STRUCTURAL STUDIES ON ISOLEUCYL-tRNA SYNTHETASE FROM *E. COLI*

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1. Introduction

Aminoacyl-tRNA synthetases play an important role in the translation of the genetic message. Very little is known about the protein chemistry of these enzymes and studies so far have been limited to the quaternary structures [1]. The only sequence data are published by Bruton and Hartley [2] and cover special peptides of the methionyl-tRNA synthetase from *E. coli*. I report here sequence studies around the cysteine residues in isoleucyl-tRNA synthetase and the effect of these results on the structure of the enzyme.

2. Materials

2.1. *Chemicals*

Jodo-[2-^{3}H]acetic acid with a specific activity of 113 mCi/mM was purchased from the Radiochemical Centre (Amersham, England) and diluted with unlabeled iodoacetic acid just prior to use to give a specific activity of 10 mCi/mM. Phenylisothiocyanate was obtained from Fluka (Buchs, Swiss), redistilled and kept under nitrogen. Pyridine and trifluoroacetic acid were products from Merck (Darmstadt, Germany) and redistilled under nitrogen when used for sequence studies. Aquasol and omnifluor were obtained from NEN Chemicals (Dreieichenhain, Germany) and used for counting liquid samples or radioactivity on papers respectively. Micro-polyamide layers F 1700 and chromatography paper grade 2316 from Schleicher und Schüll (Dassel, Germany) were used for chromatography. Solvents and other chemicals used were purchased from Merck (Darmstadt, Germany) reagent grade or the highest purity available.

2.2. *Proteins*

Isoleucyl-tRNA synthetase (EC 6.1.1.5) was purified from *E. coli* MRE 600 as described previously [3] and used as a homogeneous preparation. TPCK-treated trypsin (EC 3.4.4.4) was obtained from Worthington (Freehold, N.J., USA).

2.3. *Equipment*

High voltage electrophoresis at pH 6.4 was carried out on a flat plate at 5°C using model HVE 10 K 10045A from Hölzel (Dorfen, Germany). High voltage electrophoresis at pH 3.5 was performed in a tank using the electrophorator model D from Gilson (obtained through Techmation, Düsseldorf, Germany). Tap water served to keep the temperature at about 20°C during electrophoresis. Amino acids were analysed using a Biocal BC 200 amino acid analyser equipped with the photometer BC 201, automatic sample additioner and an integrator CRS 100A from Infotronics. Elution was carried out under conditions that separated carboxymethyl cysteine clearly from aspartic acid. For sequence studies a jacketed desiccator was employed thermostated at 60°C. Phase separation was achieved using a desk top centrifuge model Simplex from Hereus Christ. Radioactive samples were counted in a liquid scintillation counter model 3380 from Packard.

2.4. *Carboxymethylation*

Two hundred mg isoleucyl-tRNA synthetase (1.96 μmoles) were dialysed exhaustively against distilled water and freeze dried. The sample was dissolved in 25 ml 6 M guanidinium hydrochloride, 200 mM Tris, 0.1 mM EDTA pH 8.4 and reduced under nitrogen with 10 mg dithioerythritol (65 μmoles) at 37°C for

4 hr. After cooling to room temperature 160 μmoles iodoacetic acid were added under nitrogen. The reaction proceeded for 2.5 hr at room temperature and was terminated by adding 15 mmoles β-mercaptoethanol. The protein was dialysed at 4°C against several charges of 1 mM hydrochloric acid and finally against distilled water until the dialysate became free of radioactivity. The protein precipitated during dialysis and was recovered by freeze drying.

2.5. *Tryptic digest*

The carboxymethylated protein was triturated with 30 ml 100 mM ammonium bicarbonate pH 8.5 and finely dispersed. Digestion was carried out at 37°C by adding 4 mg trypsin in aliquots over a period of 3 hr. The digestion was continued for further 3 hr. By this time a fine white precipitate remained in the otherwise clear solution. The precipitate was spun down in a centrifuge at 9000 rpm for 20 min. The supernatant was recovered and the precipitate washed twice with 2 ml 100 mM ammonium bicarbonate. The combined supernatants contained 2.2×10^8 cpm (uncorrected for quench). The precipitate could be dissolved in glacial acetic acid and contained less than 1×10^6 cpm. This core material showed only traces of carboxymethyl cysteine on the amino acid analyser and appeared to be a rather long peptide with a high content of leucine. The clear digest soluble in 100 mM ammonium bicarbonate was lyophilised to reduce the volume.

2.6. *Gel chromatography*

The soluble part of the digest was redissolved in 10 ml 100 mM ammonium bicarbonate and applied to a column (2.5 × 170 cm) packed with Sephadex G-50 superfine and equilibrated with 100 mM ammonium bicarbonate pH 7.5. Elution was carried out using the same buffer at a flowrate of 19 ml/hr. Fractions of 9.5 ml were collected. The column was operated at 4°C. Aliquots of the eluate were counted in the liquid scintillation counter and the optical density of a 1:11 dilution was measured at 235 nm and 280 nm. The results are presented in fig. 1. Eleven fractions were combined according to the radioactivity and optical density profile.

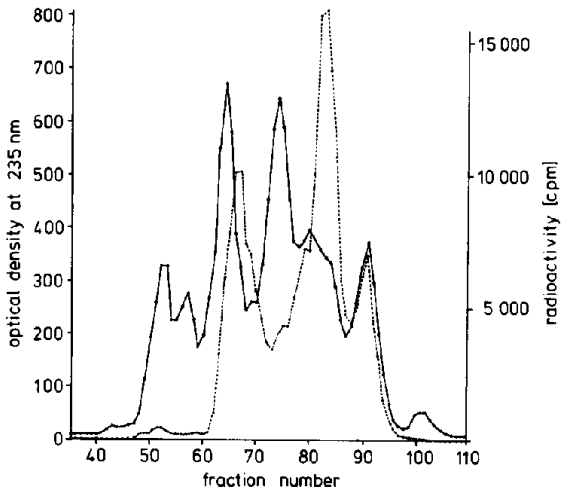

Fig. 1. Filtration on Sephadex G-50 of a tryptic digest (soluble fraction) of S-[^{3}H]carboxymethylated isoleucyl-tRNA synthetase. The soluble peptides of 200 mg protein were applied to a column (2.5 × 170 cm) packed with Sephadex G-50 superfine and eluted with 100 mM ammonium bicarbonate pH 7.5 at a flow rate of 19 ml/hr at 4°C. Fractions of 9.5 ml were collected and analyzed: (●—●—●) Represents the optical density at 235 nm of a 1:11 dilution of the eluate; (●···●···●) shows the radioactivity measured with a 10 μl aliquot. The originally collected fractions were pooled according to the following scheme: I (fractions 41–47); II (fractions 48–55); III (fractions 56–60); IV (fractions 61–65); V (fractions 66–68); VI (fractions 69–72); VII (fractions 73–76); VIII (fractions 77–80); IX (fractions 81–87); X (fractions 88–95); XI (fractions 96–105).

2.7. *Fractionation of peptides on paper*

Each pooled fraction of the previous step was divided into two equal parts and the peptides separated independently in a set of 2 experiments. The radioactive peptides were further purified by high voltage paper electrophoresis at 60 V/cm at pH 6.4, followed by electrophoresis at 50 V/cm at pH 3.5. The electrophoresis buffers consisted of pyridine and acetic acid. Chromatography of peptides was then carried out in butanol-1, acetic acid, pyridine, water (15:3:10:12, v/v/v/v) and if necessary also in butanol-2, formic acid, water (70:9:21, v/v/v). Appropriate guide strips were cut each time, scanned for radioactivity and stained with ninhydrin followed by Ehrlich's reagent to detect tryptophan. Samples were transferred to the next purification step by the 'sewing method' [4]. After the final step the peptides were eluted

Table 1
Amino acid analysis of purified cysteine containing peptides of isoleucyl-tRNA synthetase from *E. coli* MRE 600.

	IV/4	V/3	VI/64	IX/8	IX/9	X/1	X/2
Cys(cm)	1.0	0.87	0.81	0.92	0.99	0.71	1.37
Asp	5.1	2.08				1.12	
Thr						0.85	
Ser	4.1	0.88					0.87
Glu	3.0	1.05	2.00			1.12	1.02
Pro	6.2				0.99		1.00
Gly	7.8	2.02	1.00	1.02		0.95	
Ala	0.8	1.02	1.80	1.95			
Val	2.2	2.00	0.66	1.02		0.88	
Met	0.6						0.77
Ile	2.1		0.97				
Leu	6.7						
Tyr	0.7					0.70	0.71
Phe							
His	1.5		0.85			1.10	1.10
Lys	2.0	1.00		1.00		1.00	
Arg			0.90		1.00		1.00
Trp	+					+	+

from paper using 100 mM acetic acid containing 1% β-mercaptoethanol. Aliquots were taken for amino acid analysis and hydrolysed for 22 hr. This way 7 peptides were isolated with unique compositions as compiled in table 1.

2.8. *Sequence studies*

The sequence of the peptides was determined by the Edman–dansyl method described by Gray [5]. Care was taken to exclude oxygen during manipulations. Dansyl-amino acids were separated on polyamide layers 5 × 5 cm using the conditions given by Hartley [6]. The sequences established are listed in table 2. Peptide no. IV/4 is quite large and not very suitable for paper techniques. It tends to tail during electrophoresis and elution from the paper is difficult Therefore the yields were low and the complete sequence could not be established for lack of material. The *N*-terminal part was found by Edman degradation of the whole peptide, seven residues could be identified but no carboxymethyl cysteine was found in these positions. From a thermolysin digest of peptide no. IV/4 a short radioactive fragment was isolated containing a Cys(cm)–Ser sequence. This sequence is unique to ptptide no. IV/4 and rules out the possibility, that this long peptide is derived as an unsplit fragment incorporating one of the smaller radioactive peptides.

3. Discussion

Eight cysteine residues were found in unique sequences in isoleucyl-tRNA synthetase. This compares with 14 cysteine residues calculated by the amino acid composition [3]. During the purification one peptide may be lost but certainly not six peptides in two independent separations. There is strong evidence that isoleucyl-tRNA synthetase is a single chain protein with the high molecular weight of 102 000 daltons. The enzyme does not dissociate upon treatment with sodium dodecylsulfate under conditions which dissociate most other proteins [3,7,8]. If we assume that the enzyme is a single chain then the low number of cysteine-containing peptides can only be explained by homologous sequences in the chain as the result of a gene duplication and fusion. Data on other peptides of isoleucyl-tRNA synthetase containing methionine, histidine and tryptophan support this

Table 2
Amino acid sequences of cysteine containing peptides of isoleucyl-tRNA synthetase from *E. coli* MRE 600.

IX/9	Cys(cm)–Pro –Arg
IX/8	Ala–Val–Gly–Cys(cm)–Ala–Lys
VI/64	Val–Ala–Glx–His–Ala–Glx–Ile –Cys(cm)–Gly–Arg
X/1	Cys(cm)–Trp–His–Tyr–Thr–Glx–Asx–Val–Gly–Lys
X/2	Met–Glx–His–Ser–Tyr–Pro –Cys(cm)–Cys(cm) –Trp–Arg
V/3	Cys(cm)–Val–Ser–Asx–Val–Ala–Gly–Asx–Gly–Glx–Lys
IV/4	Gly–Leu–Ser–Gly–Tyr–Asx–Ser –(Cys(cm)–Ser)– Asx_4, Ser , Glx_3, Pro, Gly_6, Ala, Val_2, Ile_2 Leu_6, His_2, Trp , Lys)–Lys

conclusion (M.-R. Kula, unpublished results). The asymmetric unit of isoleucyl-tRNA synthetase appears to be roughly half the molecular weight of the chain. This surprising finding has been reported recently also for the subunit chain of phosphofructokinase [9] and may be a more general phenomenon of protein architecture which has not been recognized previously.

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