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Labelling of L-Isoleucine tRNA Ligase from *Escherichia coli* with L-Isoleucyl-bromomethyl Ketone

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L-Isoleucine : tRNA ligase from *Escherichia coli* could be irreversibly inactivated by L-isoleucyl-bromomethyl ketone but not by L-isoleucyl-chloromethyl ketone. The inactivation rate exhibited a saturation concentration dependence typical for an affinity reagent. L-Isoleucine provided 100% protection against inactivation at saturating concentration, whereas ATP, AMP, and pyrophosphate offered partial protection and $tRNA^{Ile}$, no protection.

The ligase was labelled in preparative scale with L-[^{14}C]isoleucyl-bromomethyl ketone. The molar ratio of label incorporated to enzyme inactivated was close to unity. The protein was subsequently subjected to tryptic digestion and the radioactive peptide isolated and identified. The labelled amino acid proved to be the same cysteine previously reported as being labelled with *N*-[^{14}C]ethylmaleimide [Kula, M.-R. (1974) *FEBS Lett. 46*, 130–133].

Recently there have been a number of reports describing the use of aminoacyl-halomethyl ketones as affinity labels [1,2], especially for amino-acid : tRNA ligases [3–6]. We report here the inactivation of L-isoleucine : tRNA ligase with L-isoleucyl-bromomethyl ketone and the identification of the alkylated moiety as the same cysteine previously reported to react with *N*-ethylmaleimide [7].

MATERIALS AND METHODS

ATP and AMP were purchased from Boehringer Mannheim (Mannheim), 2-*p*-toluidinyl-naphthalene-6-sulfonate from Serva (Heidelberg), [^{32}P]PP_i and uniformally labelled L-[^{14}C]isoleucine from Amersham Buchler, *N*-*t*-butyloxycarbonyl-L-isoleucine and *N*-carbobenzoxy-L-isoleucine from ICN Nutritional Biochemicals Corp. (Cleveland). All other chemicals were of analytical grade and were obtained from Merck (Darmstadt). Trypsin (treated with L-1-tosyl-amido-2-phenylethyl chloromethyl ketone) was a product from Worthington.

L-Isoleucine : tRNA Ligase

The enzyme used in the preliminary kinetic and protection studies was obtained from *Escherichia coli* K10 in electrophoretically homogeneous form by affinity chromatography as described previously [8], except that the crude extract was prepared in the presence of phenylmethylsulfonyl fluoride following the procedure of Hanke *et al.* [9] and exhaustively dialyzed before loading onto the affinity column. The enzyme used for the radioactive labelling studies was prepared from *E. coli* MRE600 as described [10] and was of the highest possible purity (spec. act. of freshly prepared enzyme was 35–40 μmol Ile-tRNA $\cdot$ mg^{-1} $\cdot$ h^{-1}).

Enzyme concentration was routinely determined from the absorbance at 280 nm, based on a value of 1.52 units for a 1-mg/ml solution and an optical path of 1 cm. This value was determined from the A_{280} of a solution which was subsequently lyophilized and weighed. Values for protein concentration determined by the method of Lowry *et al.* [11] using bovine serum albumin as a standard were regularly 5–10% higher than those determined from the absorbance. The molecular weight of the enzyme from *E. coli* MRE600 was taken as 102000 [10].

Preparation of Inactivators

N-*Carbobenzoxy-L-isoleucyl-diazomethyl ketone* was prepared by a modification of the procedure of Frolova *et al.* for the valine derivative [4]. *N*-Carbobenzoxy-L-isoleucine (2.65 g, 10 mmol) was dissolved

Abbreviation. NMR, nuclear magnetic resonance.

Enzyme. L-Isoleucine : tRNA ligase (AMP) (EC 6.1.1.5).

in 20 ml of dry ethyl acetate. Triethylamine (1.4 ml, 10 mmol) was added and the solution chilled in an ice/acetone bath. Isobutyl chloroformate (1.36 ml, 10 mmol) was added and the mixture stirred 10 min under a drying tube, during which time a gel of triethylammonium chloride formed. Then 130 ml of a dry, distilled solution of diazomethane (≈ 3 mmol) in ether was directly added and the mixture stirred 2 h in the ice/acetone bath. It was held overnight in an ice bath, then washed with water (2×50 ml), 5% sodium bicarbonate (3×50 ml), water (2×50 ml) and saturated sodium chloride solution (50 ml). The ethereal solution was dried for 1 h over sodium sulfate, filtered, and evaporated under reduced pressure to a yellow oil. Recrystallization from ether/petroleum ether at -15 °C yielded 1.59 g (55%) of yellow needles, m.p. 67–69 °C. *N-t*-Butyloxycarbonyl-L-isoleucyl-diazomethyl ketone was similarly prepared to give a 54% yield of yellow needles m.p. 90.5–92 °C.

N-*Carbobenzoxy-L-isoleucyl-bromomethyl ketone* was prepared by adding 7.5 ml of a solution of 0.4 N HBr in ether to a cold (0–5 °C) stirred solution of *N*-carbobenzoxy-L-isoleucyl-diazomethyl ketone (0.8 g, 2.8 mmol) in 20 ml of dry ether. After 15-min stirring under a drying tube the solvent was evaporated under reduced pressure. Recrystallization of the residue from ether/petroleum ether at -15 °C yielded 743 mg (79%) of white needles, m.p. 86–87 °C. Analysis: calcd for $C_{15}H_{20}NO_3Br$: C, 52.7; H, 5.88; N, 4.10; Br, 23.3%. Found: C, 53.0; H, 5.86; N, 4.17; Br, 23.3%. NMR (C^2HCl_3): δ, 7.38 (s, 5H); 5.38 (broad d, $J = 8$, 2H); 5.15 (s, 2H); 4.8–4.4 (m, 1H); 4.07 (s, 2H); 2.4–1.6 (m, 1H); 1.6–1.0 (m, 2H); 1.2–0.7 (m, 6H).

L-*Isoleucyl-bromomethyl ketone* was prepared by adding 0.25 ml of 40% HBr in acetic acid to *N*-carbobenzoxy-L-isoleucyl-bromomethyl ketone (205 mg, 0.6 mmol) under nitrogen. The material dissolved with foaming. After 15 min, 2 ml of dry diethyl ether was added followed by 2 ml of petroleum ether. The mixture was held under nitrogen at -15 °C for 30 min. The supernatant was decanted and the thick, oily residue was dissolved in 0.3 ml of chloroform and reprecipitated with 1 ml of diethyl ether followed by 2 ml of petroleum ether. After 30 min at -15 °C the supernatant was decanted. Solution and precipitation of the material was repeated twice more. The residue was then dried over phosphorus pentoxide *in vacuo* at 4 °C overnight. Thereby was obtained 120 mg (62%) of a stiff yellow gum. Analysis: calcd for $C_7H_{15}NOBr_2$: C, 29.10; H, 5.24; N, 4.85; Br, 55.3%. Found: C, 29.16; H, 5.31; N, 4.78; Br, 54.6%. NMR ($C^2H_3O^2H$): δ, 4.5 (d, $J = 3$, 1H); 4.4 (s, 2H); 2.6–2.0 (m, 1H); 1.9–0.8 (m, 8H). NMR (C^2HCl_3): δ, 8.4–7.7 (apparent broad s, 3H); 5.3–4.6 (m, 1H); 4.38 (AB q, 2H); 2.8–2.0 (m, 1H); 2.0–0.9 (m, 8H). Thin-layer chromatography on silica gel using butan-1-ol/acetic acid/water (4/1/5, by vol.) gives a main spot with $R_F = 0.52$ and an attached streak. Isolation and rechromatography of the latter indicated an $R_F = 0.22$. This material appears to be at least in part the result of decomposition during chromatography. Both spots may be visualized with iodine, ninhydrin, and fluorescein/hydrogen peroxide. The last gives a pink spot characteristic of organic bromides [12].

N-*Carbobenzoxy-L-[^{14}C]isoleucine* was prepared from L-[^{14}C]isoleucine (10 mCi/mmol, diluted with unlabelled L-isoleucine to 1 mCi/mmol). The procedure of Greenstein and Winitz [13] was followed and gave in 88% yield a clear viscous oil. After drying 5 days over P_2O_5 *in vacuo* the specific activity was 0.99 mCi/mmol. NMR and thin-layer chromatography, behaviour was identical to that of commercial *N*-carbobenzoxy-L-isoleucine.

N-*Carbobenzoxy-L-[^{14}C]isoleucyl-bromomethyl ketone* was prepared in similar manner to the unlabelled compound, except that the *N*-carbobenzoxy-L-[^{14}C]-isoleucyl-diazomethyl ketone was not recrystallized. Instead the yellow oil was treated directly with ethereal HBr. The yield was 46% based on *N*-carbobenzoxy-L-isoleucine. NMR, m.p., and thin-layer chromatography behaviour were identical to that of the unlabelled material.

L-*[^{14}C]Isoleucyl-bromomethyl ketone hydrobromide* was prepared in small batches (from about 10 mg of *N*-carbobenzoxy-L-[^{14}C]isoleucyl-bromomethyl ketone) in the same manner as the unlabelled compound. Yields varied from 60–80%. The material was stored at 3 °C over P_2O_5 *in vacuo* and used within 5 days of preparation.

L-*Isoleucyl-chloromethyl ketone hydrochloride* was prepared by dissolving 26 mg (0.3 mmol) of *N-t*-butyloxycarbonyl-L-isoleucyl-diazomethyl ketone in 1 ml of 2.25 N HCl in dry ether. There was vigorous foaming which quickly subsided. Reaction was allowed to proceed 8 h under a drying tube, during which time the product separated as a gel. The gel was collected with suction filtration and washed with 3×3 ml of ether. It was twice recrystallized from chloroform/ether and dried over phosphorus pentoxide *in vacuo*. This gave 25 mg (42%) of fluffy white crystals, m.p. 143–145 °C. Analysis: calcd for $C_7H_{15}NOCl_2$: C, 42.02; H, 7.56; N, 7.00; Cl, 35.43%. Found: C, 41.87; H, 7.54; N, 7.00; Cl, 35.35%. NMR (C^2HCl_3): δ, 9.2–7.8 (apparent broad singlet 3H); 4.9–4.5 (m, 1H); 4.47 (ABq, 2H); 2.6–1.9 (m, 1H); 1.9–0.8 (m, 8H).

Inactivation and Protection Experiments

Inactivations were carried out at 27 °C in 20 mM potassium phosphate, pH 7.5, and 0.1 mM EDTA.

Before use, the enzyme was reduced for 30 min at 37 °C in the same buffer containing 20 mM dithioerythritol and was subsequently dialyzed thrice against inactivation buffer. L-Isoleucyl-bromomethyl ketone hydrobromide was dissolved in cold water and brought to pH 7.5 with 0.5 equivalent of sodium hydroxide immediately prior to adding it to the enzyme solution. Aliquots of 10 µl of the reaction mixture were removed at various times and quenched in 1 ml of ice-cold buffer containing 20 mM Tris·HCl, pH 7.5, 0.1 mM EDTA, 0.2 mM dithioerythritol, and 0.1 mg/ml of bovine serum albumin. Residual activity was determined from the rate of aminoacylation of unfractionated tRNA with L-[^{14}C]-isoleucine following the procedure of Kosakowski and Boeck [14] with twice the concentration of crude tRNA. Values of k_{obs} for inactivation were determined from the initial slopes of semilogarithmic plots, of residual activity *versus* time.

Protection studies were also carried out with covalent blocking of cysteinyl-SH by reaction with 5,5′-dithio-bis(2-nitrobenzoic acid). 450 µg enzyme, exhaustively dialysed against 0.05 M potassium phosphate buffer pH 7.5 and 0.2 mM EDTA, was completely inactivated after 25 min at 28 °C in 3.0 ml buffer containing 0.035 M 5,5′-dithio-bis(2-nitrobenzoic acid). Upon reincubation in 0.01 M dithioerythritol (20 min at 37 °C) activity was fully recovered. 1.0 ml solution was then loaded onto a Sephadex G-75 column (1.3 × 20 cm), eluted with 0.05 M potassium phosphate buffer pH 7.5, 0.1 mM EDTA, and kept as reference. A second 1.0-ml portion of the blocked enzyme was incubated with 20 µl L-isoleucyl-bromomethyl ketone at a final concentration of 2.0 mM. After 90 min at 28 °C, this mixture was applied to the same column and eluted with the same buffer. Protein concentrations in the eluates were followed by the intrinsic protein fluorescence (280 nm excitation, 340 nm emission). Solutions were diluted until the fluorescence intensity was a linear function of the protein concentration. The enzyme treated with the reagent and the inactivator was finally reactivated at 37 °C for 30 min in 90 mM dithioerythritol and compared with the reference.

Radioactivity Labelling and Measurement

Radioactivity incorporation during inactivation with L-[^{14}C]isoleucyl-bromomethyl ketone was measured by quenching a 20-µl aliquot of the reaction mixture in 80 µl of 1 mM L-isoleucine, 20 mM 2-mercaptoethanol. 50 µl of this solution was placed on a disc of DEAE-cellulose filter paper (Whatman DE 81) which had previously been washed with 10 ml of 20 mM potasium phosphate, pH 7.5, 0.1 mM EDTA by suction filtration.

The loaded filter disc stood 1 min without suction. Then suction was reapplied and the filter washed with 40 ml more of buffer. Discs were dried under a heat lamp for 20 min, then counted in 5 ml of 0.5% diphenyloxazole in toluene using a Nuclear Chicago Isocap 300 counter. A counting efficiency of 73% was determined within internal standard. The blank was made by adding 20 µl of the enzyme solution before labelling to 60 µl of the quench buffer followed by 20 µl of 1 mM L-isoleucyl-bromomethyl ketone.

Preparative labelling with L-[^{14}C]isoleucyl-bromomethyl ketone (1 mCi/mmol) was carried out at 27 °C using 183 mg of L-isoleucine : tRNA ligase (from *E. coli* MRE 600) in 16.8 ml of solution, freshly dialyzed against 50 mM potassium phosphate, pH 7.5, 0.1 mM EDTA. A 1 mM concentration of the inactivator was used. During the reaction aliquots were removed and quenched for measurement of loss of enzyme activity and incorporation of radioactivity. At 30 min the reaction was terminated by the addition of 0.2 M L-isoleucine and 2-mercaptoethanol to give final concentrations of 1 mM and 20 mM respectively.

5 ml of 1 mM L-isoleucine in 1% ammonium bicarbonate were run onto a column (1.5 × 90 cm) of Biogel P-6 equilibrated in 1% ammonium bicarbonate. This was followed by the quenched reaction mixture. The column was then eluted with the equilibrium buffer. The function of the L-isoleucine was to displace all non-covalently bound label from the enzyme. The enzyme-containing peak emerged well ahead of a second radioactive peak which corresponded to small molecules. The pooled enzyme-containing fractions, comprising 24 ml, were heated at 50 °C for 15 min to effect denaturation and cooled to 37 °C. The pH was 8.45. 4 mg of trypsin were than added to the stirred solution in four portions over 2.5 h. Digestion proceeded for a total of 5 h. The solution was then lyophilized.

The lyophilizate was dissolved in 3 ml of 1% ammonium bicarbonate. A small amount of insoluble material was removed by centrifugation and washed twice with 1 ml of the buffer. This residue contained less than 2% of the total radioactivity and was discarded. The combined supernatants were chromatographed on Sephadex G-50 superfine as described previously [7]. Isolation and sequence determination of the labelled peptide was also performed as described [7, 15].

RESULTS AND DISCUSSION

Inactivation Kinetics

When L-isoleucine : tRNA ligase was treated with L-isoleucyl-bromomethyl ketone, the enzyme was irreversibly inactivated. The inactivation initially fol-

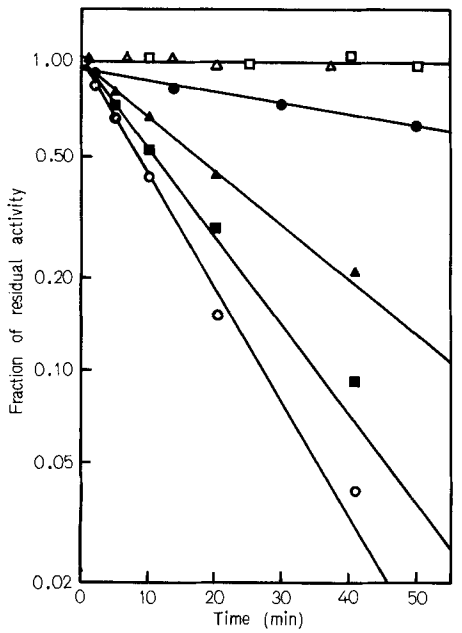

Fig. 1. *Semilogarithmic plot of the inactivation of* L-*isoleucine : tRNA ligase by* L-*isoleucyl-halomethyl ketones.* Inactivations were carried out at 27 °C in 50 mM potassium, pH 7.5, 0.1 mM EDTA containing 7 μg/ml of ligase. Portions were quenched and assayed for residual aminoacylation activity as described in Methods. Inactivation was followed in the presence of no inactivator (□), of 2.3 mM L-isoleucyl-chloromethyl ketone (△), of 0.07 mM (●), 0.34 mM (▲), 0.65 mM (■) and 1.0 mM (○) L-isoleucyl-bromomethyl ketone

lowed a single exponential time dependence (Fig. 1). With longer reaction time, especially at higher concentrations of either the inactivator or the ligase the reaction rate decreases. This was apparently due to loss of L-isoleucyl-bromomethyl ketone by side reactions yielding materials which do not alkylate. When the enzyme was separated from the reactants on a column or by dialysis and then again treated with the reagent under initial conditions the inactivation proceeded further with a rate constant similar to that seen initially.

Interestingly, L-isoleucyl-chloromethyl ketone was not an irreversible inhibitor even at prolonged incubation times (> 1 h) but is competitive with respect to L-isoleucine in the pyrophosphate exchange reaction [16] having a K_i of 0.3 mM. However, when this compound was prepared from the *N*-carbobenzoxy-protected material, the product gave inactivation, but it was shown that all inactivation could be attributed to some bromomethyl ketone which was produced by nucleophilic displacement during deblocking with hydrogen bromide (unpublished results).

When the observed rate constants for inactivation with L-isoleucyl-bromomethyl ketone were plotted as a function of the inactivator concentration (Fig. 2), a saturation dependence was seen of the form $k_{obs} = k/(1 + K_d/[I]_0)$. Symbols refer to: $[I]_0$, initial concentration of inactivator (in excess of enzyme); K_d, concentration at half-maximum rate constant; k, maximum rate constant of the inactivation reaction. The data could be linearized in accordance with the rearranged equation $k_{obs} = k - K_d \cdot (k_{obs}/[I]_0)$ (Fig. 2, inset). From the slope of the graph, a value for K_d of 7×10^{-4} M was determined. The observed saturation phenomenon is consistent with the formation of a reversible enzyme-inactivator complex prior to reaction. Therefore, the concentration of the inactivator at half-maximum rate constant, K_d, can be assumed to represent the dissociation constant. The value is in 'good agreement with the dissociation constant of 6×10^{-4} M which has been determined for the complex formation between the isosteric L-isoleucine methyl ester and the ligase [18].

Protection Studies

Protection studies were carried out in the presence of the various substrates of the ligase. In those cases where protection was seen it was observed to follow a saturation dependence on the concentration of the protector. This indicates that protection was afforded by the binding of the ligand to the enzyme. It was thus possible to calculate the maximum protection at extrapolated infinite protector concentration from Eadie plots [17]. The fact that only L-isoleucine, which is structurally analogous to the inactivator, offers a maximum protection of 100% suggests that it protects by competitive displacement of the inactivator. An apparent dissociation constant could then be calculated from the slope of the Eadie plot according to

$$K_{d\,(Ile)} = -\text{slope}/(1 + [I]_0/K_d).$$

The other protective ligands were assumed to provide protection in some non-competitive manner (*e.g.*, induction of a conformation change upon binding, resulting in a lowering of enzyme reactivity). Protection constants were taken to be equal to the negative slopes of the Eadie plots. The results are summarized in Table 1. Worthy of note is the lower value of the dissociation constant for ATP (and to a lesser extent AMP) when determined in the protection experiment. Apparently, these experiments represent enzyme-ATP complexes similar to those observed with this enzyme in the presence of L-isoleucine analogs, such as L-isoleucinol or 2-methyl-1-butylamine [18].

The ligase could be protected against L-isoleucyl-bromomethyl ketone by previous blocking of cysteinyl-SH with 5,5′-dithio-bis(2-nitrobenzoic acid) (Table 2). This resistance is in agreement with such a group being the target of alkylation by the inactivator.

The alkylating inactivator L-isoleucyl-bromomethyl ketone shows in its reaction with L-iso-

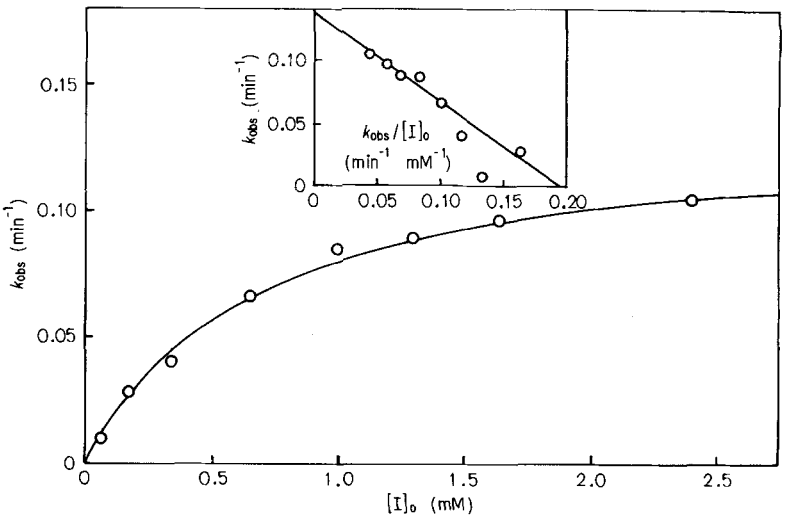

Fig. 2. *Concentration dependence of the rate constants for the inactivation of* L-*isoleucine: tRNA ligase by* L-*isoleucyl-bromomethyl ketone.* Values of k_{obs} were determined from the initial slopes of semilogarithmic plots as in Fig. 1. Inset: results replotted according to Eadie [17]

Table 1. *Protection of* L-*isoleucine: tRNA ligase against inactivation by* L-*isoleucyl-bromomethyl ketone*
Protection studies were done at 27 °C in 20 mM potassium phosphate, pH 7.5, 0.1 mM EDTA using 7 μg/ml of the ligase, 1 mM L-isoleucyl-bromomethyl ketone and varying concentrations of the protecting agent. Values of k_{obs} were determined from the initial slopes of semi-logarithmic plots as in Fig. 1. The maximum protection values were determined from the intercepts of plots of $k^0_{obs} - k_{obs}$ *versus* $(k^0_{obs} - k_{obs})$/[protector], where k^0_{obs} is the observed rate constant in the absence of a protecting agent. The protection constants were determined as described in the text

Protecting compound	Maximum protection	Protection constant	Dissociation constant	
	%	μM		
L-Isoleucine	100	4.8	5.8	[18]
ATP	91	15	120	[18]
AMP	46	2300	7500	[18]
Pyrophosphate	74	470	260	[18]
tRNA[a]	none	–	0.83[b]	[19]

[a] tRNA was unfractionated and prepared according Zubay [20]. Final concentration of $tRNA^{Ile}$ was up to 2.5 μM.
[b] pH 7.0 and 17 °C.

Table 2. *Protection of* L-*isoleucine: tRNA ligase against* L-*isoleucyl-bromomethyl ketone by blocking with 5,5-dithio-bis(2-nitrobenzoic acid)*

Enzyme	Specific activity
	μmol Ile-tRNA · mg^{-1} · h^{-1}
Native	35
Blocked with 5,5′-dithio-bis(2-nitrobenzoic acid) (A)	< 1
As (A) (but deblocked with dithioerythritol)	36
Inactivated with L-isoleucyl-bromomethyl ketone	2
As (A) (but subsequently reacted with L-isoleucyl-bromomethyl ketone, and then deblocked with dithioerythritol)	32

leucine: tRNA ligase the characteristics which are considered indicative of reaction as an affinity label: (a) the inhibition increases progressively with time and is not reversed by subsequent removal of the inhibitor; (b) saturation kinetics are seen for the inactivation, with the reagent exhibiting an apparent dissociation constant comparable to the isosteric methyl ester and chloromethyl ketone of L-isoleucine, both of which are competitive inhibitors of the amino acid in reactions catalyzed by the ligase; (c) L-isoleucine protects against inactivation in a competitive manner and offers 100% protection at saturating concentration.

Labelling

We therefore decided to label the enzyme with L-[^{14}C]isoleucyl-bromomethyl ketone and to identify the labelled residue. This was done as described in Methods. The enzyme from *E. coli* MRE600 was used in these experiments because it was available in larger quantities. The enzyme appeared to be identical with that of strain K10 in regard to inactivation kinetics. The loss of aminoacylation activity and the incorporation of radioactivity as followed by the filter assay are shown in Fig. 3A. A plot of the amount of radioactive label incorporated *versus* the amount of enzyme inactivated in an equivalent sample (Fig. 3B) was linear and indicates that 1.2 mol of label are bound per mol of enzyme inactivated. However, after the labelled enzyme was passed over

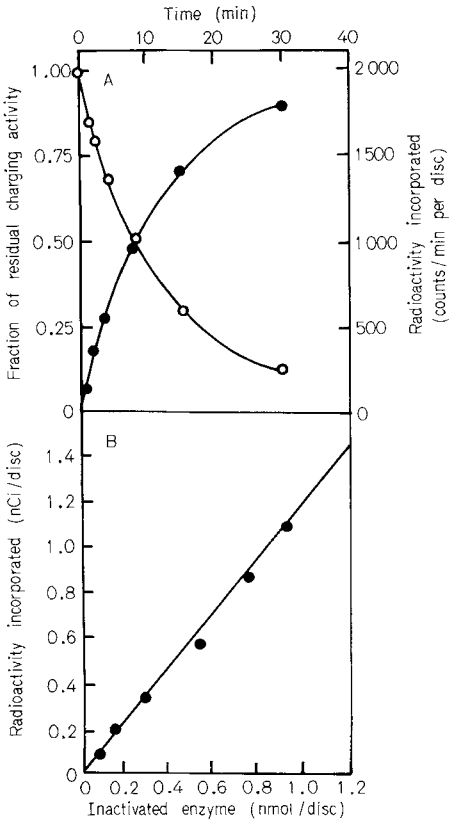

Fig. 3. *Inactivation of L-isoleucine : tRNA ligase with L-[^{14}C]isoleucyl-bromomethyl ketone (1 μCi/μmol).* (A) Loss of aminoacylation activity (○) and incorporation of radioactivity (●), as measured by retention on DEAE-cellulose filter discs. Details are given in Methods. (B) Plot of radioactivity incorporated *versus* amount of ligase inactivated

a Biogel P-6 column, direct measurement of radioactivity and protein concentration indicated a ratio of 0.91 : 1. Since the enzyme was 87% inactivated at this point, a stoichiometry of 1.05 is indicated. In a subsequent experiment, labelled enzyme was adsorbed on a DEAE-cellulose column and after thorough washing eluted with 100 mM phosphate, pH 7.5. The ratio of radioactivity to protein in the eluate pool which contained the labelled ligase was 1.13 : 1 (Ci/mol). However, part of this radioactivity was separable from the protein in the pool by ultrafiltration. After correcting for this, the overall ratio was 0.74 : 1 and the stoichiometry based on the percentage inactivation was 1.03. Thus it appears that some of the radioactivity retained by DEAE-cellulose is not covalently bound to the ligase and the stoichiometry of 1.2 determined by retention of radioactivity on the DEAE-cellulose filter discs is an upper limit. The true value would appear to be close to unity.

Identification of the Labelled Sequence

Chromatography of the tryptic digest of the labelled enzyme on Sephadex G-50 (Fig. 4) gave an elution pattern very similar to that found with ligase labelled with *N*-[^{14}C]ethylmaleimide [7]. Excepting the first two peaks in the absorbance tracing, which are incompletely digested fragments, the correspondence is nearly perfect.

Fractions of the major peak of radioactivity (fractions 90–98) were combined and the peptides

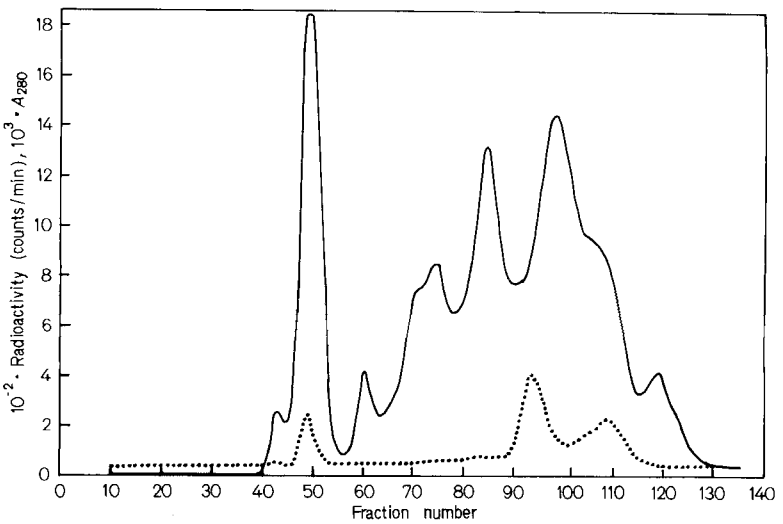

Fig. 4. *Gel filtration on Sephadex G-50 of the tryptic digest of L-isoleucine : tRNA ligase modified with L-[^{14}C]isoleucyl-bromomethyl ketone.* The soluble peptides from 183 mg of protein were applied to a column (2.5 × 180 cm) of Sephadex G-50 superfine and eluted with 1% ammonium bicarbonate at a flow rate of 19 ml/h at 4 °C. Fractions of 9.5 ml were collected. The solid line represents the absorbance at 235 nm; the broken line represents the radioactivity of a 50-μl aliquot

further purified on paper by high-voltage electrophoresis and chromatography under various conditions [15]. The radioactive peptide migrated in the first dimension at pH 6.4 with an R_F of 0.17 relative to lysine which is in agreement with the gain of one extra positive charge by alkylation with isoleucyl-bromomethyl ketone [21]. The labelled peptide was most difficult to separate from a peptide with the composition: Asp, Thr_2, Glu_2, Gly_3, Val_2, Leu, Phe, His, Arg. The final yield of the purified radioactive peptide was low (31 nmol ≙ 1.7%).

The amino acid composition was determined from an aliquot and found to be the same as peptide N/56 isolated previously [7] as the peptide which can be specifically labelled with *N*-[^{14}C]ethylmaleimide (Table 3). The identity of the sequence was established by manual Edman degradation carried through 10 residues, which were found to be identical (Table 4). In an attempt to verify that the label was attached to the cysteine residue, 5 nmol of peptide were subjected to chymotryptic digestion and the products were separated by gel filtration [7]. The results suggest that cysteine is indeed the labelled residue, but for lack of material no definite proof could be obtained. Several other radioactive fractions were isolated besides peptide IP/63, all were shown to contain a mixture of two peptides, the minor component always starting with the sequence Ile-Glu-Ser-Met-Val. Therefore it appears that the label is attached predominantly if not exclusively to the same peptide described above. The reasons leading to the isolation of several chromatographically and electrophoretically different products have been discussed previously [7]. Based on the analysis of the fragments labelled with *N*-[^{14}C]ethylmaleimide and analytical data obtained now, the third peak of radioactivity (fractions 100–115) is the result of a secondary tryptic cleavage between methionine and valine, as has been observed before [7].

In summary, the sequence work together with the cysteinyl-SH specific protection studies using 5,5′-dithio-bis(2-nitrobenzoic acid) quite definitely indicate that the target of the active-site-directed labelling with L-isoleucyl-bromomethyl ketone is the same cysteinyl residue that reacts rapidly and specifically with *N*-ethylmaleimide [7, 22]. This explains the similar patterns of protection offered by the various substrates against both inactivators. The behaviour as an affinity label together with these protection effects suggests that the labelled residue is positioned at or close to the catalytic site of L-isoleucine : tRNA ligase.

Table 3. *Amino acid analysis of purified peptides of* L-*isoleucine : tRNA ligase*
Values for N/56 were obtained previously [7]. The cysteine content was calculated from the radioactivity of the peptides

Amino acid	Amount in peptide	
	IP/63	N/56
	mol/mol peptide	
Cysteine	0.91	0.75
Aspartic acid	1.99	(2.56)
Threonine		
Serine	1.90	2.09
Glutamic acid	1.06	1.05
Proline	1.00	0.80
Glycine		
Alanine	1.08	0.91
Valine	0.98	1.05
Methionine	0.70	0.72
Isoleucine	1.99	1.73
Leucine		
Tyrosine		
Phenylalanine		
Histidine		
Lysine		
Arginine	1.82	1.83
Tryptophan	+	+

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Table 4. *Amino acid sequences of peptides from* L-*isoleucine : tRNA ligase labelled with [*^{14}*C]isoleucyl-bromomethyl ketone (IP/63) and with* N-*[*^{14}*C]ethylmaleimide (N/56)*
The sequence of peptide N/56 was determined previously [7]

Peptide	Sequence
N/56	Ile-Glu-Ser-Met-Val-Ala-Asp-Arg-Pro-Asn- Trp-Cys-Ile-Ser-Arg
IP/63	Ile-Glu-Ser-Met-Val-Ala-Asp-Arg-Pro-Asn-(Trp-Cys-Ile-Ser-Arg)

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