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INHIBITION OF SERINE PROTEASES BY STEROIDS

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Abstract—Proteolysis of ^{14}C-labeled globin, as well as the hydrolysis of the specific substrate benzoyl tyrosine ethyl ester, by purified bovine chymotrypsin was found to be inhibited by several steroid hormones. The inhibition of chymotrypsin by the steroids was of a competitive nature, with K_i values of 9.9×10^{-5} M for triamcinolone (9-fluoro-11β,16α,17,21-tetrahydroxy-1,4-pregnadiene-3,20-dione), 1.6×10^{-4} M for cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione), 3.7×10^{-4} M for testosterone (17β-hydroxy-4-androsten-3-one), 5.0×10^{-4} M for dexamethasone (9-fluoro-11β,17,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione), and 1.0×10^{-4} M for epicortisol (11α,17,21-trihydroxy-4-pregnene-3,20-dione). The activity of purified bovine trypsin on its specific substrate, TAME (tosyl arginine methyl ester), also showed a similar pattern of inhibition by steroids. Both chymotrypsin and trypsin were found to bind ^{3}H-labeled dexamethasone and cortisol. This binding was markedly inhibited by the general protease inhibitor, PMSF (phenylmethanesulfonyl fluoride), whereas the chymotrypsin-specific inhibitor, TPCK (L-[1-tosyl-amido-2-phenyl]ethylchloromethyl ketone), inhibited only the steroid binding to chymotrypsin but not to trypsin. These observations indicate that serine proteases recognize steroid hormones in a fashion similar to the recognition of their specific substrates and that the steroids inhibit activity of these enzymes at their binding sites.

The proteolytic activity of trypsin, chymotrypsin and other proteases is known to be modulated by small ligands, including certain cations, anions, detergents, methyl orange, tryptophan and steroids [1–3]. Ryan [4] and Ryan and Baraff [5] have studied extensively the inhibition of tryptic hydrolysis of albumin by testosterone and concluded that the inhibition is exerted through a testosterone-induced modification of conformation of the protein substrate. In these studies, in which the protease inhibition was ascribed to an effect of the ligand on the substrate rather than on the enzyme, protein substrates of large molecular weight were utilized.

We wished to determine whether the inhibition of trypsin and chymotrypsin activity by steroid molecules is also observed with small molecular weight ester substrates on which there are small, if any, conformational effects. In this connection, the present work was undertaken to study the effects of steroid on the catalytic activity of serine proteases and to determine the binding of steroid ligands to chymotrypsin and trypsin.

MATERIALS AND METHODS

Dexamethasone, triamcinolone, BTEE (*N*-benzoyl-L-tyrosine ethyl ester), TAME (tosyl arginine methyl ester), TPCK (L-[1-tosyl-amido-2-phenyl]ethylchloromethyl ketone), PMSF (phenylmethanesulfonyl fluoride), bovine trypsin and bovine chymotrypsin were obtained from the Sigma Chemical Co., St. Louis, MO. Cortisol and testosterone were from Makor Chemicals, Jerusalem. 1,2(n)-[^{3}H]Dexamethasone, 26 Ci/mmole, and 1,2-[^{3}H]cortisol, 40–50 Ci/mmole, were purchased from the Radiochemical Center, Amersham, U.K. Radioactivity measurements were done in 5.0 ml of Insta Gel (Packard) scintillator in a liquid scintillation spectrometer. Protein was determined according to Lowry *et al.* [6].

Assay of esterolytic activity. Chymotrypsin-like and trypsin-like esterolytic activities were measured by spectrophotometric methods using BTEE and TAME as substrates respectively [7, 8]. The activity was assayed at 25° and is given in μmoles/min of substrate hydrolyzed per mg of protein.

Proteolysis of labeled globin. ^{14}C-labeled globin substrate was prepared from hemoglobin. The hemoglobin was first prelabeled according to Roth *et al.* [9]. The globin chains were separated from the ^{14}C-labeled hemoglobin by cold (−70°) acid acetone containing 25 mM 2-mercaptoethanol. The precipitate was washed three times in cold acetone and dissolved in water to yield [^{14}C]globin at a concentration of 18 mg/ml and specific activity of 163×10^3 cpm/mg protein. Degradation of the [^{14}C]globin was followed by incubating, in triplicate, 50 μl of the labeled substrate, in 1.1 ml of 10 mM Tris–HCl buffer, pH 7.8, containing 50 mM $CaCl_2$ and 2.5 μg bovine chymotrypsin, at 37° for 10 min, and determination of the trichloroacetic acid-soluble radioactivity. The steroids were dissolved in ethanol, and 50 μl of the solutions were added to give a final concentration of 10^{-3} M.

Steroid binding assay. Binding of labeled steroids to purified bovine proteases was measured by incubating trypsin or chymotrypsin, 1 mg in 0.1 ml of 10 mM Tris–HCl buffer, pH 7.8, containing 1.5 mM

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Table 1. Effect of steroids on hydrolysis of ^{14}C-labeled globin by purified bovine chymotrypsin*

Steroid (10^{-3} M)	Proteolytic activity (mean cpm ± S.E.)	% of control
None (ethanol)	9180 ± 170	100
Dexamethasone	6830 ± 20†	74
Triamcinolone	7860 ± 10†	86
Cortisol	7210 ± 180†	79
Testosterone	5530 ± 80†	60
Epicortisol	7800 ± 90†	85

* Proteolytic activity is expressed as cpm released by 2.5 μg of the purified enzyme during 10 min of incubation at 37°. Proteolytic activity was assayed as described in Materials and Methods. All steroids were soluble in the final assay mixture containing 5% ethanol.

† Significantly different from control without steroids at $P < 0.001$ (Student's t-test).

EDTA and 10 mM $CaCl_2$, and 10^{-6} M of the ^{3}H-labeled steroids. After 15 min at 37° the unbound steroids were removed by the addition of 1.0 ml of activated charcoal, 20 mg/ml, suspended in the same buffer. The contents were mixed well and centrifuged twice at 1000 g for 3 min, and the radioactivity in the supernatant fraction was counted. When the effect of TPCK or PMSF was tested, the chymotrypsin or trypsin solutions in the same buffer were preincubated with the inhibitors for 15 min at 37° before addition of the labeled steroids.

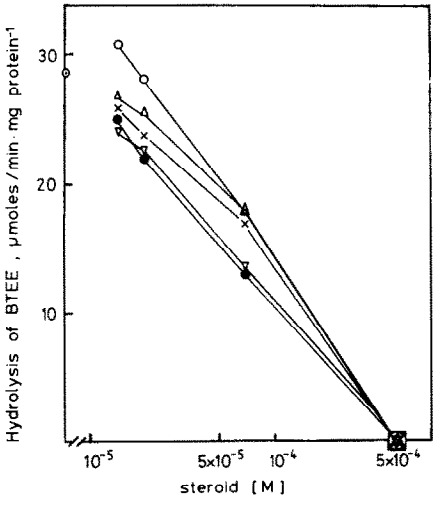

Fig. 1. Effects of steroids on hydrolysis of BTEE by chymotrypsin. Hydrolysis of BTEE was followed by measuring the increase in absorbance at 256 nm in a cuvette containing 0.5 mM BTEE solution in 50% (v/v) methanol, 0.5 ml of 40 mM Tris–HCl buffer, pH 7.8, containing 50 mM $CaCl_2$, and 2 μg/ml bovine chymotrypsin. Total volume was 1.025 ml. Controls containing 25 μl of ethanol exhibited activity of 0.31 μmoles BTEE/min·(mg protein)$^{-1}$. All steroids were dissolved in ethanol and 25 μl of the stock ethanolic solutions were added to give the final steroid concentrations as indicated. Key: (⊙) control containing 25 μl ethanol; (△) triamcinolone; (●) testosterone; (x) epicortisol; (○) dexamethasone; and (▽) cortisol.

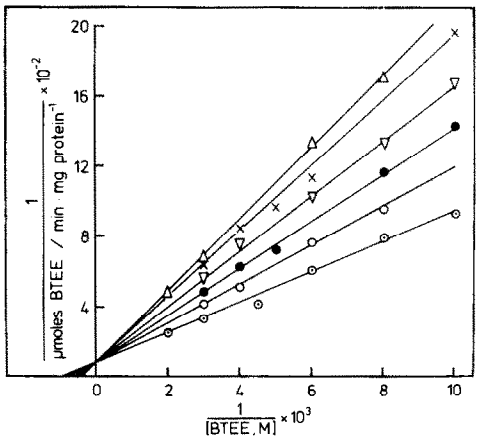

Fig. 2. Competitive inhibition of chymotrypsin activity by steroids. A Lineweaver–Burk plot was constructed from the results of experiments in which ethanolic solutions (25 μl/ml) of the steroids (1.6×10^{-4} M) were added to cuvettes containing 0.5 ml of 40 mM Tris–HCl buffer, pH 7.8, with 50 mM $CaCl_2$ and increasing concentrations of BTEE in 0.5 ml of 50% (v/v) methanol. Reaction was started with chymotrypsin, 10 μg/ml, and absorbance changes were measured at 256 nm. Symbols are defined in the legend to Fig. 1..

RESULTS

Several steroid compounds were found to inhibit the proteolysis of ^{14}C-labeled globin by bovine chymotrypsin (Table 1). Dexamethasone, triamcinolone, cortisol, testosterone and epicortisol at 10^{-3} M produced a moderate inhibition of [^{14}C]globin hydrolysis. Figure 1 shows that the same steroids also inhibited, in a concentration-dependent manner, the esterolytic activity of bovine chymotrypsin on its specific synthetic substrate BTEE. The inhibition was observed immediately upon addition of the steroids to the esterase assay medium.

The competitive nature of the inhibition of chymotrypsin by steroids is documented in Fig. 2, where the enzyme activity at various concentrations of BTEE was measured in the absence and presence of a fixed concentration of steroids. The double-reciprocal plot shows a common intercept for these activity curves, indicating the competitive type of substrate and steroid interaction with the enzyme. From the curves in Fig. 2 the following K_i values were obtained: dexamethasone, 5.0×10^{-4} M; testosterone, 3.7×10^{-4} M; cortisol, 1.6×10^{-4} M; epicortisol (11α,17,21-trihydroxy-4-pregnene-3,20-dione), 1.0×10^{-4} M; and triamcinolone, 9.9×10^{-5} M. The K_m value for the substrate BTEE was 1.1×10^{-3} M, and V_{max} was 129 μmoles/min·mg^{-1}.

Hydrolysis of TAME, a specific substrate of trypsin, by trypsin was also inhibited by the same compounds which inhibit chymotrypsin. Figure 3 shows that dexamethasone, triamcinolone, cortisol, testosterone and epicortisol produced a concentration-dependent inhibition of the hydrolysis of TAME by bovine trypsin.

The competitive nature of the inhibition exerted by the steroids on serine proteases strongly suggested

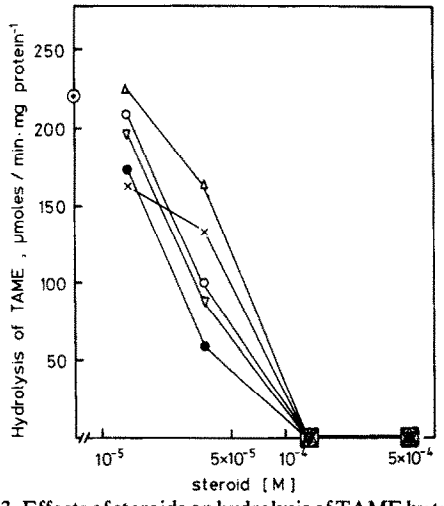

Fig. 3. Effects of steroids on hydrolysis of TAME by trypsin. Hydrolysis of TAME was followed by measuring the increase in absorbance at 247 nm in a cuvette containing 2 μg/ml of purified bovine trypsin, 0.5 mM TAME, 40 mM Tris–HCl buffer, pH 8.1, and 10 mM $CaCl_2$. Symbols as defined in the legend to Fig. 1.

that the steroids compete with the specific substrates on binding to the active site of the serine enzymes. This possibility was further investigated by study of the binding of labeled steroids to the enzymes. Incubation of bovine chymotrypsin with ^{3}H-labeled dexamethasone or ^{3}H-labeled cortisol for 15 min at 37° resulted in a significant binding of the steroids. Representative results are presented in Table 2. When the enzymes were preincubated with inhibitors, it was found that the general protease inhibitor, PMSF, inhibited steroid binding to both chymotrypsin and trypsin; on the other hand, the specific inhibitor of chymotrypsin, TPCK, inhibited steroid binding to chymotrypsin only but not to trypsin. Although in these experiments enzyme and hormone were incubated for 15 min, other experiments (not shown) revealed that binding of ^{3}H-steroids to chymotrypsin was instantaneous and could be observed immediately after mixing of chymotrypsin with the labeled steroids.

DISCUSSION

The present findings show that the proteolytic and esterolytic activities of serine proteases are inhibited by a variety of steroids, and that the inhibition of the cleavage of synthetic, specific substrates by the steroids is of a competitive nature. Previous studies have already shown that the steroids modify rates of hydrolysis of proteins by proteolytic enzymes [4, 5, 10], the effects being dependent on the nature of the steroid, the protein substrate and the proteolytic enzyme, as well as on the molar ratio steroid/substrate. Due to these multiple determinants, both steroid-induced inhibition and steroid-induced enhancement of proteolysis were described, depending on the experimental system used [5, 10]. These effects were attributed to steroid-induced conformational changes of the substrate, in analogy with other systems which showed that small ligands can alter the proteolytic digestability of proteins [3]. The present observation that steroids competitively inhibit hydrolysis of small molecular weight substrates which do not undergo conformational changes suggests that inhibition of proteases by steroids is exerted at the active site of the protease or in its vicinity rather than through a modification of the substrates. This contention is further supported by the demonstration of direct binding of steroids to the proteases, which can be displaced by active site inhibitors but not by a non-specific protease inhibitor. Thus, the steroids appear to inhibit serine proteases by interaction at or near the active site of the enzyme. The mechanism of this inhibition might be explained on the basis of interaction of hydrophobic compounds with the proteases that contain a hydrophobic binding site for the side chains of amino acid residues such as tryptophan, tyrosine and/or phenylalanine. In this context it will be of interest to study the interaction of steroids with proteinases like elastase that contain hydrophobic domains.

The binding data show that purified serine proteases were capable of low-affinity binding of labeled steroids, that steroid binding to trypsin and chymotrypsin was inhibited by the serine protease active site inhibitor, PMSF, and that the chymotrypsin-specific active site inhibitor, TPCK, selectively

Table 2. Binding of labeled steroids to bovine chymotrypsin and trypsin and effect of protease inhibitors*

Enzyme	Steroid	Binding (fmoles/mg)		
		Control	PMSF	TPCK
Chymotrypsin	[^{3}H]Dexamethasone	385	180 (205)	214 (171)
Chymotrypsin	[^{3}H]Cortisol	600	440 (160)	402 (198)
Trypsin	[^{3}H]Dexamethasone	260	150 (110)	260 (0)
Trypsin	[^{3}H]Cortisol	430	320 (110)	430 (0)

* Bovine trypsin and chymotrypsin were preincubated in the absence (control) or presence of 1×10^{-3} M PMSF or 1×10^{-3} M TPCK for 15 min at 37°. Subsequently, binding of the labeled steroids was assayed after incubation of the enzymes for 15 min at 37° with 10^{-6} M ^{3}H-labeled steroids. Numbers in parentheses show the site-specific binding, e.g. the difference between binding in the presence and absence of the inhibitor.

inhibited binding to chymotrypsin, but not to trypsin. These observations imply that the protease inhibitors, by binding to the active site of serine enzymes, interfere with binding of the steroids to the enzyme protein.

It is highly unlikely that the binding of steroids to purified trypsin and chymotrypsin was due to contamination of the commercial protease preparations with plasma corticosteroid-binding globulin as this protein does not display specific binding of synthetic, 9α-fluoro glucocorticoid hormones such as dexamethasone or triamcinolone [11].

Because of the high concentrations of steroids needed to produce a significant inhibition of the protease activity and because steroids that produce a diversity of physiological effects exhibit a similar protease inhibitory activity, the physiological importance of these interactions with proteases is not readily obvious at present. However, two points may be mentioned in this context. First, binding of androgenic steroids, which was observed in the cytosol of rat pancreas in the absence of any known biological effect of androgens in this tissue [12, 13], may be due to steroid binding to pancreatic proteases. Second, in view of recent reports that substrates and inhibitors of serine proteases also inhibit binding of steroids to specific cytosolic receptors [14–16], it is possible that intracellular steroid hormone receptors and serine proteases have a similar ligand- or substrate-recognizing site.

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