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COMPETITIVE INHIBITORS OF THE ANGIOTENSIN-CONVERTING ENZYME FROM THE VENOM OF THE VIPER *Echis multisquamatus*

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The mechanism of the inhibition of the angiotensin-converting enzyme (ACE, E.C. 3.4.15.1) by homogeneous peptides of the venom of the Central Asian viper Echis multisquamatus *Ch. has been determined, and the inhibition constants have been measured, by means of an analysis of Lineweaver-Burk graphs.*

Modern inhibitors of the angiotensin-converting enzyme used in medicine as antihypertensive agents (Captopril and analogs) have been constructed on the basis of a structural-functional investigation of the natural peptides of snake venoms [1]. At the same time, it has not hitherto been completely clear what structure is the most preferable and why [2]. This, in its turn, presupposes a fuller structural-functional study of natural inhibitors, including an analysis of the mechanism of inhibition, since it is just concrete enzyme inhibitors that are molecular models for the synthesis of new specific antihypertensive drugs.

Eight peptide inhibitors of ACE have previously been isolated in the pure form from the venom of the Central Asian viper *Echis multisquamatus* Ch. and their bradykinin-potentiating activities have been characterized [3]. In the present study we have considered the mechanisms and have determined the constants of the inhibition of ACE by these peptides.

According to the results of gel filtration, on a column of TSK HW-40F, the molecular masses of these peptides amounted to 2.1—0.7 kDa (Table 1), not differing appreciably in this parameter from the known ACE inhibitors from the venoms of the snakes *Bothrops jaracara* and *Agkistron halys blomhoffii* [4, 5]. A distinguishing feature of the structure of the peptide inhibitors of the venoms of rattlesnakes is the presence of an N-terminal Pyr residue (Pyr represents pyroglutamic acid, a cyclized form of glutamic acid: *L*-pyrrolidin-2-one-5-carboxylic acid). In view of the fact that some of the peptides of viper venom do not react with ninhydrin and dansyl chloride — i.e., they have a blocked N-end — we may also assume a certain similarity with respect to this characteristic, as well. On the other hand, the presence of N-terminal Pyr is not a necessary condition for the exhibition of ACE inhibitor activity. Thus, the peptide É-IIIb-2 does not contain a blocked N-end and its molecular mass is 0.6—0.7 kDa, which practically coincides with that of the known synthetic ACE inhibitor Val-Glu-Ser-Ser-Lys [6].

The angiotensin-converting enzyme that we have isolated from ox kidney migrates in the form of a single band on electrophoresis under denaturing conditions, which indicates a high degree of purity of the preparation. In the kinetic calculations the molecular mass of the enzyme was taken as 180 kDa, corresponding to the results of the gel filtration of the native enzyme on Sephadex G-200.

To measure ACE activity we used a continuous spectrophotometric method (in the visible region) proposed by Holmquist [7], which is based on the use of N-(3-[2-furyl]acryloyl)-Phe-Gly-Gly (FAPGG) as substrate. In relation to this substrate, the enzyme obtained was characterized by the kinetic parameters K_m and k_{cat}, with the respective values of 3.4×10^{-1} M and 26,400 min^{-1}. It is interesting to compare the values obtained for the angiotensin-converting enzyme from other sources in relation to FAPGG. Thus, K_m and k_{cat} for the ACE isolated from rabbit lungs are 3.0×10^{-4} M and 19,000 min^{-1} [7], while the parameters for the enzyme from bovine lungs are 6.4×10^{-4} M and 36,600 min^{-1} [8].

Inhibition constants (dissociation constants of the enzyme-inhibitor complex K_i) for the pure peptides from the viper venom were calculated from Lineweaver-Burk graphs. The results obtained are given in Table 1.

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TABLE 1. Inhibitory Properties of Peptides from the Viper Venom in Relation to the Angiotensin-converting Enzyme

Peptide	Mol. mass, kDa*	K_i, μM	Type of inhibition
E-IIIa-1-1-1	2.1	0.393	Noncompetitive
E-IIIa-1-2-1	1.7	1.862	Noncompetitive
E-IIIa-1-3-1	1.6	0.478	Competitive
E-IIIa-2-1	1.2	0.186	"
E-IIIa-2-2	1.2	0.109	"
E-IIIa-2-3	1.2	0.0013	"
E-IIIa-2-4	1.2	0.140	"
E-IIIb-2	0.6-0.7	0.050	"

*From the results of gel filtration on a TSK HW-40 column under conditions of high ionic strength.

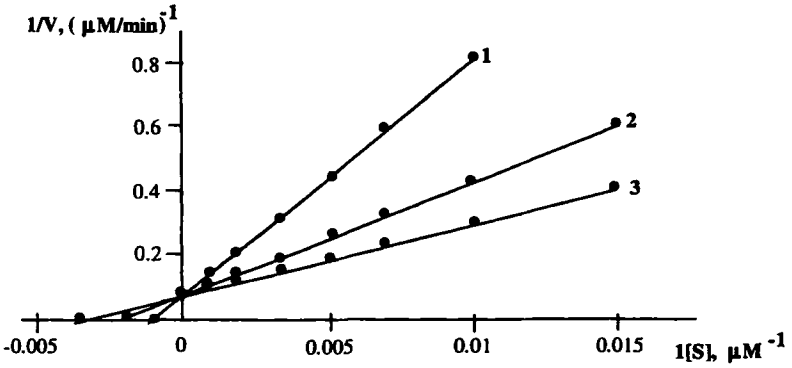

Fig. 1. Lineweaver-Burk graphs (regression lines) for the hydrolysis of FAPGG under the action of ACE from ox kidneys: *1*) noninhibited reaction; *2*) in the presence of 0.083 μM of peptide É-IIIa-2-2; *3*) in the presence of 0.830 μM of peptide É-IIIa-2-2. The point of intersection of the straight lines with the axis of ordinates does not change in the presence of different concentrations of inhibitor, which shows the competitive nature of the inhibition.

On comparing the values of K_i calculated from these results with those previously known, it is possible to draw the following conclusions. Thus, for example, for peptides É-IIIa-2-3, É-IIIb-2, and É-IIIa-2-2 we found K_i 0.0013, 0.05, and 0.109 μM, respectively. In other words, among the peptides isolated there were inhibitors somewhat exceeding in efficiency the widely used synthetic analog Captopril (K_i = 0.0034 μM) and comparable with the most powerful of the known peptide inhibitors, *D*-Cys-*L*-Pro (K_i = 0.0055 μM) [9]. It must, however be borne in mind that the latter measurements were made with the use of another ACE substrate (Hipp-His-Leu), which is hydrolyzed by enzymes considerably less well than FAPGG [7, 8]. On the other hand, the substantial activity of the inhibitors from the viper venom (see Table 1) may apparently be increased still further by their chemical modification. Such an approach, realized by Almquist et al. [10] in relation to peptide inhibitors from snake venoms, not only led to an increase in activity but also raised resistance to the proteinases of the digestive tract.

In order to study the mechanisms of the inhibition of ACE by peptides from the viper venom, we investigated the kinetics of the reaction at two different concentrations of each peptide, analyzing the changes in the full kinetic curve of the hydrolysis of the chromogenic substrate FAPGG by the angiotensin-converting enzyme and expressing the results in the form of Lineweaver-Burk graphs, where the type of inhibition is easiest to recognize. The results obtained are given in Table 1 and in Fig. 1 (as an example of competitive inhibition).

As is known, in the case of true competitive inhibition a set of straight lines with different tangents of their angles of

slope and intersecting the axis of ordinates at a single point is obtained, while the intercept cut off on the axis of abscissas corresponds to $-1/K_m(1+[I]/K_i)$, where K_m is the Michaelis constant, K_i the inhibition constant, and $[I]$ the concentration of inhibitor in the experiment. In contrast to this, in the case of noncompetitive inhibition the intersection of the straight lines is observed on the axis of abscissas.

Thus, the most active inhibitors from the venom of the viper *Echis multisquamatus* Ch. exhibited the properties of competitive ACE inhibitors, which makes an analysis of their amino acid sequences promising. The presence of noncompetitive inhibitors among the peptides is also of considerable interest in connection with the detection in the angiotensin-converting enzyme of two binding sections with different selectivities for endogenous substrates [11].

EXPERIMENTAL

We used: venom of the viper *Echis multisquamatus Ch.* from the Central Asian Zonal Zoological Combine that had been dried over calcium chloride; Sephadexes from Pharmacia (Sweden); TSK gels from Toyo-Soda (Japan); reagents for electrophoresis from Serva (Germany); and the specific chromogenic ACE substrate FAPGG from Sigma (USA).

The isolation of the peptides from the viper venom was achieved as described previously [3]. The purity of the preparations was checked by TLC on silica gel plates, using for detection either ninhydrin or the peptide reagent (tert-butyl hypochlorite—*o*-tolidine—KI).

The molecular masses of the peptide inhibitors used in the kinetic calculations were determined by the gel filtration method on a column of TSK HW-40F under conditions of high ionic strength (0.01 M ammonium formate, pH 3.6, containing 0.1 M NaCl) in order to prevent possible aggregation of the peptides and nonspecific sorption. The calibration of the column and the subsequent molecular mass calculations were carried out in accordance with the formula $(V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of the peptide, V_0 is the free volume of the column, and V_t is the volume of the gel.

Isolation of ACE from the cortical layer of ox kidneys was carried out as in [12], the purity of the enzyme being checked by electrophoresis under denaturing conditions. In the kinetic calculations the molecular mass of the enzyme was taken as 180 kDa on the basis of the results of gel filtration on Sephadex G-200.

The activity of the ACE was measured by the use of 50 μM FAPGG in 0.05 M Tris-HCl, pH 7.5, containing 0.3 M NaCl and 1 μM zinc chloride. The decrease in absorption at 328 nm was monitored with the aid of an SF-46 spectrophotometer. The enzyme (20—40 μl) was added to 2 ml of thermostated (25°C) substrate in the spectrophotometer cell to a final concentration of 1—10 μM. As the unit of activity we took the amount of enzyme that catalyzed the hydrolysis of 1 μmole of substrate per minute under the conditions given above. At the selected substrate concentration the reaction kinetics were of the first order with respect to the substrate ($[S] < K_m$) and hydrolysis reached 98% in 10—30 min.

The Michaelis constant for FAPGG was determined by measuring the initial rates of the reaction at various concentrations of substrate under conditions such that the degree of its hydrolysis did not exceed 10%. To achieve a range of adequate concentrations of substrate (below and above (K_m), the measurements were conducted at three different wavelengths (328, 345, and 352 m) using controls consisting of a single concentration of substrate that could be measured at two wavelengths.

The catalytic constant (k_{cat}) of the hydrolysis of the substrate (FAPGG) was calculated according to [7]. In the calculation of the kinetic parameters we used the method of least squares.

The inhibition constant (K_i) for the peptides from the viper venom were calculated from the Lineweaver-Burk graphs — i.e. relationships in the coordinates $1/v - 1/[S]$. The mathematical treatment of the results, including the calculation of the graphs, was effected by the use of a computer program for the analysis of regressions and correlations that we have developed the mathematical basis of which is the method of least squares.

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