ACTIVITY OF COVALENTLY BOUND TRYPSIN IN PARTLY NONAQUEOUS MEDIA

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Trypsin was covalently attached to a synthetic carrier, Enzacryl AH. The attached trypsin retains 25—30% of its original activity, its pH-optimum of cleavage of the substrate N-benzoyl-arginine-*p*-nitroanilide is slightly shifted to the alkaline region. Both free and also attached trypsin catalyze the hydrolytic reaction even in 50% dimethylformamide. The activation energy of the hydrolysis of the substrate by both forms is approximately the same and varies around 9 500 cal/mol.

Trypsin is used – because of its well known specificity – in sequence studies of protein and peptide molecules. The main advantage lies in its stability and in the ability to retain its catalytic properties even in partly nonaqueous media, a factor which considerably extends the possibilities of its application. The so far prepared forms of trypsin insoluble in water indicate that it is possible to attach trypsin to the carrier covalently[1,2] by its NH_2-groups.

In the present study we examined the basic catalytic properties of trypsin attached by a covalent bond to the synthetic polymer carrier Enzacryl AH. N-Benzoyl--D,L-arginine *p*-nitroanilide[3] was employed as substrate and the rate of its cleavage was investigated in dependence on the concentration of dimethylformamide and of hydrogen ions, and on temperature.

EXPERIMENTAL

Reagents. Three-times crystallized bovine trypsin was kindly supplied by Dr V. Holeyšovský of this Institute. Enzacryl AH was purchased from Koch-Light. N-Benzoyl-D,L-arginine *p*-nitroanilide was synthetized by Dr E. Kasafírek, Research Institute for Pharmacy and Biochemistry, Prague. All the remaining chemicals were of analytical purity grade.

Attachment of trypsin. The procedure described in the Koch-Light Catalogue was followed[4]. Enzacryl AH(100 mg) was suspended in 5 ml of 2M-HCl at 0—1°C, 4 ml of 2% $NaNO_2$ was added, and the mixture was stirred 15 min at 1—2°C. The carrier was repeatedly washed with 0·05M borate buffer at pH 8·5 and then mixed with 2 ml of borate buffer containing 2·5—4·5 mg of trypsin. The concentration of calcium chloride was 1 mM. The suspension was slowly stirred for 0·5—24 h at 1—2°C. After the reaction had been completed, the suspension was centrifuged, the sediment washed three-times with 5 ml of precooled borate buffer and finally with 10 ml

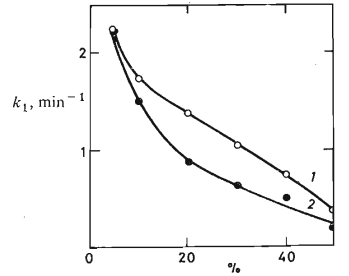

FIG. 1

Dependence of Rate of Substrate Hydrolysis on Dimethylformamide Concentration

The *p*-nitroaniline liberated was determined as described in Experimental. The composition of the reaction mixture was the following: 0·2 ml of 0·2M Tris-HCl buffer at pH 7·5, 0·2 ml of 1 mM N-benzoyl-D,L-arginine *p*-nitroanilide, 0·1 ml of trypsin solution (○—○), 10—20 μg per milliliter of 1 . 10^{-4}M-HCl, and 0·05—0·5 ml of dimethylformamide. The volume of the reaction mixture was made up to 1 ml with water. The incubation mixture for attached trypsin (●—●) was the same. The volume of trypsin added was 0·1 ml and contained 1—2 mg of the carrier with the bound enzyme. The reaction was discontinued after 10 min by the addition of 1 ml of 10% trichloroacetic acid. Ordinate, rate constant, min^{-1}; abscissa, % (v/v) of dimethylformamide.

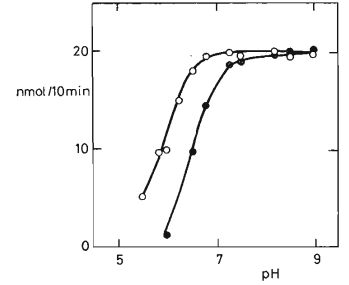

FIG. 2

Dependence of Hydrolysis of N-Benzoyl-L-arginine *p*-Nitroanilide on pH

The experimental conditions were the same as those described in the legend to Fig. 1. The concentration of dimethylformamide was 10% (v/v). 0·2M Tris-maleate buffers were used in the pH-range 5·0—7·2, 0·2M Tris-HCl buffers in the pH-range 7·2—9·0. Free trypsin (○—○), attached trypsin (●—●). Ordinate, *p*-nitroaniline (in nmol) liberated in 10 min, abscissa, pH.

of 1M-NaCl in borate buffer. The attached trypsin was stored in 0·01M Tris-HCl buffer at pH 7·5 and 2—4°C.

Determination of enzymatic activity. The course of the hydrolysis of N-benzoyl-D,L-arginine *p*-nitroanilide was examined by two methods. When free trypsin was used, the rate of liberation of *p*-nitroanilide was followed spectrophotometrically at 406 nm in a cell jacketed at 37°C. (The molar extinction coefficient of *p*-nitroanilide at 406 nm is 9 600). Since with samples of attached trypsin the direct spectrophotometric determination of *p*-nitroanilide is impossible, aliquots of the incubation mixture (without the carrier) were removed at time intervals. The quantity of *p*-nitroanilide in these aliquots was determined spectrophotometrically at 530 nm after acidification of the sample by 10% trichloroacetic acid, diazotation and coupling with N-1-naphthylethylenediamine[5,6]. The rate of the reaction was characterized by a rate constant of the first order.

RESULTS

The weight ratio of trypsin to Enzacryl AH was varied between 1 : 5 and 1 : 40. The quantity of attached trypsin in the investigated region is 85—90% of the trypsin used. The concentration of free trypsin drops during the subsequent 60 min to 3—8% of the quantity employed. The reaction can be regarded as complete in 2 to 3 h. Trypsin attached in this manner retains 25—30% of its original activity.

Dimethylformamide was used as a representative of nonaqueous media because of its more general applicability. As follows from Fig. 1, even in 50% dimethylformamide both free and attached trypsin retain still a fraction of their activity and at the same time there is no marked difference in the dependence of the rate of hydrolysis on the form of the enzyme. The pH-optima of catalysis (expressed in the quantity of *p*-nitroaniline in nmol liberated in 10/min) of the cleavage of N-benzoyl-L-arginine *p*-nitroanilide by covalently attached and free trypsin in 10% aqueous dimethylformamide lie at 7·5—9·0 and 6·5—9·0, respectively (Fig. 2).

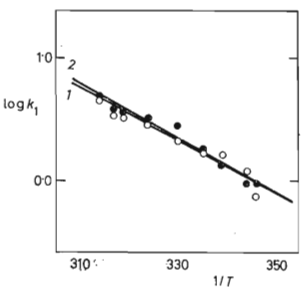

FIG. 3

Dependence of Rate Constant on Temperature

The experimental conditions are those described in the legend to Fig. 1. The concentration of dimethylformamide was 10% (v/v), temperature range 16—45°C. Ordinate, log k_1 abscissa, $1/T$ (1/K). Free trypsin (○—○), attached trypsin (●—●).

The temperature profile of the hydrolysis of N-benzoyl-L-arginine *p*-nitroanilide by trypsin was examined in the range 16–45°C. The values obtained were used for the Arrhenius plot and the activation energy of the reaction was determined. The values of activation energy at pH 8·5 varied around 9500 cal/mol for both kinds of enzymes (Fig. 3). No effects of the covalent attachment of the enzyme on the activation energy of the hydrolytic reaction were observed at the optimal pH-value and in 10% dimethylformamide.

DISCUSSION

The usual method of attachment of trypsin to high molecular weight carriers is the formation of a covalent bond between the primary NH_2-groups of the enzyme and the carrier. The catalytic activity of trypsin does not require the existence of free NH_2-groups, as has been shown with acetyl-trypsin[7]. We used as carrier a commercial product on the basis of modified acrylamide with hydrazide groups convertible into reactive azide groups. The process of the attachment, followed in terms of free trypsin decrease, was completed in 2 h under the conditions described. Covalently attached trypsin has retained its activity for 30 months when stored at 2–3°C between the individual experiments. No drop of activity has been observed even in the absence of Ca^{2+}-ions.

Trypsin is one of the few proteases capable of catalyzing reactions at higher concentrations of nonaqueous solvents. The character of the solvent can cause an irreversible decrease of the activity of the enzyme (*e.g.* trypsin is irreversibly denatured in 20–50% dioxane whereas tryptic activity can be restored by dilution of trypsin solutions in dioxane at high concentrations[8]). A part of the original esterase and peptidase activity is retained also in dimethylsulfoxide or formamide. The use of these solvents does not lead moreover to an irreversible loss of activity[9].

We observed in our experiments with dimethylformamide that the hydrolysis of the substrate takes place even in 50% aqueous solution of this solvent. When the reverse dilution was carried out we observed that even the storage of trypsin (both free or attached) in 20–50% aqueous formamide at 2–3°C did not lead to a loss of the activity of the enzyme.

The catalytic properties of trypsin were measured by the rate of release of *p*-nitroaniline from the substrate. The rate of the liberation was expressed by a rate constant of the first order. Under these conditions of the experiment, the effect of the individual variables on the rate of the acylation or deacylation reaction of the free or attached enzyme was not determined. The presence of the inhibiting D-form of the substrate, the probable inhibitory effect of the product (NH_2^+-group of the product) on the acylation reaction[3], as well as the formation of noncovalent complexes between the enzyme and the substrate which precedes in time the acylation step[10], these are facts which do not permit us to make any conclusions concerning the effect of the indi-

vidual parameters (temperature, pH, concentration of dimethylformamide) on the forms of trypsin mentioned. The attachment of trypsin to the carrier by its NH_2-groups cannot, however, be expected to bring about any marked changes in the binding and cleavage of low molecular weight substrates. This is indicated both by the characteristics of acylated trypsin and also by the identical value of activation energy of the hydrolytic reaction, determined with the free and the attached enzyme.

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