

YEAST PHENYLALANYL-tRNA SYNTHETASE: PROPERTIES OF THE SULFHYDRYL GROUPS; EVIDENCE FOR -SH REQUIREMENT IN tRNA ACYLATION

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1. Introduction

In spite of the particular importance of aminoacyl-tRNA synthetases, little is known about the role of specific aminoacid residues in the catalytic activity [1,2]. Among the aminoacid residues the most studied were the sulfhydryl groups. In most cases, modification of thiol groups resulted in the disappearance of the acylation activity of the enzymes, while the ATP-pyrophosphate exchange activity was little or not affected [3-7].

We report here the properties of the thiol groups of phenylalanyl-tRNA synthetase (PRS) and their participation in the activity of the enzyme. Two -SH groups per molecule of enzyme were found to be necessary for the acylation of tRNA, but not for phenylalanine activation. 2-mercaptoethanol was shown to inhibit the heterologous acylation of yeast tRNA^{Val} by yeast PRS, while the homologous acylation of yeast tRNA^{Phe} was unaffected. The possible existence of a thioesteracylenzyme intermediate in tRNA acylation, as proposed by Mc Elroy et al. [8], will be discussed.

2. Materials and methods

The yeast phenylalanyl-tRNA synthetase was prepared by the procedure previously described [9], modified according to [10]. In order to study thiol properties, the stock solution of enzyme was freed from 2-mercaptoethanol by extensive dialysis at 4°C against 50 mM Tris-HCl buffer pH 7.4, 0.1 mM EDTA, 10 mM MgCl₂, glycerol 10% (v/v). To prevent the oxydation of sulfhydryl groups by dissolved

oxygen, all buffers were degassed using pure nitrogen and the dialyses were performed under a continuous flow of nitrogen.

Purified yeast tRNA^{Phe} was obtained by counter-current distribution, as described by Dirheimer et al. [11]. *p*-Aminophenylmercuriacetate (PAPM) was synthesised according to [12]. *p*-Chloromercuribenzoate (PCMB) was purchased from Sigma. All other reagents used were analytical grade chemicals from Merck, Fluka and Prolabo. Radiochemicals were purchased from the Commissariat à l'Energie Atomique-Saclay - France.

2.1. Enzymatic activity determinations

The enzymatic activity was measured either by tRNA aminoacylation [13] or by ATP-pyrophosphate exchange by a technique derived from that of Calender and Berg [14], as described by Fasiolo et al. [15].

2.2. Polyacrylamide gel electrophoresis

Electrophoreses were carried out in (0.6 × 10) cm glass tubes with 7.5% acrylamide, using the system described by Davis [16]. Gels containing sodium dodecyl sulphate in 10% acrylamide were prepared and used according to the method of Laemmli [17].

2.3. Chromatography on agarose columns in the presence of SDS

Ascending chromatographies were conducted at room temperature on a (3 × 90) cm column filled with Biogel A 1.5 m equilibrated in 50 mM acetate buffer pH 5.5, 0.1% sodium dodecyl sulphate. 3 ml fractions were recovered.

2.4. Incubation of phenylalanyl-tRNA synthetase with thiol reagents

Treatment of PRS with thiol reagents (PAPM, PCMB, DTNB, iodoacetic acid iodoacetamide, cupric chloride) were performed in 50 mM phosphate or Tris-HCl buffers pH 7.5, 10 mM MgCl_2 , 10% glycerol (v/v) at 37°C. At different time intervals, aliquots were taken out and analysed for enzymatic activity after a convenient dilution in 50 mM Tris-HCl buffer pH 7.4, bovine serum albumin 1 mg/ml, 0.1 mM EDTA, 60 mM KCl, 10% glycerol (v/v). For polyacrylamide gel electrophoresis, the reaction mixture was used without dilution.

Titration of PRS thiol groups, under non dissociating conditions was performed with [^{14}C] *p*-chloromercuribenzoate (10.4 $\mu\text{Ci}/\mu\text{mol}$) in the following conditions: the enzyme (2.75×10^{-6} M) in 0.1 M Tris-HCl buffer pH 8.2 was incubated at 0°C in the presence of 2.75×10^{-4} M of labelled PCMB (final vol 400 μl). At given time intervals, 50 μl aliquots were taken out and filtered on nitrocellulose filters (0.45 μm) after dilution in 1 ml of 0.1 M Tris-HCl buffer pH 8.2. Filters were rinsed with 5 ml of the same buffer. When short incubation times were used (15 and 30 sec), the dilution buffer contained the stoichiometric amount of 2-mercaptoethanol with respect to the introduced PCMB, in order to stop immediately the reaction. Radioactivities were determined by scintillation counting, using a 4 g/l Omnifluor solution in toluene.

2.5. Study of tRNA binding by chromatography on G 200 Sephadex column

The binding of tRNA^{Phe} to organomercurial modified enzyme was studied by filtration on a G 200 Sephadex column (0.8 \times 25 cm) equilibrated at a constant concentration of tRNA^{Phe} ($7 \cdot 10^{-6}$ M) in 0.05 M Tris-HCl buffer pH 7.2, 0.01 M MgCl_2 , 0.1 mM EDTA. Fractions of 400 μl were recovered.

3. Results

3.1. Incubation of PRS with Cu^{++} ions

Fig.1 shows the modification of the acylation activity of PRS as a function of time during the incubation of the enzyme in the presence of different Cu^{++} concentrations. A strong inhibition is observed

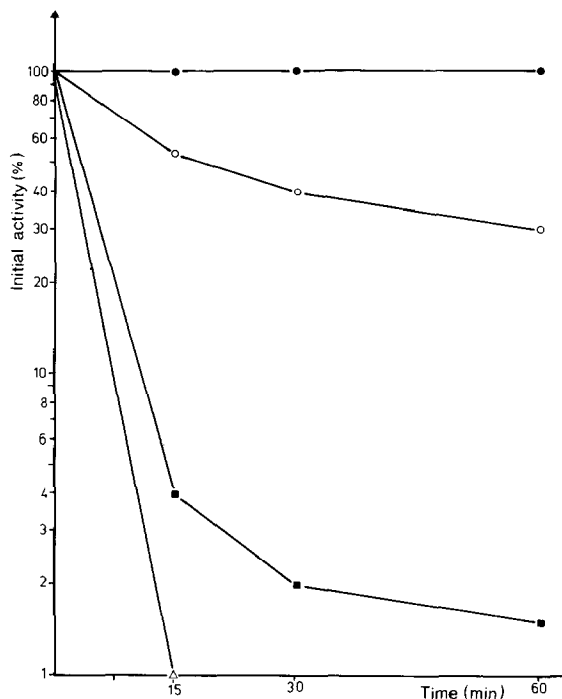


Fig.1. Modification of the acylation activity of PRS as a function of time in the presence of different concentrations of CuCl_2 . (●—●) CuCl_2 $1 \cdot 10^{-6}$ M. (○—○) CuCl_2 $5 \cdot 10^{-6}$ M. (■—■) CuCl_2 $5 \cdot 10^{-5}$ M. (△—△) CuCl_2 $5 \cdot 10^{-4}$ M.

for Cu^{++} concentrations as low as $5 \cdot 10^{-6}$ M. The analysis of the modified enzyme by SDS-polyacrylamide gel electrophoresis shows (fig.2, gel 2) the appearance of a band of high mol. wt (around 150 000). This heavy component has been separated by chromatography on Agarose in the presence of SDS (fig.3). Peaks 2 and 1 were analysed by electrophoresis on SDS polyacrylamide gels (fig.2, gel 3 and 4). The incubation of the high mol. wt compound eluted in peak 1 with 0.1 M 2-mercaptoethanol results in the formation of α and β subunits in equimolecular amounts, as can be seen from fig.2 (gel 5), thus demonstrating that a covalent α - β dimer was built upon treatment with Cu^{++} . This α - β dimer mainly results from an intramolecular condensation of -SH groups, since the analysis of the modified enzyme by acrylamide gel electrophoresis in non dissociating conditions (fig.4, gel B) does not reveal the existence of polymers $(\alpha_2\beta_2)_n$ which should

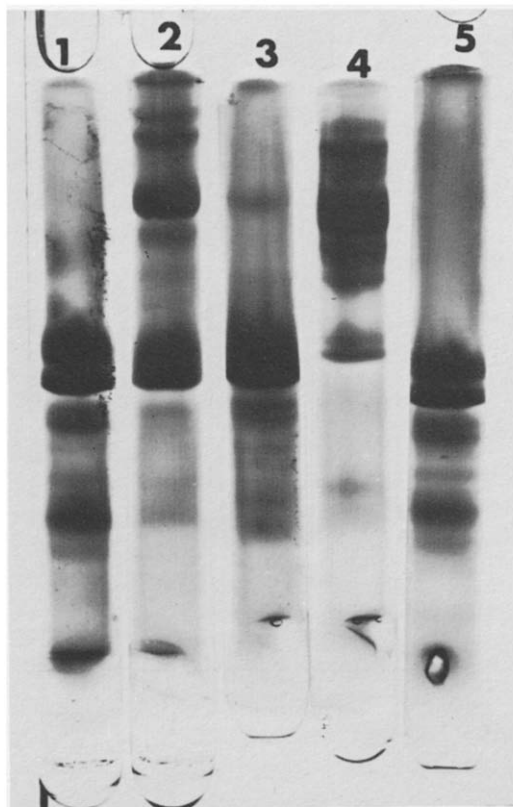


Fig.2. Electrophoretic pattern on SDS-polyacrylamide gels of the Cu^{++} modified enzymes. gel 1: native PRS. gel 2: PRS treated by $5 \cdot 10^{-4}$ CuCl_2 . gel 3: low mol. wt fraction of PRS subunits after treatment with Cu^{++} and separation on SDS-Agarose column (peak 2 of fig.3). gel 4: high mol. wt fraction of PRS subunits after treatment with Cu^{++} and separation on SDS-Agarose column (peak 1 of fig.3). gel 5: high mol. wt fraction of PRS subunits after treatment with Cu^{++} , separation on SDS-Agarose column, and treatment with 10^{-1} M mercaptoethanol.

migrate slower than the native enzyme. It can also be seen that the internal 'dimerisation' of the enzyme is accompanied by some dissociation in α - β structures.

3.2. Incubation of PRS with thiols reagents

Fig.5 shows the modification of the acylation activity of PRS during the treatment of the enzyme

Fig.4. Polyacrylamide gel electrophoresis of Cu^{++} modified PRS, in non dissociating conditions. gel A: native enzyme. gel B: Cu^{++} modified enzyme.

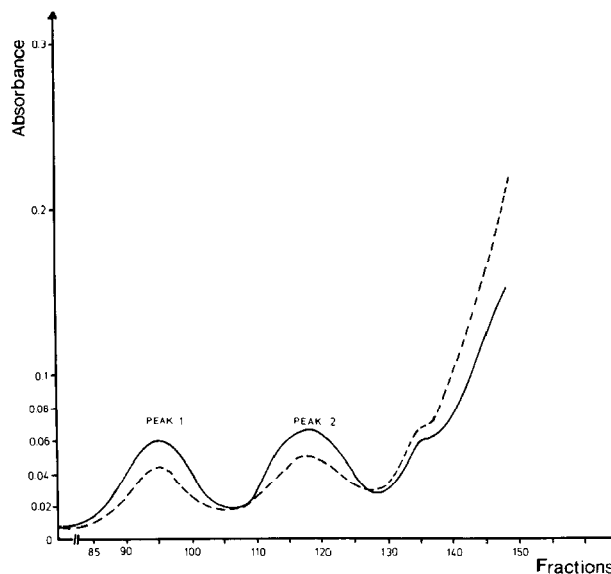
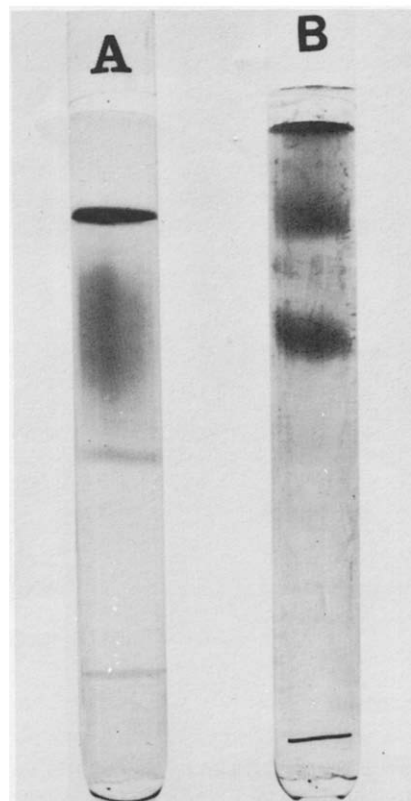


Fig.3. Filtration of Cu^{++} modified PRS on SDS-Agarose A 1.5 M column (3×90 cm). (—) optical density at 260 nm, (---) optical density at 280 nm. Fraction volume 3 ml.



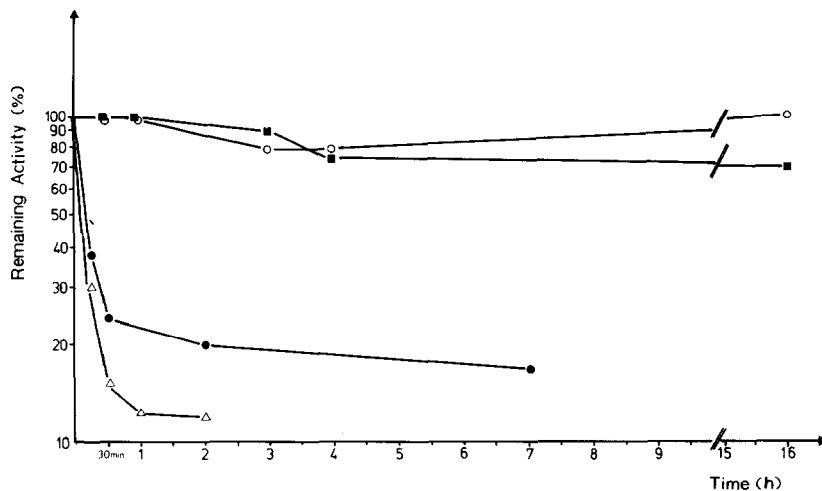


Fig.5. Modification of the acylation activity of PRS in the presence of different thiols reagents: (■—■) PRS: $4 \cdot 10^{-7}$ M ICH_2COOH : $8 \cdot 10^{-5}$ M (200-fold molar excess), (○—○) PRS: $4 \cdot 10^{-7}$ M $\text{ICH}_2\text{CONH}_2$: $8 \cdot 10^{-5}$ M (200-fold molar excess), (●—●) PRS: $2 \cdot 10^{-7}$ M *p* aminophenylmercuriacetate: $20 \cdot 10^{-7}$ M (10-fold molar excess), (△—△) PRS: $2 \cdot 10^{-7}$ M *p* aminophenylmercuriacetate: $20 \cdot 10^{-7}$ M (30-fold molar excess).

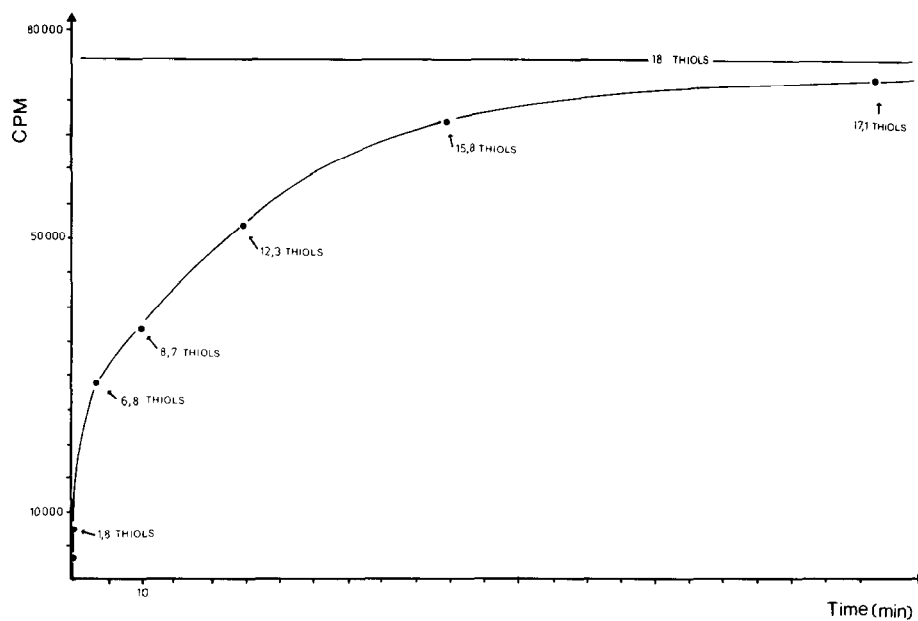


Fig.6. Titration of PRS sulfhydryl groups by $[^{14}\text{C}]p$ -chloromercuribenzoate (10.4 mCi/mmol) in non-dissociating conditions: the number of modified thiol groups per molecule of enzyme are indicated for each experimental point on the figure.

with different thiol reagents. It can be seen that iodacetamide and iodacetic acid do not inhibit the enzyme, even for molar excess of reagents as high as 200-fold. DTNB is slightly more efficient, since a slow inactivation occurs in the presence of a 30-fold excess of reagent. PAMP is much more reactive since a 30% inactivation is observed within 30 sec in the presence of a 5-fold molar excess of reagent. A 10-fold molar excess of the mercurial leads to almost complete inhibition of the enzyme. The same results are obtained with *p*-chloromercuribenzoic acid.

Fig.6 shows the titration of PRS thiol groups by [14 C]*p*-chloromercuribenzoic acid, in non dissociating conditions. On the basis of a mol. wt of 270 000 for PRS [13], the titration reveals 18 thiol groups per molecule of enzyme. It must be pointed out that after 30 sec of incubation, around two thiol groups are titrated per molecule of PRS. This result has to be compared to the inactivation study, which showed that the rapid phase of the enzyme inactivation took place within 30 sec.

As it can be seen from fig.7 the SH groups which are essential for tRNA^{Phe} acylation are not required for the activation of phenylalanine, since the ATP-

pyrophosphate exchange activity of PRS is much less inhibited by mercurials than the acylation activity.

The selective inhibition of the acylation reaction does not result from a decrease of the tRNA binding. This fact is demonstrated in fig.8. PRS, which has been inactivated to 90% in tRNA charging and which has kept 80% of the AMP-PP_i exchange activity has been applied to a G 200 Sephadex equilibrated at a constant tRNA^{Phe} concentration. The complex peak is still clearly visible. The stoichiometries measured within the peak were all found close to 2, as for the native enzyme [13].

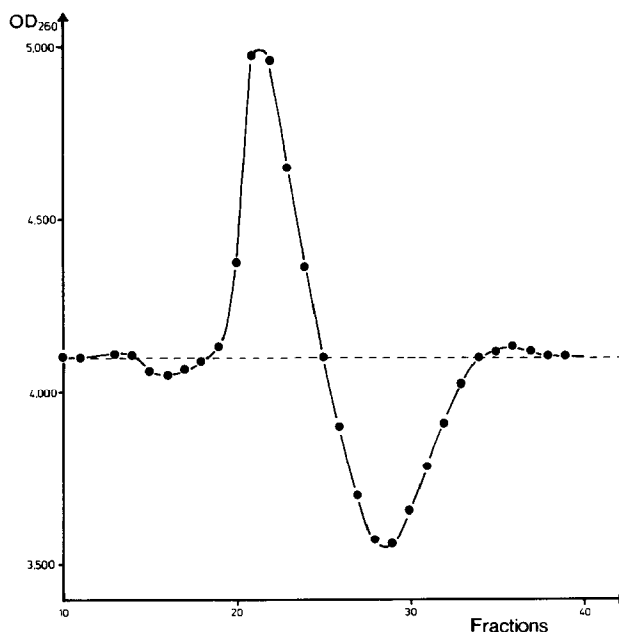
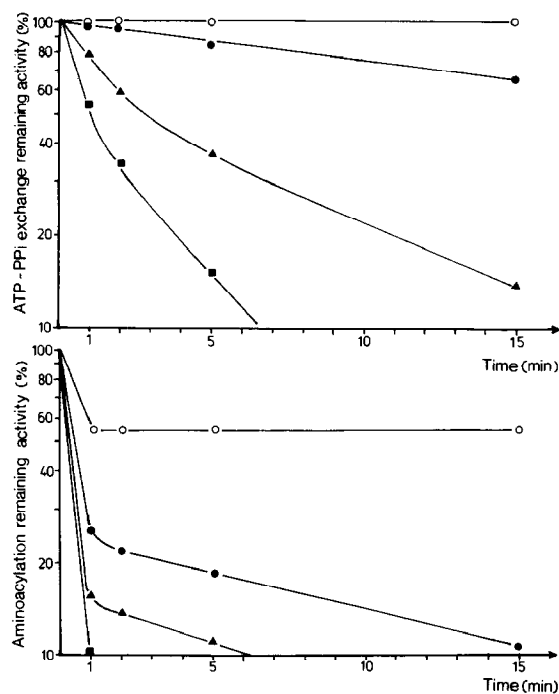


Fig.8. Filtration of *p*-chloromercuribenzoate modified PRS on a G 200 Sephadex column (0.8 × 25 cm) equilibrated at a constant concentration of tRNA^{Phe} ($7 \cdot 10^{-6}$ M).

Fig.7. Comparison of the modifications of ATP-PP_i exchange activity (A) and tRNA acylation activity (B) as a function of time in the presence of different concentrations of *p*-aminophenylmercuriacetate: PRS $2.8 \cdot 10^{-6}$ M (○—○) *p*-aminophenylmercuriacetate $14 \cdot 10^{-6}$ M (5-fold molar excess). (●—●) *p*-aminophenylmercuriacetate $28 \cdot 10^{-6}$ M (10-fold molar excess). (▲—▲) *p*-aminophenylmercuriacetate $42 \cdot 10^{-6}$ M (15-fold molar excess). (■—■) *p*-aminophenylmercuriacetate $56 \cdot 10^{-6}$ M (20-fold molar excess).

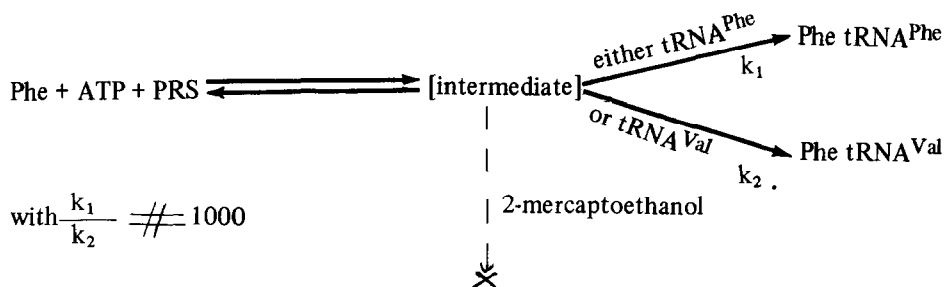
Finally, it has to be noticed that treatment of PRS with a 10-fold molar excess of PAPM completely prevents the formation of α - β dimer upon subsequent treatment with Cu^{++} ions.

3.3. Protection by the ligands against organomercurial inactivation

As can be seen from fig.8, phenylalanine affords a rather good protection against PAPM inhibition of the acylation activity of PRS. No improvement of the protection could be observed upon addition of ATP. tRNA^{Phe} has no effect on the rapid phase of this inactivation but seems to decrease the rate of the slow inhibition phase. ATP alone has almost the same effect as tRNA^{Phe} .

3.4. Effect of 2-mercaptoethanol on homologous and heterologous aminoacylations catalysed by PRS

In order to check the possible existence of some intermediate in the aminoacylation reaction, we studied the effect of increasing concentrations of a nucleophilic agent (2-mercaptoethanol) on the aminoacylation rate in two different conditions; a) homologous aminoacylation of yeast tRNA^{Phe} by PRS; b) heterologous acylation of yeast tRNA^{Val} by PRS according to the following scheme:



in which the nucleophilic agent (2-mercaptoethanol) is expected to react with the supposed intermediate.

Fig.9 shows that the homologous acylation reaction is not inhibited by 2-mercaptoethanol even in the presence of $5 \cdot 10^{-2}$ M of this reagent. On the contrary the heterologous acylation reaction is strongly inhibited for low concentrations of 2-mercaptoethanol (less than 10^{-3} M). It can also be seen that the phenylalanine activation (ATP-pyrophosphate exchange) is much less sensitive to the effect of 2-mercaptoethanol than the heterologous acylation

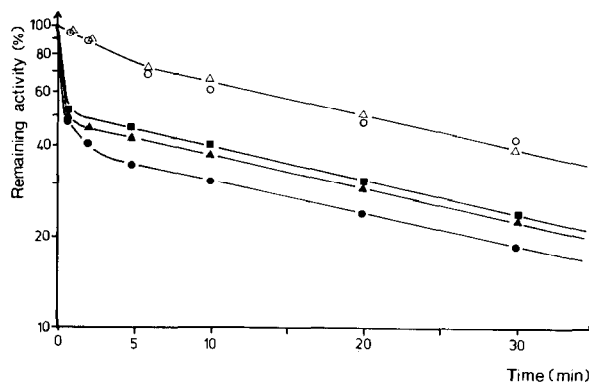


Fig.9. Protection afforded by different ligands of PRS against *p*-aminophenylmercuriacetate inactivation. PRS: $5 \cdot 10^{-8}$ M, *p*-aminophenylmercuriacetate: $5 \cdot 10^{-7}$ M. (●—●) no addition. (▲—▲) + tRNA^{Phe} $5 \cdot 10^{-7}$ M. (■—■) + ATP $1 \cdot 10^{-3}$ M. (△—△) + Phe $1 \cdot 10^{-3}$ M. (○—○) + Phe $1 \cdot 10^{-3}$ M + ATP $1 \cdot 10^{-3}$ M.

suggesting that the target of the nucleophilic agent appears later than the enzymeaminoacyladenylate complex.

4. Conclusion and discussion

The above results demonstrate that sulfhydryl

groups are essential for aminoacylation of tRNA but are not required for phenylalanine activation. Comparison of the titration of PRS thiol groups by ^{14}C PCMB and of the inactivation of the enzyme by organomercurials showed that within the time range of the fast inactivation reaction only two thiol groups are titrated by PCMB. Since other experimental evidences (see accompanying paper) show that the essential SH groups are located on the α subunit (mol. wt = 73 000), it may be concluded that one -SH group per α subunit is needed for the acylation

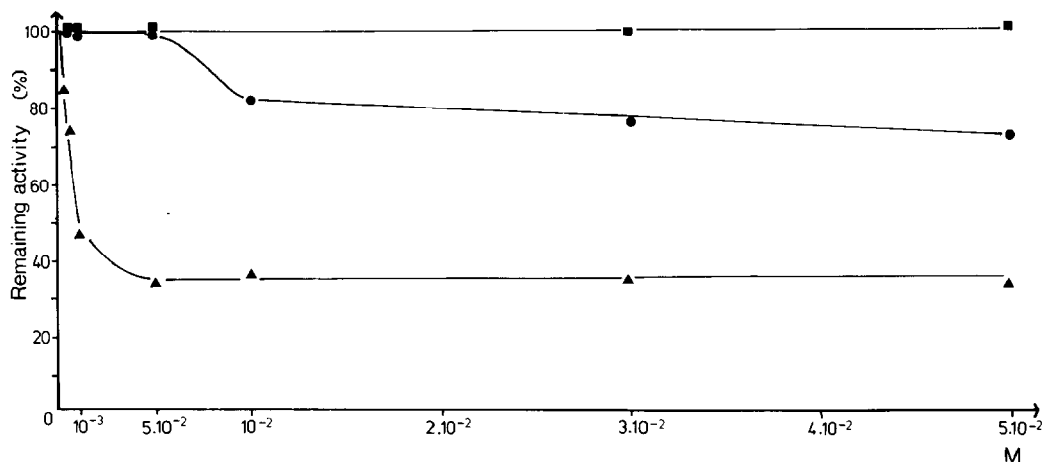


Fig.10. Effect of increasing concentrations of 2-mercaptoethanol on the homologous acylation of tRNA^{Phe} heterologous acylation of tRNA^{Val} and phenylalanine activation: (■—■) acylation of yeast tRNA^{Phe}. (▲—▲) acylation of yeast tRNA^{Val}. (●—●) ATP-PP_i exchange.

of tRNA^{Phe}. These essential -SH groups seem to be implicated in the intramolecular α - β disulfide binding catalysed by Cu⁺⁺ ions, since reacting these "fast" -SH groups with an organomercurial prevents the α -S-S- β formation upon subsequent treatment by Cu⁺⁺. So, the essential thiol groups are probably located close to the α - β interaction area. The protection afforded by phenylalanine against organomercurial inactivation suggests that the essential -SH are close to the aminoacid binding site or that, upon binding of aminoacid, some structural transconformation occurs, which buries these thiol groups.

The difference observed in the inhibition patterns by 2-mercaptoethanol of homologous and heterologous acylation reactions suggests that in the heterologous reaction, which is much slower ($K_{\text{homologous}}/K_{\text{heterologous}} \cong 1000$), some intermediate accumulates which can react with 2-mercaptoethanol, thus being no longer available for tRNA acylation. The fact that ATP-PP_i exchange is much less sensitive to 2-mercaptoethanol inhibition than the heterologous acylation reaction suggests that the supposed intermediate might be different from the enzyme-aminoacyladenylate complex. Similar results have been observed by Parin and Kisselev [18], who showed that $5 \cdot 10^{-2}$ M 2-mercaptoethanol

completely inhibited the acylation of tRNA by rat liver tryptophanyl-tRNA synthetase, while the hydroxamate formation reflecting the aminoacid-activation rate was not affected. These authors concluded that the observed effect was due to the reduction of a disulfide bond, which would obliterate the acylation activity, without altering the aminoacid activation.

Such an explanation can be ruled out in our case since the homologous acylation reaction is not affected even in the presence of $5 \cdot 10^{-2}$ M mercaptoethanol.

All the properties observed in this paper might be consistent with a catalytic model similar to that proposed by Mc Elroy et al; [8] which involves the attack of aminoacyladenylate by a thiol group of the enzyme leading to an intermediate thioacyl-enzyme. Such a model should allow an ATP-AMP exchange in the absence of tRNA. The general failure to demonstrate such an exchange could be explained, as already suggested by Mc Elroy et al. [8], by a conformational change of the enzyme upon binding of tRNA, which would trigger the attack of aminoacyladenylate by a thiol group of the protein. Further experiments are in progress to check such a possibility.

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