STRUCTURAL STUDIES ON ISOLEUCYL-tRNA SYNTHETASE FROM *E. COLI* – IDENTIFICATION OF THE CYSTEINE RESIDUE MODIFIED SPECIFICALLY WITH *N*-ETHYLMALEIMIDE

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Received 24 June 1974

1. Introduction

Iaccarino and Berg [1] have shown that one sulfhydryl group in isoleucyl-tRNA synthetase (IRS) reacts rapidly with *N*-ethylmaleimide (NEM). In the presence of isoleucine and ATP this sulfhydryl group is protected against the action of NEM. It is assumed that the cysteine residue involved is positioned at or near the catalytic site of the enzyme and must be free for maximal rate of the isoleucyl-adenylate formation and utilisation, while tRNA binding is not affected by the modification [1].

Investigating the sequences around the cysteine residues in IRS it was of interest to me to identify the sequence involved in the catalytic site of the enzyme. First labeling experiments with NEM indicated that it was very difficult to purify the modified peptide. Finally it was shown that this special peptide had been missed in the first experiment aimed to establish the sequences of the cysteine containing peptides [2].

2. Materials and methods

2.1. *Chemicals and equipment*

N-Ethyl [2,3-^{14}C]maleimide with a specific activity of 2.4 mCi/mMol was purchased from the Radiochemical Centre (Amersham, England) and used as such. Bio-Gel P 6 was obtained from Biorad Laboratories (München, Germany). All other chemicals and the equipment used were as described previously [2].

2.2. *Proteins*

Isoleucyl-tRNA synthetase (EC 6.1.1.5) was purified from *E. coli* MRE 600 as described [3] and used as a homogeneous preparation. Chymotrypsin (EC 3.4.4.5) and TPCK-treated trypsin (EC 3.4.4.4.) were obtained from Worthington (Freehold, N.J., USA).

2.3. *Labeling with ^{14}C-NEM*

Conditions for the specific labeling of IRS with NEM were chosen according to Iaccarino and Berg [1]. The enzyme was first reduced with an excess of dithioerythritol and dialysed in the coldroom against 30 mM potassium phosphate buffer, pH 7.0. 124 mg IRS (1.22 μmoles) were reacted with 20.6 μmoles ^{14}C-NEM in a total volume of 20 ml at 23°C. The reaction was terminated after 20 min by adding 30 mmoles β-mercaptoethanol. The protein was dialysed at 4°C against 10 mM potassium phosphate buffer, pH 7.0, until the dialysate became free of radioactivity and than freeze-dried.

Since it was intended to isolate other peptides besides the radioactive one from the digest of the experiment, the protein was carboxymethylated with nonradioactive iodoacetate as described previously [2].

2.4. *Tryptic digest*

The labeled and carboxymethylated protein was triturated with 18 ml 100 mM ammonium bicarbonate, pH 8.5, and finely dispersed. 2.4 mg trypsin was added in three aliquots over a period of 2 hr. The digest was maintained with stirring at 36°C for a total of 6 hr. The remaining fine white precipitate was centrifuged and washed twice with 100 mM ammonium bi-

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carbonate. The combined supernatants contained 5.1×10^6 cpm. The precipitate was analysed for radioactivity and found to contain less than 0.1% of the soluble part. From these data it was calculated that 1.14 μmoles ^{14}C-NEM were incorporated into the protein, corresponding to a ratio of 0.94 moles of NEM per mole of enzyme.

2.5. *Gel chromatography of the tryptic digest*

The soluble part of the tryptic digest was lyophilised to reduce the volume and subjected to gel filtration on Sephadex G-50 superfine using the same conditions as described [2]. 12 ml fractions were collected and the radioactivity of the eluate measured in a 10 μl aliquot. The optical density of a 1:11 dilution was determined at 235 nm. The results are presented in fig. 1. In comparing fig. 1 with a similar figure in ref. [2] one can see, that the distribution of the optical density is almost identical in both experiments, indicating that the tryptic digest and the separation of peptides was very reproducible. The distribution of the ^{14}C-label is very different from the ^{3}H-label of a uniformly carboxymethylated IRS [2] and demonstrates that the reaction with NEM was quite specific.

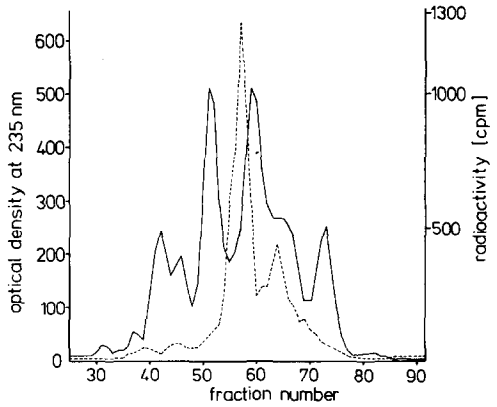

Fig. 1. Filtration on Sephadex G-50 of a tryptic digest (soluble fraction) of isoleucyl-tRNA synthetase modified with *N*-ethyl [2,3-^{14}C]maleimide. The soluble peptides of 124 mg protein were applied to a column (2.5 × 170 cm) packed with Sephadex G-50 superfine and eluted with 100 mM ammonium bicarbonate, pH 7.5, at a flowrate of 19 ml/hr at 4°C. Fractions of 12 ml were collected and analysed: the unbroken line represents the optical density at 235 nm of a 1:11 dilution, the broken line shows the radioactivity measured with a 10 μl aliquot.

Under the conditions employed approximately 80% of the label appeared in a single peak. The radioactive fractions were combined and freeze-dried.

2.6. *Fractionation of peptides on paper and sequence analysis*

The further fractionation and purification of the radioactive peptides, amino acid analysis and sequence studies were carried out as described previously [2]. Under the elution conditions used *S*-(2-succinyl)-cysteine did not separate from aspartic acid, while *S*-carboxymethyl-cysteine did. In peptides labeled with ^{14}C-NEM the value found for aspartic acid therefore is the sum of aspartic acid and *S*-(2-succinyl)-cysteine. The cysteine content in this case was calculated from the radioactivity of the peptide. Carboxymethyl-cysteine was determined as such on the aminoacid analyser. From the main peak of radioactivity 5 peptides were finally isolated in different yields (20–65 nmoles), all 5 peptides gave the same composition as shown in table 1 for peptide N/56. All 5 peptides revealed an identical sequence by Edman degradation. The established sequence is shown in table 2. Radioactive peptides with a different sequence have not been isolated from the tryptic digest. Random reactions

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

	N/56	N/92	VI/52	VII/1
Cys*	0.75	0.81	0.82	
Asp*	2.56	2.48	2.02	
Thr				
Ser	2.09	1.00	1.80	1.00
Glu	1.05		1.09	1.03
Pro	0.80	0.91	0.89	
Gly				
Ala	0.91	0.91	1.08	
Val	1.05	0.81	1.00	
Met	0.72			0.86
Ile	1.73	1.00	1.89	0.89
Leu				
Tyr				
Phe				
His				
Lys				
Arg	1.83	1.81	1.80	
Trp	+	+	+	

* For explanation see text.

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

N/56	Ile–Glu–Ser–Met–Val–Ala–Asp–Arg–Pro–Asn–Trp–Cys–Ile–Ser–Arg
VI/52	Ile–Glu–Ser–Met–Val–Ala–Asp–Arg–Pro–Asn–Trp–Cys–Ile–Ser–Arg
N/92	Val–Ala–Asp–Arg–Pro–Asn–Trp–Cys–Ile–Ser–Arg
VII/1	Ile–Glu–Ser–Met

* For explanation see text.

with NEM at other sites of the enzyme must have been small. After identification of the peptide labeled with ^{14}C-NEM the same peptide was also isolated from a tryptic digest of IRS uniformly labeled with H^3-iodoacetate (tables 1 and 2, VI/52) using the same methods.

2.7. *Chymotryptic digest of peptide N/56*

To establish the correct sequence of the C-terminal part of the peptide, especially the order Trp–Cys versus Cys–Trp, that was difficult to decide during Edman degradation of the whole peptide a chymotryptic digest was carried out. Twenty nmoles of peptide N/56 were incubated in 200 µl 100 mM ammonium bicarbonate, pH 8.0, with 20 µg of chymotrypsin for 16 hr at room temperature. The digest was diluted to 500 µl and loaded onto a column (1 × 50 cm) packed with Bio-Gel P 6 equilibrated with 100 mM ammonium bicarbonate, pH 7.6. The column was eluted with the same buffer at a flow rate of 1.4 ml/hr. 700 µl fractions were collected, lyophilised and redissolved in 100 µl water. Aliquots of 5 µl were taken to count the radioactivity of the sample and to test for tryptophan on paper by staining with Ehrlich's reagent. The remaining part was used for sequence analysis. The following fragments were identified in the order of their elution from the column: the decapeptide Ile–Glu–Ser–Met–Val–Ala–Asp–Arg–Pro–Asn, the hexapeptide Val–Ala–Asp–Arg–Pro–Asn, two tetrapeptides eluting together Ile–Glu–Ser–Met and Cys–Ile–Ser–Arg and free tryptophan. These fragments are derived from the parent peptide by splitting the C-terminal bond of tryptophan, methionine and asparagine. The latter residue is inferred to be asparagine rather than aspartic acid by comparison with bonds in other proteins susceptible to chymotryptic cleavage. The asignment of glutamic acid and aspartic acid to the other positions was made according to the electrophoretic mobility at pH 6.4 of the different peptides isolated (table 1).

3. Discussion

Inspection of the sequence reveals that there are two sulfur-containing amino acids present, one methionine and one modified cysteine. Each can independently and reversibly be oxidized by air to the sulfoxide, giving rise to chromatographically and electrophoretically different products. Such oxidations occurred quite rapidly on paper. Because of the tryptophan present in the peptide it appeared not to be advisable to oxidize the sulfur to the sulfone stage using performic acid or another agent to simplify the isolation of the peptide. There are two more peculiarities in the sequence which tend to complicate the analysis and lower the yield of this unique peptide. The Arg–Pro bond appears to be resistent to trypsin. Split fragments have not been isolated but may be present in low yield. On the other hand the Met-Val sequence has been split to some extent. A radioactive peptide N/92 was isolated from the minor peak of radioactivity (fig. 1) with the correct composition and sequence starting at Val (table 1 and 2) A nonradioactive tetrapeptide VII/1 was also found with the sequence Ile–Glu–Ser–Met comprising the N-terminal part of the original peptide. The reason for this proteolytic split is unclear. The trypsin used was of the highest purity available and had been treated with TPCK by the manufacturer. Chymotrypsin does split this bond but much more slowly than the Trp–Cys bond and the Asn–Trp bond as demonstrated by the chymotryptic digest of peptide N/56. But no radioactive tetrapeptide has been isolated from the tryptic digest of ^{14}C-NEM labeled IRS. The secondary split between methionine and valine and the difficulties arising from oxidation

during the purification of the peptide on paper are the main reasons for the severe losses in yield and explain why the peptide was lost during the first experiment [2].

It is too early to speculate which of the amino acids of this sequence are involved in the catalytic site of the enzyme. There are a number of specific interactions possible between the sidechains of the amino acids and an isoleucyl-adenylate. We are exploring other affinity labels and also K_m-mutants of IRS to get a better understanding of the complex catalytic process.

Acknowledgements

This work has been supported by the technology program of the Deutsche Bundesministerium für Forschung und Technologie. I am indebted to Miss S. Granda and Miss F. Köhler, to Mr A. Durekovic and Mr J. Gottschlich for the preparation of isoleucyl-tRNA synthetase. I thank Miss S. Löslein for her excellent technical assistance during the purification of peptides and the sequence studies.

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