POST-PROLINE ENDOPEPTIDASE. INTERACTION OF THE ENZYME WITH SUBSTRATES CONTAINING DISULFIDE AND THIOETHER BOND

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The free thiol group of post-proline endopeptidase (EC 3.4.21.26) can interact with the disulfide bridge contained in some of the substrates of this enzyme (neurohypophysial hormones and some of their analogues). The influence of these interactions on the activity of this enzyme was studied using several substances modelling individual types of interactions: thiol-disulfide exchange, catalytic interaction and a complex interaction including the two preceding types. Deamino-1-carba-oxytocin is catalytically hydrolysed in the concentration range up to 10^{-3} mol/l, oxytocin and arginine-vasopressin are catalytically hydrolysed in concentrations of 10^{-5} to 10^{-8} mol/l. A reaction leading to inactivation of the enzyme prevails at concentrations of 10^{-3} to 10^{-4} mol/l. When inactivated by lower concentrations of arginine-vasopressin (up to a molar ratio of 1 : 1), the enzyme can be reactivated by incubation with dithiothreitol, higher concentrations of arginine-vasopressin cause irreversible enzyme inactivation.

The physiological function of post-proline endopeptidase in the organism is not yet known. Two hypotheses were recently proposed[1]: *a*) Post-proline endopeptidase participates in processes of non-specific catabolism of proteins and *b*) post-proline endopeptidase specifically inactivates proline-containing peptide hormones, thus taking part in the process of regulating their effects. Up to now, neither of the two hypotheses has been either proved or disproved in a convincing manner. Admitting that the physiological role of post-proline endopeptidase consists in inactivating proline-containing peptide hormones, the question of neurohypophysial hormones must be answered, which in addition contain a disulfide bridge. How will thio-disulfide exchange between the thiol group of the enzyme and the disulfide bridge of the substrate, which has to be assumed, influence the final hormone inactivation?

Results of experiments dealing with the above topic are presented in this communication.

EXPERIMENTAL

Materials

Oxytocin*, [8-lysine]vasopressin, deamino-1-carba-oxytocin[3], Cys-Tyr-Ile-Gly-Gly-Cys* (ref.[4]), benzyloxycarbonylglycyl-proline 4-nitranilide[5] and benzyloxycarbonylglycyl-proline 2-naphthylamide[6] were synthesised by one of us (K.J.). Sephadex G-50 was purchased from Pharmacia (Uppsala, Sweden), DTT and Triton X-100 from Serva (Heidelberg, Germany), Fast Garnet GBC and DTNB from Fluka AG (Buchs, Switzerland). Arginine-vasopressin (AVP) and diiodo--AVP were kindly provided by Ferring AB (Malmö, Sweden). [$^3H_2$]-AVP was prepared from diiodo-arginine-vasopressin by catalytic tritiation (Pd) using gaseous tritium. Diiodo-AVP was likewise prepared by iodating AVP using elementary iodine[7] or Iodogen[8]. Pure diiodo-AVP and the resulting [$^3H_2$]-AVP were isolated from the mixture by HPLC with a reverse phase of Separon SIX C18 in the water–methanol system (6 : 4) containing 0·1% TFA. Final specific radioactivity was $2{\cdot}2 \times 10^{14}$ Bq/mol (6Ci/mmol).

Prolylendopeptidase was isolated from porcine kidney by a method published previously[9,10]. The final preparation contained about 95% of the enzyme, homogeneous by criteria of electrophoretic and sedimentation analysis and determination of the N-terminal amino acid. Activity of the preparation was 20 nmol cleaved Z-Gly-Pro-2-naphtylamide per minute per mg enzyme at 30°C and pH 7·0.

Methods

The incubation mixture (1 ml) contained 0·05—4 mg enzyme (0·75—60 nmol), 10^{-6}—10^{-9} mol peptide, 0·02 mol/l Tris-HCl buffer, pH 7·5 and in some cases also 1 μmol DTT. Incubation proceeded at 37°C generally for 120 min. Samples of the incubation mixture for analysis of the intact peptide content (by a biologic test) or enzyme activity were taken at intervals of 0, 15, 30, 60, and 120 min.

Enzyme assays: a) 40 μl incubation mixture containing 0—200 pkat prolylendopeptidase was added to 3·0 ml of the following solution: 0·156 mmol/l benzyloxycarbonylglycyl-proline 4-nitranilide, 0·03 mol/l Tris-HCl, pH 7·5, 10% dimethyl sulfoxide. The time relationship of absorbance at 406 nm was measured at 25°C and the initial rate of hydrolysis of the substrate was read from the plot.

b) The incubation mixture (1 ml) contained Na-phosphate buffer (20 mmol/l, pH 7·0), benzyloxycarbonylglycyl-proline 2-naphthylamide (250 μmol/l), 15% dimethyl sulfoxide and 0—20 pkat enzyme activity. Incubation proceeded for 0—40 min and was terminated by adding 2 ml of the following solution: 0·05% Fast Garnet GBC solution in Na-acetate, pH 4·0, 0·5 mol/l, containing 5% Triton X-100. In some cases, the enzyme was activated in the presence of DTT (1 mmol/l) before addition of the substrate; the incubation mixture contained in these cases in addition DTT (1 mmol/l) and the Fast Garnet solution moreover contained 2·5 mmol/l $HgCl_2$. The precipitate formed was removed by centrifugation and the absorbance of the clear solution was measured at 525 nm. The amount of 2-naphthylamide liberated was read from a calibration curve.

* The terminology employed complies with the recommendation[2]. Amino acids are of L-configuration. Abbreviations: DTT dithiothreitol, DTNB dithiobis-(dinitrobenzoate), AVP [8-L-arginine]vasopressin, OT oxytocin, HPLC high-performance liquid chromatography, Tris tris-(hydroxymethyl)aminomethane, DCOT-1 deamino-1-carba-oxytocin.

The activity of AVP after incubation with prolylendopeptidase was determined by a biologic test on a despinalised rat, using a method reported by Krejčí and coworkers[11].

Analysis of thiol groups. For thiol group analysis, 100 nmol of the enzyme was incubated with 100 nmol and 1 nmol, resp., AVP under the above-described conditions. The mixture was lyophilised and the amount of thiol groups determined by the Ellman method[12].

Incubation of prolylendopeptidase with [$^3H_2$]-AVP. Incubation was carried out under the above-described conditions. Each sample contained, in addition, 3 μg [$^3H_2$]-AVP.

Chromatography on Sephadex G-50. A sample of 0·5 ml was separated on a Sephadex G-50 column (27 × 1·5 cm) in 0·02 mol/l Tris-HCl buffer, pH 7·5. Fractions of 0·5 ml were taken at a flow-rate of 6 ml/h. For radioactivity measurements, samples of 0·1 ml were taken.

Reductive cleavage with dithiothreitol: 100 μl dithiothreitol solution (100 mmol/l) was added to 1 ml of the sample containing the enzyme-[$^3H_2$]-AVP complex and the mixture was incubated for 30 min at 37°C. The entire sample was then separated on a Sephadex G-50 column (27 × 1·5 cm) in 0·02 mol/l Tris-HCl buffer pH 7·5, containing 1 mmol/l dithiothreitol. Fractions of 0·5 ml were taken at a flow-rate of 6 ml/h. Samples of 0·1 ml were taken for radioactivity measurements. Enzyme activity was likewise measured after incubation of the reaction mixture with dithiothreitol.

RESULTS AND DISCUSSION

From the peptides employed in the study oxytocin (*I*) and [8-L-arginine] vasopressin (*II*) contain proline and a disulfide bridge, and were therefore employed to model the complex interaction, *i.e.* catalytic in conjunction with thiol-disulfide exchange. DCOT-1 (*III*) contains proline and served as model of the substrate with respect to the carba modification, however, interaction with the thiol group of the enzyme was not expected. The cyclic peptide (*IV*) was used to model thiol-disulfide exchange; since it contains no proline, interaction with the catalytic site of the enzyme was not expected.

I OT Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

II AVP Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

III DCOT-1 CH_2—CH_2—CO-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
CH_2———————————

IV Cys-Tyr-Ile-Gly-Gly-Cys

OT and AVP were hydrolysed in concentrations of $10^{-3}-10^{-8}$ mol/l, hydrolysis stopped at concentrations of 10^{-4} mol/l and higher. Table I gives the results of the experiment, in which hydrolysis of AVP in the presence of post-proline endopeptidase was studied using a biologic test on despinalised rats. DCOT-1 was hydrolysed up to concentrations of 10^{-3} mol/l (higher peptide concentrations were not employed). Results of incubation experiments with all peptides studied are summarised in Table

II. This table also includes the results of measuring the activity of post-proline endopeptidase in incubation mixtures (Fig. 1). The activity of the enzyme was measured on the basis of the rate of hydrolysis of a synthetic substrate (see Enzyme assay) in the presence of different concentrations of the peptides *I*–*IV*. From the results of the measurements follows, that while DCOT-1 is hydrolysed in practically the entire concentration range employed and does not influence the activity of the enzyme, oxytocin and [8-L-arginine]vasopressin are hydrolysed at lower concentrations, the enzyme is inactivated at concentrations of 10^{-4} mol/l and higher and hydrolysis stops. The peptide *IV*, which contains no proline, is not hydrolysed, although it inactivates post-proline endopeptidase at concentrations as low as 10^{-5} mol/l.

It becomes evident that enzyme hydrolysis taking place in mixtures with lower substrate concentrations competes with thiol-disulfide exchange between the enzyme and substrate. At higher substrate concentrations the latter reaction prevails and leads to inactivation of the enzyme. If thiol-disulfide interaction actually is the

TABLE I

Inactivation of AVP by post-proline endopeptidase

Concentration mol/l	Inactivation of AVP (%)	
	30 min incubation	60 min incubation
10^{-6}	90	96
10^{-5}	50	65
10^{-4}	0	0

TABLE II

Interaction of peptides with post-proline endopeptidase

	Peptide	Concentration range of peptide hydrolysis mol/l	Concentration range of enzyme inactivation mol/l
I	OT	10^{-5}–10^{-8}	10^{-3}–10^{-4}
II	AVP	10^{-5}–10^{-6}	10^{-4}
III	DCOT-1	10^{-3} and lower	–
IV		–	10^{-5} and higher

cause of enzyme inactivation, it should be possible to activate post-proline endopeptidase in the presence of low-molecular thiols. The results of experiments aimed at this fact are summarised in Fig. 2. It is possible to activate the enzyme even in incubation mixtures containing enzyme and substrate in molar concentrations in the ratio of 1 : 1. With an excess of the substrate (molar concentration ratio 1 : 10) irreversible inactivation of the enzyme takes place, and this process cannot be reversed. The amount of free thiol groups in the enzyme, reacting in a titration reaction, also correlates with enzyme activity: the active enzyme was found to contain 2 groups per molecule, in the partially inactivated enzyme (enzyme to substrate ratio 1 : 1) 0·4 groups per molecule were found and the irreversibly inactivated enzyme contained none.

In order to find out whether a covalent bond is actually formed between the substrate with the disulfide bridge and the thiol group of the enzyme we incubated the enzyme with radioactively labelled AVP and separated the incubation mixture containing different component ratios on a column of Sephadex G-50. We then

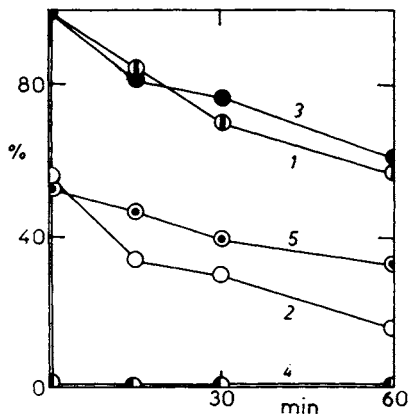

FIG. 1

Inactivation of prolylendopeptidase in the presence of peptides *I*, *III*, *IV*. % of prolylendopeptidase activity in % of maximal activity, min time of incubation, *1* prolylendopeptidase with no peptide, *2* prolylendopeptidase in the presence of oxytocin (10^{-4} mol/l), *3* prolylendopeptidase in the presence of DCOT-1 (10^{-3} mol/l), *4* prolylendopeptidase in the presence of peptide *IV* ($3 . 10^{-4}$ mol/l), *5* prolylendopeptidase in the presence of peptide *IV* ($1{\cdot}5 . 10^{-5}$ mol/l)

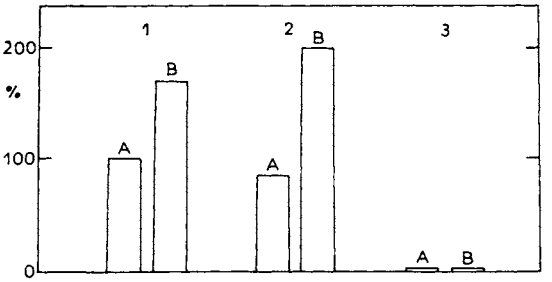

FIG. 2

Activity of prolylendopeptidase in the presence of [8-L-arginine]vasopressin and dithiothreitol. *1* Prolylendopeptidase with no AVP, *2* prolylendopeptidase with AVP (molar ratio 1 : 1), *3* prolylendopeptidase with AVP (molar ratio 1 : 10); A in the absence of dithiothreitol, B in the presence of dithiothreitol ($2 . 10^{-3}$ mol/l), % of enzyme prolylendopeptidase activity in the % of activity in the absence of AVP and DTT

reductively cleaved the isolated enzyme-[$^3H_2$]-AVP complexes in the presence of DTT and again separated on Sephadex G-50 under the same conditions. The elution profiles of the two separation procedures are shown in Fig. 3 and 4. The results of chromatography confirmed that the enzyme-[$^3H_2$]-AVP complex is formed in amounts proportional to the AVP concentration when the enzyme is incubated with [$^3H_2$]-AVP. After reaction with DTT the complex dissociated in part. Comparison of the elution volumes leads to the conclusion that at least two enzyme molecules take part in formation of the complex. Likewise, the labelled peptide formed after dissociation of the complex has a molecular mass higher than would correspond to AVP alone. The possibility cannot be excluded that we obtained a product of partial enzyme autolysis which takes place in the presence of thiol compounds. The identity of the isolated peptide is the subject of our further work.

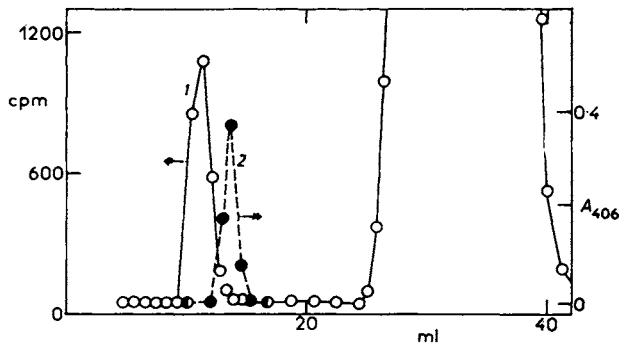

FIG. 3

Elution profiles of prolylendopeptidase on Sephadex G-50. *1* - Enzyme in the presence of [$^3H_2$]-AVP (cpm evaluation), *2* - non-treated enzyme (evaluated according to the hydrolytic activity)

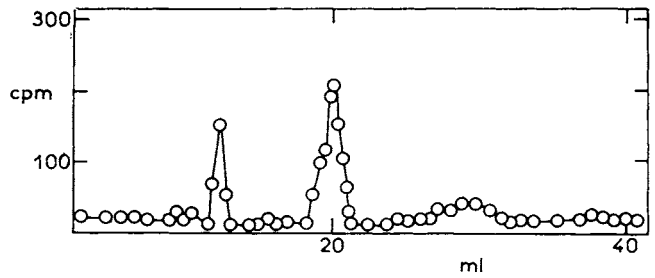

FIG. 4

Elution profile of the enzyme-tritiated vasopressin complex after incubation with dithiothreitol on Sephadex G-50. The same conditions as indicated in Fig. 3

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