IRREVERSIBLE INHIBITION OF BEEF LIVER VALYL-tRNA SYNTHETASE BY AN ALKYLATING DERIVATIVE OF L-VALINE

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1. Introduction

Pseudosubstrates containing chemically reactive groups allow valuable information about the catalytic centres of enzymes to be obtained. Halogenomethylketones, inhibitors which retain the main structural features of substrates as well as possessing pronounced alkylating properties, have given good results. They have been used to demonstrate the role of histidine residues in trypsin and α-chymotrypsin [1, 2] and the essential nature of SH-groups in papain and carbamoylphosphate synthetase [3, 4].

Because of these and other observations it looked promising to use this type of irreversible inhibitor to investigate the active centres of aminoacyl-tRNA synthetases (EC 6.1.1). The carboxyl group of the substrate amino acid is not essential for binding to these enzymes [see 5] and it may therefore probably be substituted by an alkylating group without preventing binding.

It is shown here that L-3-amino-1-chloro-4-methylpentan-2-one (chloromethylketone), an analogue of L-valine, is an irreversible inhibitor of valyl-tRNA synthetase.

2. Materials and methods

2.1. *Enzymes and tRNA*

Total tRNA was isolated from beef liver [6]. Highly purified beef liver valyl-tRNA synthetase was obtain by the procedure in [7] with modifications to be described elsewhere. The preparation contained traces of some other synthetases and some other proteins.

2.2. *Activity measurement*

Enzyme activity was measured by the initial rate (5 min, 30°C) of aminoacylation of total tRNA with [^{14}C]valine. The incubation mixture (0.2 ml) contained (in μmoles): Tris–HCl, pH 7.5, 10; $MgCl_2$, 4; KCl, 4; ATP, 0.6; L-valine, 0.01: in addition, human serum albumin 0.05 mg; tRNA 0.05–0.1 mg and a rate-limiting amount of valyl-tRNA synthetase. L-[^{14}C]-valine (100–130 Ci/mole) was from the Institute for Isotope Research (Czechoslovakia), ATP (Na-salt) from Reanal (Hungary). The reaction was stopped by addition of 0.2 ml cold 10% trichloroacetic acid, precipitates were collected on nitrocellulose filters (RUFS, Chemapol, Czechoslovakia), washed with cold 5% trichloroacetic acid and counted in an Intertechnique SL-30 scintillation counter.

Activity in the presence of L-valine methyl ester and of L-3-amino-4-methylpentan-2-one (methylketone) was measured in the same way.

2.3. *Inhibition*

The rate of irreversible inhibition was followed by assaying activity (as in 2.2.) in samples at different times after mixing enzyme and inhibitor. The inhibitors, chloromethylketones, L-valine and L-aspartate analogues, were incubated with enzyme in 0.05 M K-phosphate buffer, pH 7.5 at 25°C. The kinetic constants of inhibition were determined with a 100-fold concentration excess of inhibitor over enzyme.

2.4. *Test for possible reactivation*

Enzyme (3 mg/ml) was incubated for 5 h in the presence of 5×10^{-4} M chloromethylketone or methyl ketone as in 2.3. Aliquots were diluted 100-fold and

assayed as in 2.2. or aliquots were assayed after passing through a Sephadex G-25 (fine) column (1 × 20 cm) equilibrated with 0.05 M K-phosphate buffer, pH 7.5, to remove excess inhibitor. The activities were compared with that of enzyme subjected to the same procedure but without inhibitor.

2.5. *Syntheses*

For the synthesis of 3-amino-1-chloro-4-methylpentan-2-one (chloromethylketone), *N*-carbobenzoxy-L-valine [8], isobutyl chloroformate [9] and diazomethane [10] were prepared. A solution of 1.2 g (5 mmol) of *N*-carbobenzoxy-L-valine in 10 ml dry ethyl acetate was treated at −5°C with 0.7 ml (5 mmol) triethylamine and 0.68 ml (5 mmol) isobutyl chloroformate. After 5 min the triethylammonium chloride formed was removed and 10 ml (0.88 N) of a solution of diazomethane in dry ether were added. The mixture was shaken for 3 hr at 0°C and left to stand for 10 hr. It was then washed at 4°C with water, 5% $NaHCO_3$, washed again and dried over anhydrous Na_2SO_4. After removal of half the solvent, light petroleum was added to initiate crystallization of diazoketone, the yield of which was 0.66 g (50%), m.p. 77–78°C, IR spectrum (in KBr) had bands at 2120 cm^{-1} (−N_2) and 1715 cm^{-1} (−C=O).

Hydrogen chloride gas was passed into a solution of 0.265 g (1 mmol) diazoketone in 10 ml dry ether for 30 min at 4°C. After removal of the ether, the residue was dried *in vacuo* over NaOH. Two ml 37% HBr in glacial acetic acid was added and the mixture shaken for 40 min at 20°C. After addition of 5 ml dry ether the flask was allowed to stand for 12 hr at 4°C. The solution was then decanted and the precipitate washed several times with petroleum in the cold and dried *in vacuo* over P_2O_5 and NaOH. Yield 0.15 g (55%), m.p. 80–82°C, IR spectrum (in KBr) had a band at 1733 cm^{-1} (−C=O), electrophoretic mobility 1.05×10^{-4} cm^2/sec/V.

L-3-amino-4-methylpentan-2-one (methylketone) was prepared by reduction of chloromethylketone on Pd/C with close to a quantitative yield, m.p. 99–100°C, electrophoretic mobility 1.4×10^{-4} cm^2/sec/V.

2-Amino-4-keto-5-chloropentanoic acid was prepared starting from the α-benzyl ester of *N*-carbobenzoxy-L-aspartic acid [12] by the method used above for chloromethylketone L-valine analogue. To effect the simultaneous removal of the α-benzyl group and the carbobenzoxy group, treatment with 37% HBr in glacial acetic acid was extended to 12 hr. The product was purified by preparative electrophoresis, m.p. 151–152°C [4], electrophoretic mobility 1.3×10^{-4} cm^2/sec/V. L-valine methyl ester was prepared as in [11].

Analytical electrophoresis was in pyridine–acetic acid–water (1:10:189, v/v), pH 3.5, potential gradient 100 V/cm, FN12 paper, with cooling. The same system was used for preparative electrophoresis but with Whatman 3 MM paper and 80 V/cm for 2 hr. All substances prepared were characterized by elemental analysis; the values found corresponding with theoretical ones to within the limits of accuracy of the methods used.

3. Results

The dependencies of the reaction velocities on L-valine concentration with various concentrations of carboxyl-substituted L-valine analogues, plotted in Dixon's co-ordinates [13], are shown in fig. 1. The inhibition is evidently competitive and reversible and in accord with similar data for *E. coli* valyl-tRNA synthetase with some other analogues [14]. The K_i values, obtained graphically from the data in fig. 1, are 4×10^{-4} M for the methyl ester and 3×10^{-4} M for the methylketone, whereas with L-valine K_m = 7×10^{-6} M. Since both methylketone and L-valine methyl ester have high affinities for the enzyme, low K_i values, it is reasonable to expect that chloromethylketone will also have a considerable affinity and,

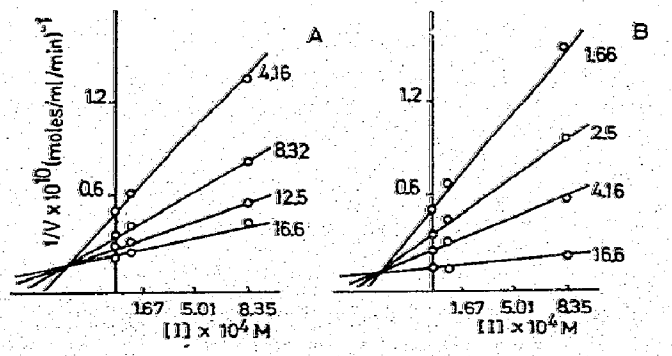

Fig. 1. The effect of methylketone, an analogue of L-valine (A) and L-valine methyl ester (B) on the formation of valyl-tRNA catalyzed by valyl-tRNA synthetase from beef liver. Concentrations of L-valine are μM.

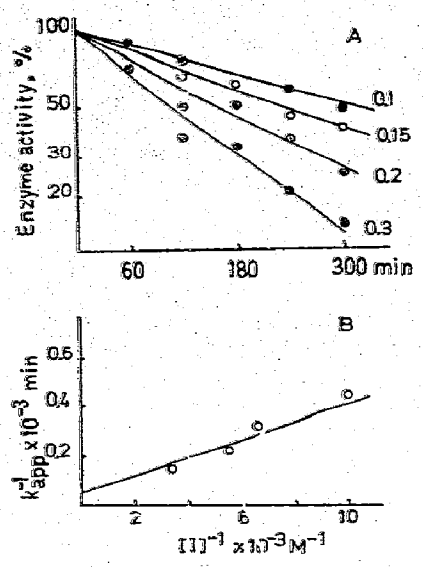

Fig. 2. The dependence of activity of valyl-tRNA synthetase on the time of incubation with chloromethylketone, an analogue of L-valine: A) Inhibitor concentrations are mM. Y-axis is logarithmic. Concentration of the protein, 0.6 mg/ml; B) The dependence of k_{app} on concentration of chloromethylketone, an analogue of L-valine (calculated from fig. 2A).

because of its alkylating ability, will act as an irreversible inhibitor. As is shown in fig. 2, chloromethylketone does indeed cause a progressive inhibition of the enzyme, even in low concentrations. During the first hours L-valine has a protective action against chloromethylketone inhibition (table 1).

No reactivation was observed after gel-filtration of the enzyme–chloromethylketone complex and treatment with excess L-valine. A 100-fold dilution of the enzyme–methylketone complex led to almost complete restoration of enzyme activity while it had no effect on the enzyme–chloromethylketone complex. The chloromethylketone analogue of aspartic acid, 2-amino-4-keto-5-chloropentanoic acid, at 10^{-4} M caused no inactivation of the enzyme. Since all the evidence points to an irreversible inhibition by the chloromethylketone analogue of L-valine, a further kinetic analysis by the method of Kitz and Wilson [15] was made. The reaction scheme for the formation of the alkylated enzyme (E'I) is:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E'I$$

Table 1

The influence of preincubation with chloromethylketone, an analogue of L-valine, on the activity of valyl-tRNA synthetase in the absence or presence of L-valine.

Chloromethylketone (M) CH_3>CH–CH(NH_2)–$COCH_2Cl$ CH_3	L-Valine (M)	Residual activity, %		
		Time of preincubation with inhibitor (hr)		
		1	2	4
5×10^{-5}		90	85	73
5×10^{-5}	5×10^{-5}	100	100	80
5×10^{-4}		35	16	10
5×10^{-4}	5×10^{-4}	100	45	35

where EI is a reversible enzyme–inhibitor complex and k_2 the rate constant for the conversion of EI into E'I.

Semilogarithmic plots of the degree of inactivation with time (fig. 2A) are straight lines, showing pseudo-first-order kinetics. The half-time of conversion of EI into E'I ($t_{1/2}$) and the first-order rate constants for irreversible inactivation (k_{app}) at each inhibitor concentration may be calculated from fig. 2A. A reciprocal plot of k_{app} against inhibitor concentration is shown in fig. 2B, the positive intercept on the Y-axis shows that formation of the reversible complex EI predeeds that of the irreversible complex E'I. From fig. 2B, k_2 (0.02 min^{-1}) and K_i may be calculated. The approximate value of $K_i \approx 7 \times 10^{-4}$ M found is close to the values for methylketone and L-valine methyl ester.

With high concentrations of chloromethylketone ($\geq 10^{-3}$ M), deviations from the pattern characteristic of simple irreversible inhibition were observed, probably due to alkylation reactions with additional SH-groups.

4. Discussion

The data presented here provide evidence that chloromethylketone, and alkylating analogue of L-valine, is an irreversible inhibitor of beef liver valyl-tRNA synthetase. The inhibition develops progressively with time, no reactivation occurs after removal of excess inhibitor and addition of high concentrations

of substrate and the kinetic pattern is characterstic of irreversible inhibition.

Alkylation involves a region of the molecule important for its catalytic function, probably the substrate-binding site because L-valine protects against inactivation and the kinetics of inhibition show involvement of the active site, inhibition occurs even at low inhibitor concentrations at which the rate of alkylation outside the catalytic centre is usually low and another alkylating derivative, the analogue of L-aspartic acid, does not inactivate the enzyme at similar concentrations.

The present work opens up the way to specific affinity labelling of aminoacyl-tRNA synthetases with halogenmethylketone analogues of their substrate amino acids.

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