fluid and the peptide and the products of its cleavage were collected after a single passage through the pulmonary circulation. The products of the hydrolysis of labeled Ang I were separated either in thin-layer chromatography[17] or in high voltage electrophoresis at pH 2.1.

The conversion of Ang I to Ang II in the lung was shown by the appearance of His-Leu-^{14}C and by the decrease in the concentration of labeled Ang I. After a single passage through the lung, 45% of the added (0.87 µg/ml) Ang I was hydrolyzed to Ang II and to His-Leu-^{14}C. Only 3% of the total count was due to free Leu-C^{14}. The conversion in the lung was inhibited by various added endogenous and exogenous compounds (Table III). Since much more Ang I was perfused than found usually in blood, the inhibitors also had to be employed in higher than physiological concentrations. Bk was a remarkably potent inhibitor, probably because it is a competitive substrate of DH. Insulin, glutathione and the nonapeptide SQ 20881 inhibited the conversion, but the synthetic pentapeptide SQ 20475 was much less effective.

In other studies ^{14}C-DNS-Gly-Gly-Gly (45 *p*mole per ml) was perfused through lung. The perfusate was collected and desalted on a Dowex 50W-×8 column. The radioactive, fluorescent substrate and the products of its enzymic hydrolysis were separated in thin-layer chromatography. ^{14}C-DNS-Gly was the major radioactive hydrolysis product. About 10 to 20% of this substrate was hydrolyzed during a single passage through the rat lung as determined in the scintillation counter. When the Ac-Ser-Pro-Phe-Arg tetrapeptide fragment of Bk was perfused through the lung at a rate of 0.4 µmole per min, Phe-Arg was the major product of hydrolysis detected. This reaction was also inhibited by SQ 20881.

Conclusions. The details of the experiments summarized here will be published elsewhere. We conclude, however, that the properties of DH coming from plasma, kidney or lung are quite similar[2-4,18,19]. The enzyme purified from hog lung cleaved dipeptides in vitro from the C-terminal end of a variety of substrates including Ang I, Bk, the B chain of insulin and ^{14}C-DNS-Gly-Gly-Gly. The last substrate is radioactive and fluorescent, to be used for very sensitive assays.

In lung perfusion experiments Ang I was converted to Ang II by the release of His-Leu-^{14}C Gly-Gly was liberated from ^{14}C-DNS-Gly-Gly-Gly, and C-terminal Phe-Arg from a Bk derivative.

The enzyme was inhibited by several agents as determined with various techniques in vitro and in the perfused lung in situ. Specific inhibitors blocked the inactivation of the hypotensive peptide (Bk), the release of the hypertensive one (Ang II) and the hydrolysis of the B chain of insulin by DH. The results indicate that DH has an important role in the lung in metabolizing active peptides by the hydrolysis of C-terminal peptidyl dipeptide bonds.

Zusammenfassung. Ein Enzym (Angiotensin I «converting enzyme» oder Kininase II; DH) wurde aus Schweinelungen isoliert und gereinigt. DH spaltet Dipeptide vom Carboxyterminus der Peptidsubstrate mit Einschluss von Bradykinin, Angiotensin I, B-Kette von Insulin und ^{14}C-DNS-Gly-Gly-Gly. Zu den besten Inhibitoren des Enzyms gehören zwei kürzlich synthetisierte Peptide sowie Glutathion und Insulin. Die Experimente deuten auf ein einziges Enzym hin, das alle erwähnten Substrate hydrolysiert.

Table II. Inhibition of the conversion of angiotensin I and the inactivation of bradykinin in vitro by DH of the lung

Inhibitor	Substrate		
	Concentration (*M*)	Angiotensin I	Bradykinin
Peptide C	1×10^{-6}	++	++
SQ 20881	3×10^{-6}	++	++
Insulin	1×10^{-6}	++	++
B chain of insulin	2×10^{-6}	++	++
Glutathione	1×10^{-4}	++	N.t

++, greater than 75% inhibition. N.t, Not tested.

Table III. Inhibition of the conversion of (Asp^1-Ile^5-^{14}C Leu^{10}) angiotensin I during a single passage through the rat lung in situ

Inhibitor	Concentration (*M*)	Inhibition (%)
Insulin	2×10^{-5}	51
Bradykinin	1×10^{-6}	43
Glutathione	1×10^{-3}	57
SQ 20881 (nonapeptide)	1×10^{-5}	61
SQ 20475 (pentapeptide)	1×10^{-4}	14
EDTA	1×10^{-3}	45

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