THE SUBUNITS OF YEAST PHENYLALANYL-tRNA SYNTHETASE : A NEW FRACTIONATION PROCEDURE BASED UPON THEIR CYSTEINE CONTENTS

F. FASIOLO and Y. BOULANGER

Laboratoire de chimie biologique, Institut de biologie moleculaire et cellulaire – 15 Rue Descartes, 67000 Strasbourg, France

Received 9 October 1975

1. Introduction

Earlier studies have shown that yeast phenylalanyl-tRNA synthetase* has an $\alpha_2\ \beta_2$ structure [1,2]. In a recent paper Remy et al. described the isolation of the two subunits on organomercurial-Sepharose columns [3]: upon incubation each α-subunit of the native enzyme reacted with the organomercurial-Sepharose gel whilst the β-subunits did not bind at all and could be selectively eluted from the gel with guanidine hydrochloride (the α-chain was then washed off with 2-mercaptoethanol). Preparative SDS-gel electrophoresis was also used to separate these two subunits. Aminoacid analyses showed that only 20% of the cysteine residues of the enzyme were located on the β-subunits (S. Robbe-Saul, F. Fasiolo and Y. Boulanger, unpublished). Taking advantage of this property we have developed a new method to separate these two subunits: it relies on the alkylation of thiol groups with *p*-chloromercury benzoate or 5, 5′ dithiobis-(2 nitrobenzoate) under dissociating conditions, followed by ion-exchange chromatography on DEAE-cellulose columns. The distribution of the reporter group between the two subunits is discussed with respect to their aminoacid compositions.

2. Materials and methods

PheRS was prepared as previously described [4]. The enzyme was stored at a concentration of 22 mg/ml in 50% glycerol in the presence of 1 mM dithiothreitol, at −20°C; pCMB was from Sigma, the labelled compound was from the CEA, Saclay France. The following reagents were purchased from: DNTB, Merck-Schuchardt, urea, Riedel de Häen, Hannover. DEAE-cellulose type DE 52 was obtained from Whatman. All other compounds were reagent grade.

2.1. *Preparation of the pCMB solution*

A solution of 5 mM pCMB was prepared in 0.1 M Tris–HCl buffer pH 8.0. The purity of the reagent was checked spectrophotometrically by absorbance measurements at 232 nm at pH 7 using a molar absorptivity of 1.69×10^4.

2.2. *Purification of urea*

6 M urea solutions used in the chromatographic steps were filtered through a column of packed charcoal (5 × 1.5 cm). Up to 10 litres of solution could be filtered without releasing any absorbing material. For enzyme dissociation urea was recrystallised before use from ethanol–water (v/v, 1:1).

2.3. *Titration of the native enzyme with pCMB*

The time course of the reaction was followed in an ice-bath with 1.2 nmol of PheRS and 100 nmol of labelled pCMB (1000 cpm/nmol) in a total volume of 0.5 ml (unless otherwise stated the buffer system contained 10% glycerol (v/v) and 10^{-4} M EDTA). At given time intervals aliquots of 60 μl were taken out, filtered onto nitrocellulose filters and counted as previously reported [3].

2.4. *Titration with DTNB*

Titration with DTNB was followed at room tempera-

* *Abbreviations:* phenylalanyl-tRNA synthetase, PheRS; *p*-chloromercury benzoate, pCMB; 5,5′-dithiobis-(2 nitrobenzoate) DTNB; 5-thio-2-nitrobenzoate, TNB.

North-Holland Publishing Company – Amsterdam

ture in 0.05 M Tris–HCl buffer pH 7.5, containing 10^{-4} M EDTA, using 7 mg protein and 6500 nmol of DTNB. The colour was developed for 45 min and read in a Zeiss PMQII spectrophotometer at 412 nm against a reagent blank. The highest plateau value was obtained under dissociating conditions in the presence of 5 to 7 M urea and was stable for 20 min. Calculations of the sulfhydryl content were made using a molar absorptivity of 13 600 M^{-1} cm^{-1} for TNB [5] and a mol. wt of 260 000 for the enzyme.

2.5. *Alkylation of the enzyme with pCMB under dissociating conditions*

PheRS (11 mg/ml) was freed from dithiothreitol by dialysis at 4°C (for 12 h against 2 × 1 litre of 0.1 M Tris–HCl buffer pH 8.2, containing 10% glycerol (v/v) and 10^{-4} M EDTA under a continuous flow of nitrogen. Alternatively the enzyme was reduced with fresh 2-mercaptoethanol (5% v/v) for 30 min at 20°C and filtered through a column of Sephadex G-50 (1.3 × 19.5 cm); the whole procedure could be achieved in 2 h.

Reaction with pCMB was performed in 0.1 M Tris–HCl buffer pH 8.2 (containing 10% glycerol, v/v, 10^{-4} M EDTA and 5 mM $MgCl_2$) using about 7 mg enzyme (27 nmol) and 2.5 μmol of labelled pCMB (1000 cmp/nmol). The excess of reagent over total enzyme SH groups was 3.7-fold. The reaction was carried out at 0°C for 1 h; urea was then added to a final concentration of 8 M (final vol 4.5 ml) and the incubation was continued for 2 h at room temperature.

Excess of pCMB was removed by filtration at 4°C through a column of Sephadex G50 (1.5 × 36 cm) equilibrated with 10 mM potassium phosphate buffer pH 7.2 containing 6 M urea. Up to 8 ml could be applied to this column without affecting the resolution. Fractions of 2 ml were collected at a flow-rate of 13 ml/h. The protein was recovered in the void volume and monitored either by scintillation counting (aliquots of 25 μl in 5 ml of Bray's solution) or by spectrophotometric measurements at 280 nm.

2.6. *Chromatography of the dissociated pCMB labelled enzyme on DEAE-cellulose*

The enzyme fraction was loaded onto a column of DEAE-cellulose DE 52 (1.2 × 65 cm) equilibrated with 10 mM potassium phosphate buffer pH 7.2, 6 M urea. The column was eluted at 4°C with a linear gradient from 0 to 0.3 M KCl in the same buffer (total vol 400 ml). Fractions of 4 ml were collected at a flow-rate of 15 ml/h. Protein was monitored by scintillation counting and absorbance measurements at 280 nm.

2.7. *Polyacrylamide gel electrophoresis*

Aliquots of the fractions eluted from the DEAE-cellulose column were analysed directly by disc electrophoresis using the SDS-phosphate system [5].

3. Results and discussion

3.1. *Titration of thiol groups*

The reaction with pCMB was studied under several conditions in the native enzyme a maximum of 15 SH groups reacted (fig.1). In 8 M urea 21 to 22 SH groups reacted as was calculated from the protein-bound radioactivity (fig.2). As shown in fig.1 the reactivity of SH groups at pH 8.2 is lower than at pH 7.5 in the Tris-buffer system but in the latter case the plateau value decreases rapidly. This is not due to some splitting of C–Hg bonds since no release of labelled pCMB could be detected during the Sephadex filtration experiments (fig.2) but more likely to a labilization of the quaternary structure caused by the alkylation thus decreasing the amount of protein retained by the nitrocellulose filter. Indeed it must be pointed out that no radioactivity stuck to the filter when the [^{14}C]pCMB labelled enzyme was filtered in the presence of 6 M guanidine hydrochloride.

Titration with DTNB proceeds at a much slower

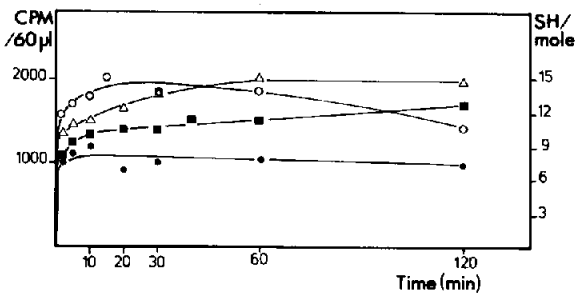


Fig.1. Titration of the native enzyme with pCMB. (△) In 0.1 M Tris–HCl buffer pH 8.2. (■) In the same buffer with 5 mM $MgCl_2$. (○) In 0.05 M Tris–HCl buffer pH 7.5. (●) In 0.05 M potassium phosphate buffer pH 7.6.

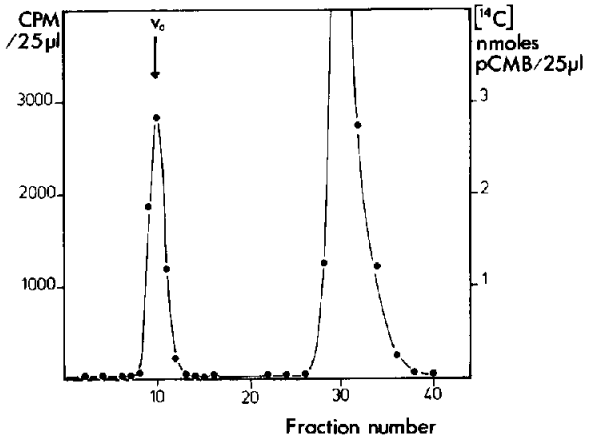


Fig.2. Filtration of pCMB-PheRS on a column of Sephadex G50.

rate and 20–21 SH groups could be detected in the presence of 8 M urea (15 in the native enzyme).

These values are in fairly good agreement with aminoacid analyses: 24 cysteic acid were found after hydrolysis of the performic-oxidised enzyme.

3.2. *Chromatography of the dissociated pCMB-labelled enzyme on DEAE-cellulose*

Two protein peaks were eluted from the column respectively at 0.08 M Cl^- corresponding to pure β (63 000 daltons) and 0.11 M Cl^- corresponding to pure α (72 000 daltons) as was shown by SDS-gel electrophoresis (fig.3).

The absorbance ratio of the two peaks was 1:1 at 280 nm. It must be emphasized that such a ratio was a good indication of the purity of each fraction: when alkylation was not complete (for instance 12 SH blocked per mole) the first peak always contained both β and α and the absorbance ratio peak 1: peak 2 was 2.6.

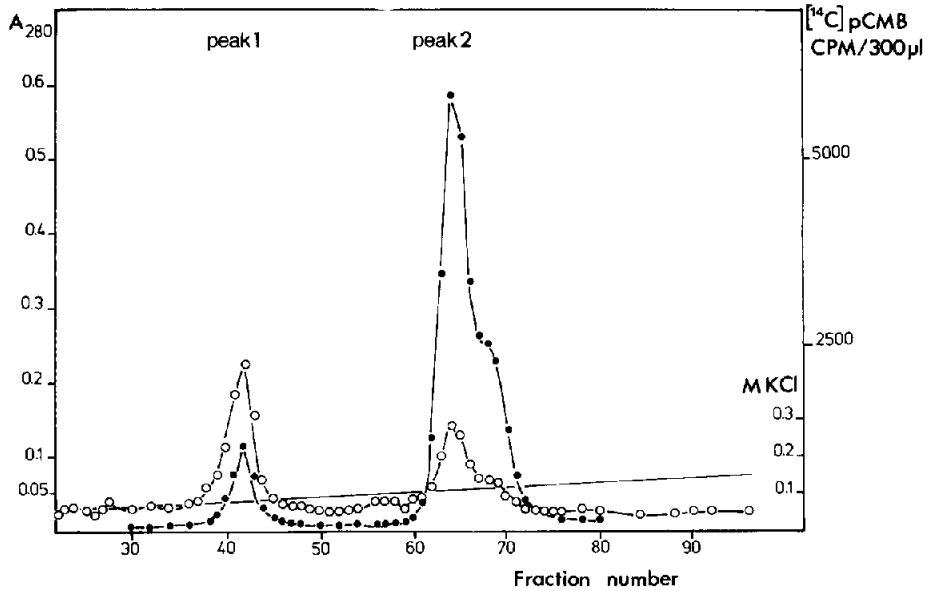
In contrast to the normal absorbance ratio of 1 the radioactivity ratio was about 9. This value is much higher than the ratio of about 5 given by aminoacid analyses. It could be due to incomplete labelling of β.

3.3. *Chromatography of the dissociated TNB-enzyme*

Under the same conditions two peaks were obtained corresponding to β and α respectively with an absorbance ratio of 1:1. Both fractions were incubated with an excess of 2-mercaptoethanol and from the amount of TNB released a ratio $\alpha : \beta$ of about 5 was calculated. (Which is in good agreement with amino acid analyses).

The SDS gel pattern showed that each fraction was contaminate by a small amount of the other.

Uncomplete alkylation could result in such a contamination as was observed with the pCMB-labelled enzyme. But an additional effect can also account



(A)

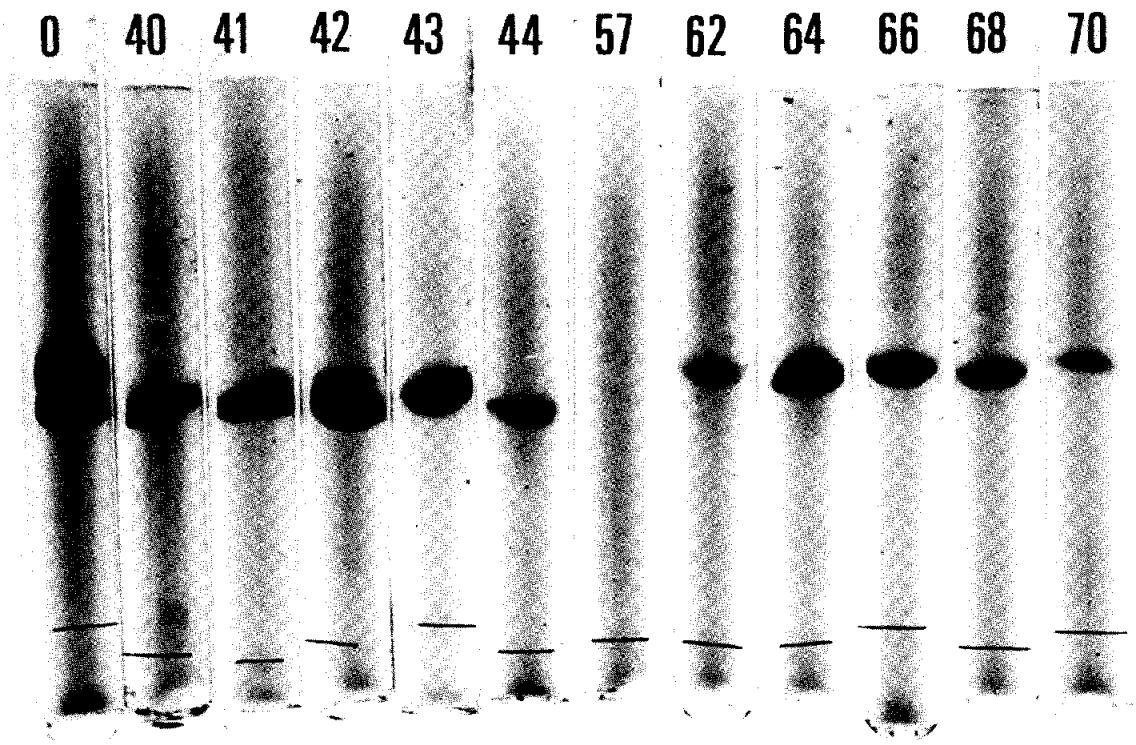


Fig.3.(A) Chromatography of pCMB-PheRS on a column of DEAE-cellulose. (○) Absorbance at 280 nm. (●) radioactivity measurements. (—) gradient of KCl. (B) SDS-gel electrophoresis of fractions 40 to 44, 57, 62 to 70 and native enzyme (0).

for this double contamination: a non-blocked SH group located on one subunit, e.g. β, could cross-react with the highly reactive S-substituted derivative of the other, TNB-α, giving rise to a disulphide bridge α–S–S–β.

3.4. *Restoration of enzyme activity*

Removal of the blocking reagent was obtained by incubation of the modified enzyme with 1% (v/v) 2-mercaptoethanol at 0°C for 30 min (removal of TNB occurs in less than 1 min). Mixing stoichiometric amounts of unfolded α and β chain prior to dialysis of urea yielded an enzyme with a specific activity of 3300 U/mg identical with that of the native one (acylation test). The overal yield of protein recovery was 75%. After dialysis of urea isolated α or β subunits exhibited very little activity either in the activation reaction or in the acylation one (7 to 14 U/mg in the latter).

It remains to be demonstrated whether this very low activity is a true feature of isolated subunits or a result of a very small contamination of each subunit by the other.

4. Conclusion

The present study clearly demonstrates that ion-exchange chromatography of the pCMB modified PheRS under dissociating conditions, provides the best way of preparing pure subunits with a high resolution and good yields. The technique is both simple and quick. The results reported here (and those of amino acid analyses) show that cysteine residues are unequally distributed between the two subunits since 80% of them are located on the α-subunits (10 Cys per α). This big difference between the two subunits makes unlikely the hypothesis according to which native PheRS is a tetramer $\alpha_4,\alpha_2,\beta_2$ being a proteolysed form [6]: if this were true 8 of the 10 cysteine resi-

dues would be located in the fragment of 10 000 daltons supposedly released from α to yield the β chain.

Acknowledgements

The authors thank Professor Ebel for his help and advice and his critical reading of the manuscript.

The excellent technical assistance of Mrs Nussbaum is gratefully acknowledged.

References

[1] Fasiolo, F., Befort, N., Boulanger, Y. and Ebel, J. P. (1970) Biochem. Biophys. Acta 217, 205–318.
[2] Fasiolo, F., Remy, P., Pouyet, J. and Ebel, J. P. (1974) Eur. J. Biochem. 50, 227–236.
[3] Murayama, A., Raffin, J. P., Remy, P. and Ebel J. P. (1975) FEBS Lett. 53, 23–25.
[4] Fasiolo, F. and Ebel, J. P. (1974) Eur. J. Biochem. 49, 257–263.
[5] Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70.
[6] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[7] Hossain, A. (1974) Fed. Proc. 33, 1421.