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## On the interaction of trypsin with neutral substrates and inhibitors

The classic view of trypsin (EC 3.4.4.4) as a simple enzyme containing only one catalytic site and a single binding site specific for positively charged molecules is challenged by a number of experiments. First, with some substrates, especially $N^{\alpha}$-toluenesulfonyl-L-arginine methyl ester, substrate[1,2] and product[3] activation have been observed. Secondly, a number of investigators have noted "chymotryptic" activity in purified[4] trypsin preparations, that is, the ability of trypsin to bind and hydrolyze neutral substrates[5,6].

The first type of evidence cited above indicates that trypsin contains a second, auxiliary, binding site. The second type of evidence requires that the specificity pattern of trypsin include neutral molecules. We have obtained evidence which suggests a connection between these two types of observations. For some neutral substrates and modifiers binding appears to involve primarily the second site on the enzyme.

We have prepared $N^{\alpha}$-benzoyl-L-citrulline methyl ester (BCME) and tested it as a substrate of trypsin in aqueous and mixed solvent systems. The compound was prepared form L-citrulline by esterification with thionyl chloride, followed by benzoylation[7], m.p. 151–152°, $[\alpha]_D$ — 19.3° (c, 1% 6 M HCl). [Found: C, 57.3; H, 6.5; N, 14.2. Calc. for $C_{14}H_{19}N_3O_4$: C, 57.5; H, 6.2; N, 14.4.] The trypsin catalyzed hydrolysis of this ester was measured by standard pH-stat techniques. The observed kinetic constants in water at 25° (pH 7.0) in the presence of 0.1 M KCl were $K_0 = 4.1 \cdot 10^{-2}$ M and $k_0 = 0.14$ sec$^{-1}$. Diisopropylphosphoryl-trypsin (Worthington) did not catalyze the hydrolysis of the ester. This control experiment demonstrates that catalysis involves the enzyme active site. Trypsin incubated with L-(1-tosylamido-

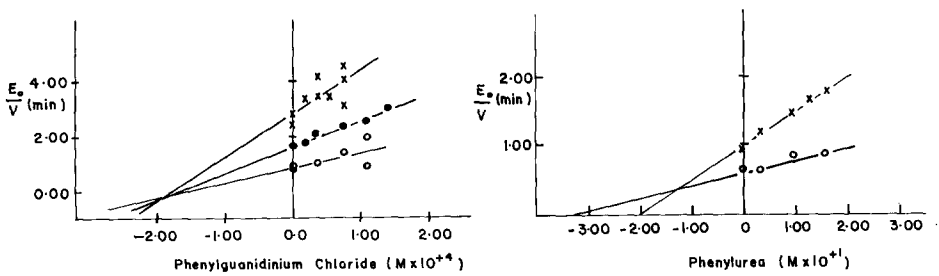


Fig. 1. The effects of phenylguanidinium chloride and phenyl urea on the trypsin catalyzed hydrolysis of BCME. Both experiments were carried out at pH 7.0, 25 ± 0.1°, in 0.1 M KCl and 25% dioxane. Substrate concentrations: left: ×, $2.89 \cdot 10^{-2}$ M; ●, $5.79 \cdot 10^{-2}$ M; ○, $8.57 \cdot 10^{-2}$ M. Right: ×, $5.72 \cdot 10^{-2}$ M; ○, $11.4 \cdot 10^{-2}$ M. The lines in the left-hand graph were generated by means of a computer program which determines the three best lines through the experimental points which also have a common intercept. Competitive, $K_i$, and non-competitive, $K_i'$, inhibition constants can be calculated from the x and y coordinates of the intersection point of the three lines and the intercepts of any of the three lines on the ordinate axis[11]. Under the conditions of this experiment, $K_i = 2.0 \pm 0.3 \cdot 10^{-4}$ M and $K_i' = 2.3 \pm 0.7 \cdot 10^{-4}$ M.

Abbreviations: BCME, $N^{\alpha}$-benzoyl-L-citrulline methyl ester; BAME, $N^{\alpha}$-benzoyl-L-arginine methyl ester.

2-phenyl)ethylchloromethylketone to inactivate any chymotrypsin impurity present hydrolyzed both $N^{\alpha}$-benzoyl-L-arginine methyl ester (BAME) and BCME at the same rate as the control trypsin.

The trypsin catalyzed hydrolysis of a neutral substrate is not novel. However, we have noted that BCME hydrolysis is affected differently by modifiers than are the hydrolyses of typical, positively charged substrates. First, phenylguanidinium chloride, a competitive inhibitor of $N^{\alpha}$-benzoyl-L-arginine *p*-nitroanilide[7] with $K_i = 7.4 \cdot 10^{-5}$ M, is a non-competitive inhibitor of BCME hydrolysis, with a comparable dissociation constant, $K_i = 5.0 \cdot 10^{-5}$ M. Secondly, phenylurea, which we have observed to be an activator at $10^{-2}$ M of the trypsin catalyzed hydrolysis of BAME at a substrate concentration of $10^{-5}$ M is a competitive inhibitor of the trypsin

TABLE I

TRYPSIN CATALYZED HYDROLYSIS OF BAME IN THE PRESENCE OF BCME

Experiments were carried out at pH 7.0, 25.0 ± 0.1°, 0.1 M KCl. [BAME] = $1.93 \cdot 10^{-5}$ M, [*E*] = $3.92 \cdot 10^{-9}$ M in 100-ml volume.

| *BCME concentration (M)* | *Rate observed (equiv/l per min)* | *Rate expected assuming competitive behavior (equiv/l per min)* |
|---|---|---|
| 0.00 | $1.46 \cdot 10^{-6}$ | $1.46 \cdot 10^{-6}$ |
| $2.89 \cdot 10^{-3}$ | $1.53 \cdot 10^{-6}$ | $1.43 \cdot 10^{-6}$ |
| $8.67 \cdot 10^{-3}$ | $1.63 \cdot 10^{-6}$ | $1.34 \cdot 10^{-6}$ |
| $14.4 \cdot 10^{-3}$ | $1.87 \cdot 10^{-6}$ | $1.31 \cdot 10^{-6}$ |

catalyzed hydrolysis of BCME with $K_i = 3.0 \cdot 10^{-2}$ M (12% acetonitrile). Typical results of these modifiers with BCME are presented in Fig. 1. Finally, (in experiments patterned after those of BECHET, GARDINNET AND YON[8]) it was observed that BCME does not act as a competitive inhibitor of the trypsin catalyzed hydrolysis of BAME. Instead, the rate of BAME hydrolysis is increased in the presence of BCME in concentrations equal to or below the observed $K_0$ value of the neutral modifier (Table I).

It appears that both the neutral substrate BCME and the neutral modifier phenylurea bind primarily at the secondary[2] binding site of trypsin. The generality of this phenomenon remains to be determined, but the possibility that the "chymotryptic" activity of trypsin involves the secondary, rather than the primary binding site must be considered in any studies involving neutral substrates and modifiers of trypsin. Activation of the trypsin catalyzed hydrolysis of acetyl glycine ethyl ester by amines[9] which cannot be explained completely by INAGAMI's mechanism[10] is consistent with the view that neutral substrates bind primarily at the auxiliary specificity site.

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1 C. G. TROWBRIDGE, A. KREHBIEL AND M. LASKOWSKI, JR., *Biochemistry*, 2 (1963) 843.
2 J.-J. BECHET AND J. YON, *Biochim. Biophys. Acta*, 89 (1964) 117.
3 S. M. HOWARD AND J. W. MEHL, *Biochim. Biophys. Acta*, 105 (1965) 594.
4 V. KOSTKA AND F. H. CARPENTER, *J. Biol. Chem.*, 239 (1964) 1799.
5 T. INAGAMI AND J. M. STURTEVANT, *J. Biol. Chem.*, 235 (1960) 1019.
6 S. MAROUX, M. ROVERY AND P. DESNUELLE, *Biochim. Biophys. Acta*, 122 (1966) 147.
7 M. MARES-GUIA AND E. SHAW, *J. Biol. Chem.*, 240 (1965) 1579.
8 J.-J. BECHET, M-C. GARDIENNET AND J. YON, *Biochim. Biophys. Acta*, 122 (1966) 101.
9 B. F. ERLANGER AND H. CASTLEMAN, *Biochim. Biophys. Acta*, 85 (1964) 507.
10 T. INAGAMI AND T. MURACHI, *J. Biol. Chem.*, 239 (1964) 1395.
11 G. E. HEIN AND K. POWELL, *Biochem. Pharmacol.*, 16 (1967) 567.

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## Allosteric fine control of citrate synthase in *Escherichia coli*

Citrate synthase (citrate oxaloacetate-lyase (CoA acetylating), EC 4.1.3.7) occupies a key position in the tricarboxylic acid cycle in that it effects the entry of carbon, in the form of acetyl-CoA, into the cycle. The combustion of this carbon in later steps of the cycle provides the energy which is subsequently trapped as ATP. The metabolic regulation of citrate sythase activity would thus also regulate energy production.

Previous studies[1] suggested that citrate synthase activity may be regulated by the level of NADH. Furthermore, the observation that the enzyme may be desensitized towards the inhibitor without loss of catalytic activity indicated that NADH acts as an allosteric effector[2]. Since NADH may be considered both a "product" of the tricarboxylic acid cycle and an intermediate in the formation of ATP, its effect on citrate synthase activity may constitute an essential feedback control mechanism in *Escherichia coli*, especially since (unlike the similar enzyme from mammals[3,4], plants[5] and yeast[6]) the *E. coli* enzyme is essentially insensitive to inhibition by ATP (ref. 1). In order to study further the nature and mechanism of the metabolic control of citrate synthase, the enzyme has been isolated from *E. coli* strain K 12 in a highly purified state. This has permitted a study of the action of possible allosteric effectors both in stimulating and inhibiting enzymic activity.

The organisms were grown up in 15 l of medium[7] in a carboy at 30° with 50 mM acetate as sole carbon source. After washing the cells with water and disrupting them by ultrasonication, the purification procedure[8] consisted of the following steps: (1) treatment of the sonic extract with protamine sulphate (1 mg per

Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).