1 G. A. LEVVY AND C. A. MARSH, *Advan. Carbohydrate Chem.*, 14 (1959) 381.
2 G. A. LEVVY AND C. A. MARSH, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 4, Academic press, New York, 1960, p. 397.
3 P. TALALAY, W. H. FISHMAN AND C. HUGGINS, *J. Biol. Chem.*, 176 (1946) 757.
4 E. BUDDECKE AND E. WERRIES, *Z. Physiol. Chem.*, 340 (1965) 257.
5 T. W. KWON AND H. S. OLCOTT, *Bioch. Biophys. Res. Commun.*, 19 (1965) 300.
6 S. SCHWIMMER, *J. Theoret. Biol.*, 3 (1962) 102.
7 H. HAUSS AND G. G. JUNGE-HÜLSING, *Deut. Med. Wochschr.*, 86 (1961) 763.
8 E. ODEBLAD AND H. BOSTRÖM, *Acta Chem. Scand.*, 7 (1953) 233.
9 E. BUDDECKE AND O. HOEFELE, *Z. Physiol. Chem.*, in the press.
10 E. BUDDECKE AND D. PLATT, *Z. Physiol. Chem.*, 343 (1965) 61.

Received August 11th, 1966

BBA 63228

Inhibition of the trypsin-catalyzed activation of chymotrypsinogen by *N*-acetyl-L-3,5-dibromotyrosine

Chymotrypsinogen readily forms a stoichiometric equilibrium complex with reversible chymotrypsin (EC 3.4.4.5) inhibitors[1–4]. The great similarity of all the binding characteristics suggested that the binding sites are identical in chymotrypsinogen and chymotrypsin[1,4]. Thus, at least part of the active center of chymotrypsin is preformed in the zymogen molecule. In the trypsin (EC 3.4.4.4)-catalyzed activation of chymotrypsinogen an N-terminal isoleucine is formed which has been shown to control through its ionization the activity of chymotrypsin[5]. The localization of this isoleucine residue with respect to the binding site of chymotrypsinogen and hence the active center of chymotrypsin, is of primary importance in the elucidation of the mechanism of the control process. Studying the activation of the zymogen in the presence of inhibitors combining with its binding site is one possible tool for the determination of the relative position of the catalytically important isoleucine residue. If the latter is in close proximity to the binding site then the binding of an inhibitor to this site certainly would inhibit the activation of the zymogen. If, on the contrary, the isoleucine is widely separated from the binding site, then the binding of an inhibitor to the zymogen would only have a small secondary effect on its activation.

In order to decide between these two possibilities, we determined the influence of *N*-acetyl-L-3,5-dibromotyrosine on the trypsin-catalyzed activation of chymotrypsinogen. This particular inhibitor was chosen for the reason that its binding characteristics to the zymogen are accurately known[1]. The dissociation constant of the inhibitor–zymogen complex, as measured by equilibrium dialysis at pH 5 has a value of $0.83 \cdot 10^{-2}$ M at 5° and $0.985 \cdot 10^{-2}$ M at 20°.

If the activation process would occur at close proximity to the binding site and the zymogen–inhibitor complex would be totally unable to react with trypsin, then the activation reaction should be described by the following kinetic scheme:

$$Chtg \cdot I \underset{K_i}{\overset{+I}{\rightleftarrows}} Chtg + Try \overset{K_s}{\rightleftarrows} Try \cdot Chtg \xrightarrow{k_{cat}} Try + Cht$$

where *Chtg*, *I*, *Try* and *Cht* designate chymotrypsinogen, *N*-acetyl-L-3,5-dibromotyro-

sine, trypsin and chymotrypsin, respectively. Then with $[Try] \ll [Chtg]$ and $[Try] \ll [I]$ the rate equation is

$$\frac{d\,Chtg}{dt} = \frac{k_{cat}\,Try_o\,Chtg}{Chtg + K_s(1 + [I]/K_i)}$$

Since the reaction is first order to at least 90% of completion even at $Chtg = 1 \cdot 10^{-3}$M, K_s must be much greater than $Chtg$(ref. 6). Then the experimental first order rate constant is $k_{exp} = \frac{k_{cat}\,Try_o}{K_s(1 + [I]/K_i)}$ and in the absence of the inhibitor $k_{exp_o} = \frac{k_{cat}\,Try_o}{K_s}$

Then $K_i = \frac{I}{k_{exp_o}/k_{exp} - 1}$ (Equation 1).

We measured the rate of the trypsin-catalyzed activation of chymotrypsinogen in presence of various amounts of *N*-acetyl-L-3,5-dibromotyrosine by determining the amount of chymotrypsin formed at various time intervals in the reaction mixture. The rate assays were carried out spectrophotometrically at 300 mμ and pH 7, at 25°, using methyl *N*-acetyl-L-tryptophanate as substrate, after dilution of the reaction mixture[7]. From the data first order rate constants have been calculated which are reported in Table I together with the values of K_i calculated on the basis of Equation 1. The inhibitor constant determined in this manner is independent within experimental error of the inhibitor concentration, indicating that the data are at least compatible with Equation 1.

TABLE I

THE TRYPSIN-CATALYZED ACTIVATION OF CHYMOTRYPSINOGEN (*Chtg*) IN PRESENCE OF *N*-ACETYL-L-3,5-DIBROMOTYROSINE (*I*)

The zymogen was activated at 25°, pH 5 (0.2 M acetic acid–sodium acetate buffer with KCl), ionic strength 0.187 M; using Worthington twice crystallized, lyophilized bovine trypsin, Lot TRL6263 and Worthington chromatographically homogenous bovine chymotrypsinogen, Lot CGC763A, $Try_o = 1.3 \cdot 10^{-5}$ M; $Chtg = 8 \cdot 10^{-5}$ M. *p*-Nitrophenyl-N^a-benzyloxycarbonyl-L-lysinate was hydrolyzed under identical conditions, with $[Try_o] = 1.9 \cdot 10^{-7}$ M; $[S_o] = 8.6 \cdot 10^{-5}$ M and 1.6% acetonitrile.

$[I] \cdot 10^2$ (*M*)	$k_{exp} \cdot 10^5$ (sec^{-1})	$k_{exp}/[Try_o]$ ($M^{-1} \cdot sec^{-1}$ for *Chtg*)	$K_i \cdot 10^2$ (*M*)	$(k_{cat}/K_m) \cdot 10^{-5}$ ($M^{-1} \cdot sec^{-1}$ for *p*-nitrophenyl-N^a-benzyloxycarbonyl-L-lysinate)
—	47.9	36.8	—	7.5
1.57	21.8	16.7	1.31	9.5
3.13	11.5	8.86	0.99	9.0
4.70	8.4	6.46	1.00	8.5

We find an average $K_i = 1.1 \cdot 10^{-2}$ M, while from the equilibrium dialysis data[1] we calculate for 25° $K_i = 1.04 \cdot 10^{-2}$ M, in excellent agreement. Under identical conditions the second-order rate constant (k_{cat}/K_m) of the trypsin-catalyzed hydrolysis of *p*-nitrophenyl-N^a-benzyloxycarbonyl-L-lysinate, as determined spectroscopically[8] is independent of the inhibitor concentration within experimental error (Table I). Similarly, 10^{-2} M *N*-acetyl-L-3,5-dibromotyrosine had no observable effect on the

trypsin-catalyzed hydrolysis of N^{α}-benzoyl-L-arginine ethyl ester at pH 5, 25°. Thus *N*-acetyl-L-3,5-dibromotyrosine is not a trypsin inhibitor under these conditions.

On the basis of the rate assay measurements and using the kinetic constants determined previously[7], one can calculate the amount of chymotrypsin produced at the end of the reaction (5 to 6 half-lives). In all it was $95 \pm 2\%$ of the amount calculated from the dry weight of the zymogen used. Therefore, the total amount of enzyme produced in the reaction is independent of the inhibitor concentration.

Our data thus show that the formation of the inhibitor–zymogen complex competitively inhibits the activation of the zymogen.

The process of formation of active chymotrypsin from the zymogen consists of the hydrolysis of one single peptide bond, namely that Arg-15–Ile-16 bond of the single-chain zymogen molecule[9,10]. Competitive inhibition of the zymogen activation then means that the inhibitor prevents the formation of the appropriate complex between the active site of trypsin and the Arg-15 of the zymogen. The hypothesis that the inhibition would prevent the binding of the zymogen to a "secondary binding site" of trypsin seems unlikely, since chymotrypsinogen is readily activated by other enzymes also, *e.g.*, by subtilisin[11,12]. Equally unlikely is the possibility that the presence of the inhibitor would prevent a conformational change of the zymogen, since the inhibitor used is a virtual substrate of chymotrypsin and it would rather stabilize the conformation of the active enzyme.

The most likely interpretation of our results, therefore, is that the inhibitor shields the Arg-15 by direct contact and thus exerts its action by steric hindrance. Therefore, since the binding site of the zymogen is identical to the binding site of the active chymotrypsin[4], the Arg-15–Ile-16 bond of the zymogen molecule must be part of the region from which the active center of chymotrypsin is formed.

It is then quite probable that Ile-16 is one of the constituents of the active center of chymotrypsin and that it controls the activity of the enzyme by a mechanism requiring direct contact between the substrate and the isoleucine side-chain, *e.g.*, by competitively inhibiting the binding of the substrate, instead of an "action at distance", *e.g.*, by conformational changes.

The author wishes to thank Professor M. L. BENDER and Professor T. NAKAMOTO for helpful discussions. This work was supported by U.S. Public Health Service Medical Research Grant No. GM 13885-01.

Department of Biochemistry,
University of Chicago,
Chicago, Ill. (U.S.A.)

F. J. KEZDY

1 F. VASLOW AND D. G. DOHERTY, *J. Am. Chem. Soc.*, 75 (1953) 928.
2 H. WEINER AND D. E. KOSHLAND, JR., *J. Biol. Chem.*, 240 (1965) 2764.
3 A. N. GLAZER, *Proc. Nat. Acad. Sci. U.S.*, 54 (1965) 171.
4 D. A. DERANLEAU AND H. NEURATH, *Biochemistry*, 5 (1966) 1413.
5 H. L. OPPENHEIMER, B. LABOUESSE AND G. P. HESS, *J. Biol. Chem.*, 241 (1966) 2720.
6 J. A. V. BUTLER, *J. Am. Chem. Soc.*, 63 (1941) 2968.
7 B. ZERNER, R. P. M. BOND AND M. L. BENDER, *J. Am. Chem. Soc.*, 86 (1964) 3674.
8 M. L. BENDER AND F. J. KEZDY, *J. Am. Chem. Soc.*, 87 (1965) 4954.
9 M. ROVERY, M. POILROUX, A. CURNIER AND P. DESNUELLE, *Biochim. Biophys. Acta*, 16 (1955) 590 and 17 (1955) 565.
10 B. S. HARTLEY, *Nature*, 201 (1964) 1284.
11 A. ABRAMS AND C. F. JACOBSEN, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, 27 (1951) 447.

12 A. BRONFENBRENNER, K. LINDENSTRØM-LANG AND M. OTTESEN, *Biochim. Biophys. Acta*, 20 (1956) 408.

Received September 9th, 1966

Biochim. Biophys. Acta, 132 (1967) 197–200

BBA 63229

A constitutive aldolase for 4-hydroxy-2-ketoglutarate in soil bacteria

4-Hydroxy-2-ketoglutarate (HKG), a product of mammalian enzymes[1–3], has also been isolated from higher plants[4,5]. HKG aldolase, which catalyzes the reversible formation of HKG from glyoxylate and pyruvate, has been purified from rat liver[6–8] but has not been described in plants or bacteria; HKG formation in Acetobacter extracts, however, has recently been reported[9] as an enzymic product of glyoxylate and oxaloacetate. This communication reports the partial purification of a constitutive enzyme from soil bacteria which catalyzes reversible cleavage of HKG to glyoxylate and pyruvate. From $NaBH_4$ inactivation studies, the bacterial aldolase may bind both glyoxylate and pyruvate as Schiff base ligands. The enzyme also appears to catalyze a non-stereospecific cleavage of HKG. In these unusual respects it resembles the highly purified mammalian enzyme[8].

DL-HKG was made chemically by the method of RUFFO *et al.*[10], using 10 mmoles each of sodium glyoxylate and oxaloacetic acid. After incubation (3 h, 37°), the reaction mixture was acidified to pH 4.0 (formic acid) and stirred 30 min to remove CO_2 and to complete decomposition of residual oxaloacetate. HKG was isolated by Dowex 1 formate chromatography (2 cm × 40 cm column), essentially as described earlier[2] except that the formic acid gradient was made with a 200-ml mixing chamber (initially 3.8 M formic acid) and a reservoir of 7 M formic acid. Residual glyoxylate appeared at about 100 ml, pyruvate at about 280 ml, and HKG at about 370 ml. HKG-containing eluates were concentrated to dryness several times from added water in a rotary evaporator (40°) to remove formic acid. The yield of DL-HKG averaged 75%, and neutralization required 2.00 to 2.16 equivalents of NaOH, relative to the assay with glutamic dehydrogenase[11] (EC 1.4.1.2). Time of exposure to acid was minimized to avoid decomposition (see below).

Certain properties of HKG were noted in addition to those already described[2,3]. Although neutralized solutions of HKG are stable for at least several months in the cold[11], acid solutions were unstable. In one trial, a solution of HKG initially at 86 mM and pH 2.6 (formic acid eluate) showed first-order decomposition (glutamate dehydrogenase assay) at 37°, with a half-life of about 9.5 days. Appreciable decomposition also occurred in acid solutions stored at −15°. The decomposition products were not identified; however, they appeared to be acid (relatively constant titration value during decomposition), and unreactive with 2,4-dinitrophenylhydrazine. In experiments with [5-^{14}C]HKG, synthesized enzymically[11] from pyruvate and [1-^{14}C]glyoxy-

Abbreviation: HKG, 4-hydroxy-2-ketoglutarate.