SYNTHESIS AND USE OF AN AMINOQUINOLINONE DERIVATIVE FOR THE FLUOROMETRIC DETERMINATION OF OXYTOCINASE

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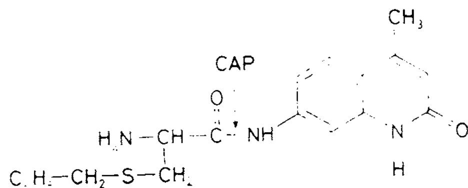
A new fluorogenic substrate for the determination of the activity of human serum oxytocinase — cystine aminopeptidase (EC 3.4.11.3), H-Cys(Bzl)-NH-Meq, has been synthesized. The affinity of H-Cys(Bzl)-NH-Meq to oxytocinase was by two orders higher than that of the usually employed chromogenic substrates. The Michaelis constant of oxytocinase for this substrate was within the range of the optimum pH (7·0–7·5) $2{\cdot}3 \,.\, 10^{-6}$ mol l^{-1}, i.e. in the region of the affinity of the natural substrate, oxytocin. The concentration of dimethylsulfoxide used for the solubilization of H-Cys(Bzl)-NH-Meq (<0·4%) did not influence adversely the course of the enzyme reaction as in the case of chromogenic substrates, where the concentrations of the organic solvent exceeded 3%.

The determination of the activity of serum oxytocinase-cystine aminopeptidase (CAP – EC 3.4.11.3) – which progressively increases during the course of normal pregnancy[1,2] is very useful in the evaluation of the placental insufficiency[3–8]. Increased CAP activity was also found in the serum of patients with serious liver diseases and in seminal plasma[9]. Recently, CAP activity has been detected in the brain synaptic membranes[10] and in a haemolysate of human erythrocytes[11].

Several colorimetric assays using cystyl or cysteinyl β-naphthylamides[3,12–15] or *p*-nitroanilides[2,6,16–20] have been developed for the determination of CAP activity in human serum. However, the utilization of these substrates is limited by their low solubility in water and an affinity to oxytocinase ($0{\cdot}5–2{\cdot}0 \,.\, 10^{-3}$ mol l^{-1}) by 2–3 orders lower than is the affinity of the natural substrate – oxytocin[21]. This leads to the necessity to use such substrate concentrations at which the concentration of the organic solvent employed for its dissolving affects the enzyme activity[20].

These drawbacks stimulated the development of new types of substrates exhibiting higher affinity to the enzyme and better solubility in the concentration region of the expected K_m. Suzuki et. al.[22] employed an aminocoumarin derivative – 7-(S-benzyl-L-cysteinyl-amido)-4-methylcoumarin – as a substrate for fluorometric ultramicrodetermination of human serum cystine aminopeptidase in clinical practice. This substrate and another fluorogenic substrate, 6-(S-butyl-L-cysteinyl-amido)quinoline,

have also been used for the fluorimetric assay of CAP in a haemolysate of human erythrocytes[11]. The work of Kokotos and Tzougraki[23] on fluorescent markers has shown that the amine 7-amino-4-methyl-2-quinolinone (AMeq)*, is an excellent



fluorophore and in the case of chymotrypsin, substrates containing AMeq permit more sensitive enzyme determination than the corresponding ones containing AMec. As an extension to this work we have prepared the fluorogenic substrate H-Cys(Bzl)-NH-Meq for the determination of oxytocinase activity. This paper deals with the synthesis and characterization of the new substrate.

EXPERIMENTAL

Melting points were determined on a Buchi apparatus and are uncorrected. Optical rotations were measured with a Perkin Elmer 141 Polarimeter. Infrared absorption spectra were taken in KBr with a Perkin Elmer 283 B spectrometer. Boc-Cys(Bzl)-OH was of L-configuration and purchased from Fluka. THF was passed through a column of aluminium oxide and distilled over CaH_2. DMF and NMM were distilled from ninhydrin. HCl was thoroughly dried prior to use. All other solvents and chemicals were of reagent grade and used without further purification. The purity of the synthesized compounds was checked by thin-layer chromatography, IR spectra and elemental analysis. Solvent systems for thin-layer chromatography on silica gel F-254 plates (Merck) were: chloroform–methanol (5 : 1) (A); 1-propanol–ammonium hydroxide 25% (67 : 33) (B); 1-butanol–acetic acid–water–pyridine (60 : 6 : 24 : 20) (C). Spots were visualized by UV light, by ninhydrin and by chlorine TDM spray. AMeq was prepared as described in literature[24]. The fluorescence spectra of the compounds AMeq and H-Cys(Bzl)-NH-Meq were taken at 37°C on a computer-controlled fluorescence spectrophotometer Fluorolog (U.S.A.) equipped with a thermostat.

7-(N^{α}-tert-butyloxycarbonyl-S-benzyl-L-cysteinyl-amido)-4-methyl-2-quinolinone

To a stirred solution of Boc-Cys(Bzl)-OH (0·62 g, 2 mmol) and AMeq (0·35 g, 2 mmol) in DMF at 0°C DCC (0·45 g, 2·2 mmol) was added. The mixture was stirred for 2 h at 0°C and overnight

* Abbreviations are generally those recommended by the IUPAC-IUB Joint Commission on Biochemical Nomenclature [Biol. Chem. Hoppe-Seyler *366*, 3 (1985)]. AMec, 7-amino-4--methylcoumarin; AMeq, 7-amino-4-methyl-2-quinolinone; Boc, tert. butyloxycarbonyl; Bzl, benzyl; DCC, N,N′-dicyclohexylcarbodiimide; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; NMM, N-methylmorpholine; Np, 4-nitrophenyl; TES, N-tris(hydroxymethyl)-methyl-2-amino-ethane sulfonic acid; THF, tetrahydrofuran.

at room temperature. The N,N′-dicyclohexylurea was removed by filtration and the solvent was evaporated under reduced pressure. The residue was taken up into ethyl acetate, the organic phase was washed consecutively with cold 0·5M-HCl, H_2O, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4 and evaporated to a small volume. Precipitation with petroleum ether provided 0·62 g (66%) of the product; $[\alpha]_D^{25}$ +22·5° (*c* 1, DMF). IR spectrum (cm^{-1}): 3 325 (NH); 1 690, 1 670, 1 660 (C=O), 1 620 (C=C). For $C_{25}H_{29}N_3O_4S.H_2O$ (485·6) calculated: 61·83% C, 6·43% H, 8·65% N, 6·60% S; found: 61·88% C, 6·45% H, 8·53% N, 6·80% S.

7-(S-benzyl-L-cysteinyl-amido)-4-methyl-2-quinolinone Hydrochloride

Boc-Cys(Bzl)-NH-Meq (0·47 g, 1 mmol) was treated with 4M-HCl in THF (12·5 ml) in the presence of 2-mercaptoethanol (0·1 ml) for 30 min at room temperature. The excess acid and solvent were evaporated and the residue re-evaporated twice from THF. After addition of dry ether, the solid was filtered and recrystallized from methanol dry ether to yield 0·37 g (92%); m.p. 218–220°C; $[\alpha]_D^{25}$ +153·5° (*c* 1, CH_3OH). IR spectrum (cm^{-1}): 3 100–2 700 (NH_3^+); 1 690, 1 645 (C=O); 1 615 (C=C). For $C_{20}H_{21}N_3O_2S.2\,HCl$ (440·4) calculated: 54·55% C, 5·26% H, 9·54% N, 7·28% S; found: 54·57% C, 5·33% H, 9·65% N, 7·24% S.

Purification of Human Serum Oxytocinase

Serum oxytocinase – cystine aminopeptidase (EC 3.4.11.3) was isolated from human retroplacental serum by means of ethanol fractionation according to the previously described method[14]. The crude enzyme preparation was purified further by means of ion-exchange chromatography on DEAE-Sephacel (elution with 0·2M-NaCl in 5 . 10^{-2}M Na-phosphate buffer, pH 5·5) and gel filtration on Sephadex G-200 (elution with 10^{-2}M Na-phosphate buffer, pH 7·0). The specific activity of the enzyme purified in this manner, determined on the basis of the hydrolysis of the chromogenic substrate H-Cys(Bzl)-NH-Np was in the range of 6–13 ncat/mg protein. The protein concentration was determined according to Lowry[25].

Hydrolysis of H-Cys(Bzl)-NH-Meq

The kinetics of the H-Cys(Bzl)-NH-Meq hydrolysis with oxytocinase was measured at 37°C in a reaction mixture containing 500 μl TES buffer (5 . 10^{-2} mol l^{-1}) of the appropriate pH, 100 μl oxytocinase (70 μg) and various concentrations of the substrate (0·5–2·5 . 10^{-6} mol l^{-1}; adjusted with water to total volume 2·8 ml). The concentration of dimethylsulfoxide was 0·1–0·4%. The measurements were performed in the pH range of 5·5–8·5.

The reaction mixture was excited at 340 nm and the fluorescence was recorded at 430 nm. The concentrations of the liberated AMeq determined from the calibration curve were used for the calculation of initial reaction rates and for the determination of the K_m by means of the Lineweaver–Burk plot. The influence of dimethylsulfoxide, used for the solubilization of the substrate, on the rate of the hydrolysis was determined at solvent concentrations within the range of 0·5–15%.

RESULTS

The new fluorogenic substrate H-Cys(Bzl)-NH-Meq was prepared in good yield and purity by deprotection of Boc-Cys(Bzl)-NH-Meq, which was obtained by coupling of the fluorescent marker AMeq with Boc-Cys(Bzl)-OH utilizing the DCC procedure.

The fluorescence spectra of the compounds AMeq and H-Cys(Bzl)-NH-Meq are shown in Fig. 1 and their fluorescence properties are summarized in Table I. Both the amine and the substrate are fluorescent compounds having emission maxima at 430 nm and 410 nm, when excited at 330 nm and 320 nm respectively. At $\lambda_{ex} = 340$ nm and $\lambda_{em} = 430$ nm, the relative fluorescence intensity of AMeq is approximately 100-fold greater than that of an equimolar solution of the substrate. Thus, the enzymic release of AMeq can be followed at these wavelengths, where the presence of the substrate does not interfere.

First of all, it was necessary to ascertain influence of the organic solvent used for the substrate solubilization on the course of its hydrolysis with oxytocinase. From Fig. 2 it follows, that already 5% of DMSO in the reaction mixture causes an approximately 50% decrease in the oxytocinase activity and at the concentration of 15% DMSO, the residual enzyme activity is lower than 20%. A most pronounced effect occurs at low pH (5·5).

Michaelis constants (K_m) of human serum oxytocinase for H-Cys(Bzl)-NH-Meq have been determined in the pH range of 5·5–8·5 and are summarized in Table II. The optimum pH for the hydrolysis was 7·0–7·5. The K_m values for various substrates at optimum pH are compared in Table III. From these data it is evident, that the affinity of H-Cys(Bzl)-NH-Meq to oxytocinase ($K_m = 2 \cdot 3\ \mu mol\ l^{-1}$ at optimum pH) is approximately by two orders higher than that of chromogenic substrates and it is also greater than the affinity of the aminocoumarin substrate to oxytocinase[22].

DISCUSSION

The aim of this work was to prepare a substrate with a high affinity to the enzyme, suitable for the determination of the activity of human serum oxytocinase.

Similarly as in the case of the use of chromogenic substrates, it is essential to employ an organic solvent (DMSO) for the solubilization of H-Cys(Bzl)-NH-Meq, which influences adversely the course of the enzyme reaction. However, the affinity of this substrate to serum oxytocinase is by two orders higher than the affinity of the chromogenic substrates, and thus, in the range of substrate concentrations used for the kinetic studies the concentration of DMSO (<0·4%) did not reach the value which affects the enzyme activity.

The pH-optimum ascertained for the hydrolysis of H-Cys(Bzl)-NH-Meq with serum oxytocinase was within the range 7·0–7·5 which corresponds to the values determined for the hydrolysis of other substrates[22,26–28].

The dependence of the K_m on pH plotted according to Dixon and Webb[29] presented in Fig. 3 shows a value of the molecular ionization constant $pK_s = 6 \cdot 25$ which slightly differs from that obtained for the chromogenic substrates H-Cys(Bzl)-NH-Np and H-Cys(Me)-NH-Np (ref.[20]). However, according to Dixon and

TABLE I

Fluorescence properties of H-Cys(Bzl)-NH-Meq and AMeq. The reagents (0·85 . 10^{-6} mol l^{-1}) were dissolved in TES buffer (5 . 10^{-2} mol l^{-1}, pH 7·0), in the presence of 0·2% DMSO and the measurements were carried out at 37°C

Parameter	H-Cys(Bzl)-NH-Meq	AMeq
Excitation maximum, nm	320	330
Emission maximum, nm	410	430
Relative fluorescence at 430 nm, excited at 340 nm	1	100
Upper limit of the concentration without self-quenching .10^6, mol l^{-1}	2	4

TABLE II

K_m of serum oxytocinase for H-Cys(Bzl)-NH-Meq at different pH values

pH	5·5	6·5	7·0	7·5	8·0	8·5
K_m, 10^{-6} mol l^{-1}	6·2	4·0	2·3	2·3	15·0	25·0

TABLE III

Michaelis constants of serum oxytocinase for various substrates at optimum pH

Substrates	Assay conditions		K_m 10^{-6} mol l^{-1}	Reference
	pH	T, °C		
H-Cys(Bzl)-NH-Meq	7·0—7·5	37	2·3	this paper
H-Cys(Bzl)-NH-Mec	7·0	37	5·4	22
H-Cys(Bzl)-NH-Np	7·0	30	360	26
	7·0	37	290	20
	7·5	25	220	27
	7·4	37	120	30
H-Cys(Me)-NH-Np	7·0	37	440	20
H-Cys-NH-Np | H-Cys-NH-Np	7·7	37	2 500	28
	7·7	37	210	27
	7·5	37	30	27

Webb[29], this value corresponds also most probably to the substrate α-amino group. Values $pK_1 = 6{\cdot}95$ and $pK_2 = 7{\cdot}75$ could reveal the presence of an α-amino group or cystine in the active site of cystine serum aminopeptidase. However, this presumption should be confirmed experimentally.

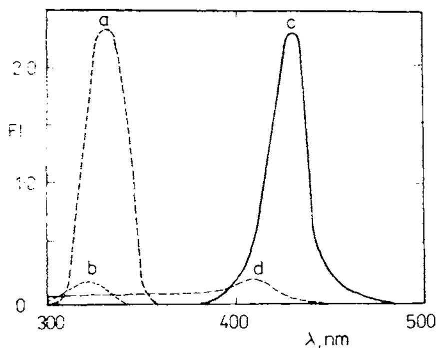


FIG. 1

Fluorescence spectra of H-Cys(Bzl)-NH-Meq and AMeq at pH 7·0—7·5 and 37°C. FI Fluorescnece intensity in arbitrary units. Excitation spectrum of AMeq (a) and H-Cys(Bzl)-NH-Meq (b); emission spectrum of AMeq (c) and H-Cys(Bzl)-NH-Meq (d). The reagents were dissolved in TES buffer ($5 \,.\, 10^{-2}$ mol l^{-1}) to the same concentration ($0{\cdot}85 \,.\, 10^{-6}$ mol l^{-1}); 0·2% DMSO

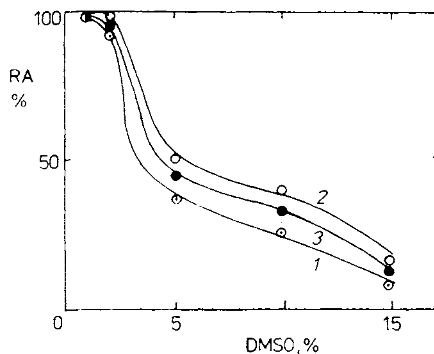


FIG. 2

Influence of dimethylsulfoxide (DMSO) on the relative activity (RA) of serum oxytocinase at pH 5·5 (*1*), 7·5 (*2*) and 8·5 (*3*)

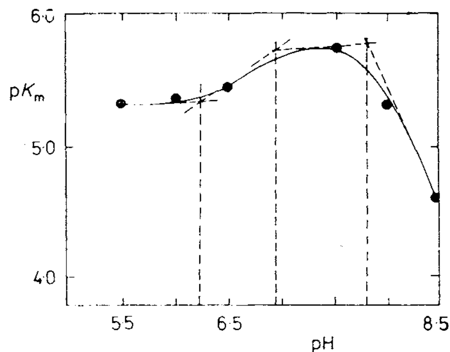


FIG. 3

Dixon–Webb's plot of the dependence of pK_m (log K_m) on pH (ref.[29]) for hydrolysis of H-Cys(Bzl)-NH-Meq with serum oxytocinase

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