'HALF-SITE' AFFINITY MODIFICATION OF TRYPTOPHANYL-tRNA SYNTHETASE LEADS TO 'FREEZING' OF THE FREE SUBUNIT

O. O. FAVOROVA, I. A. MADOYAN and V. L. DRUTSA*

Institute of Molecular Biology, USSR Academy of Sciences and **Moscow State University, Moscow V-334, USSR*

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1. Introduction

Affinity modification that combines the advantages of inhibitory analysis and chemical modification of essential residues in a protein molecule has the merit of helping to gain a better insight into the structural–functional interrelations in enzymes. The technique has been used for studying certain aminoacyl-tRNA synthetases, enzymes that catalyse specific aminoacylation of tRNA (see [1,2]). In one case, of beef pancreas tryptophanyl-tRNA synthetase (EC 6.1.1.2) (M_r 108 000–120 000, α_2-type (see [3]), affinity labelling was done using analogs of all of the 3 substrates:

(i) The tryptophanyl chloromethyl ketone [4];
(ii) The photoaffinity analog of ATP, γ-(*p*-azidoanilide)-ATP [5] and ATP analogs that contain alkylating and phosphorylating groups in the polyphosphate chain [6];
(iii) An analog containing an active group bound to the aminoacyl moiety of tryptophanyl-tRNA (*N*-chloroambucilyl–tryptophanyl-$tRNA^{Trp}$) [5].

Moreover, a relatively stable tryptophanyl enzyme derivative has been found; this made it possible to introduce an affinity label into the enzyme active centre directly from the substrate [7,8].

We have used as affinity label of this enzyme a mixed anhydride of AMP with mesitylenecarboxylic acid (mesitoyl-AMP) [10]. Here, the modified enzyme was used to evaluate the interactions between two subunits in the dimeric enzyme protein. The 'half-site' phosphorylation by an AMP residue from mesitoyl-[^{14}C]AMP is observed. The modified enzyme fully inactive in both reactions is still capable however of forming 1 mol tryptophanyl adenylate. The absence of catalytic activity of the free centre is due to the pyrophosphate release being blocked, as follows from partial restoration of the activity of the modified enzyme in tRNA aminoacylation in the presence of PP_i. Both the 'half-site' modification and 'freezing' of a non-modified subunit are considered to be the result of strong negative co-operativity between identical subunits.

2. Materials and methods

[γ-^{32}P]ATP (3000 Ci/mmol), sodium [^{32}P]pyrophosphate (33 Ci/mmol) and [^{14}C]tryptophan (52 Ci/mol) were purchased from the Radiochemical Centre, Amersham, and [^{14}C]AMP (230 Ci/mmol) was obtained from UVVVR (Czechoslovakia). Mesitoyl-[^{14}C]AMP (28 Ci/mol) was synthesized as in [9]. Inorganic pyrophosphatase from baker's yeast was from Sigma.

Preparation of the enzyme, activity assays and modification with mesitoyl-AMP were described [10]. Formation of complexes between the enzyme and aminoacyl adenylates was determined from [γ-^{32}P]-ATP consumption [4,11]. The incubation mixture contained 0.5–0.8 μM tryptophanyl-tRNA synthetase, 0.02–0.06 mM L-tryptophan, 0.05 M Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 20 mM NaCl and [γ-^{32}P]ATP at concentrations which were 5- or 10-times as high as those of the enzyme. The reaction was conducted at 37°C and stopped by addition of 0.5 ml of the suspension used in the ATP–PP_i exchange assay (see [3]) to 20–40 μl aliquots.

3. Results

We incubated tryptophanyl-tRNA synthetase with 1 mM mesitoyl-[^{14}C]AMP until the enzyme was completely inactivated, and then removed the unreacted

Table 1
Stoichiometry of [^{14}C]AMP residue incorporation into tryptophanyl-tRNA synthetase from mesitoyl-AMP

Expt. no.	Enzyme (pmol)	[^{14}C]AMP inc. (pmol)	No. inc. residues/ enzyme dimer	Residual act. (%)
1	13	13	1.0	0
2	25	25	1.0	0
3	16	15	0.95	6

mesitoyl-[^{14}C]AMP by dialysis, and estimated the molar stoichiometry of binding the radioactive residue of the inhibitor to the protein which was found to be close to 1 (table 1). Therefore, modification of tryptophanyl-tRNA synthetase consisting of two identical subunits with mesitoyl-[^{14}C]AMP was characterized by the 'half-site' stoichiometry of incorporation of the radioactive label; here, the two active sites of the enzyme, both the free and blocked ones, lost the catalytic activity in the exchange and acylation reactions.

The native and modified enzymes were compared further by measuring the number of aminoacyl adenylates formed on the enzyme molecule by 'burst' technique [11]. As was shown earlier for tryptophanyl-tRNA synthetase, the enzyme can form a complex only with 1 mol tryptophanyl adenylate under certain conditions [4] though the enzyme possesses two sites that form and bind tryptophanyl adenylate [12]. Fig.1 presents kinetic curves that make it possible to estimate the amount of [γ-^{32}P]-ATP hydrolysed as result of formation of tryptophanyl adenylate in the active centres of the native and modified enzymes.

In all the experiments rapid consumption of ATP takes place, then the adenylate formed remains stable during incubation. At 10-fold molar excess of ATP over enzyme when tryptophanyl adenylate is formed on each subunit of the native enzyme (1.8 mol adenylate/1 mol protein) the enzyme modified with mesitoyl-AMP is still capable of catalysing the formation of 0.9 mol tryptophanyl adenylate/1 mol protein. When the native enzyme displays the properties of the 'half-of-the-sites reactivity' (5–fold molar excess of ATP over enzyme) the modified enzyme does not differ from it in its capacity for the burst of [^{32}P]-ATP hydrolysis. Therefore, reaction of 1 mol mesitoyl-AMP with dimeric tryptophanyl-tRNA synthetase completely blocks only one active centre of the

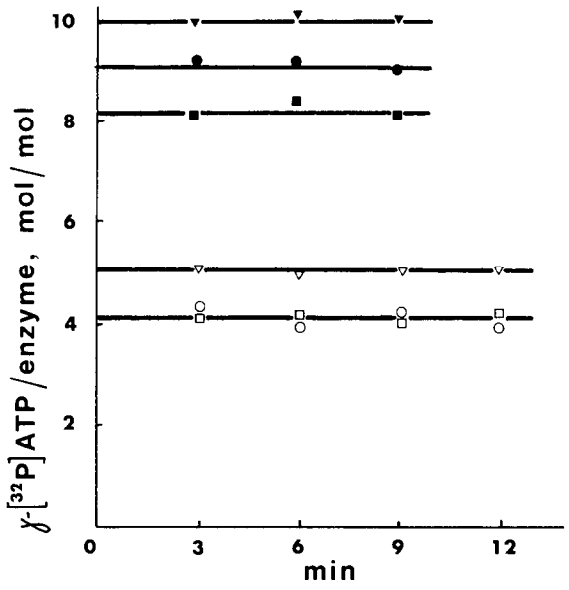

Fig.1. 'Burst' of [γ-^{32}P]ATP hydrolysis due to formation of a stable complex between tryptophanyl adenylate and the native (■,□) or modified (●,○) enzyme. Control without the enzyme (▼,▽). Molar proportions between ATP and the enzyme are 10:1 (■,●,▼) and 5:1 (□,○,▽).

enzyme; the second one retains the capacity to synthesize tryptophanyl adenylate.

However, the reactivity of this tryptophanyl adenylate against tRNA^{Trp} and pyrophosphate is impaired, as follows from the identical loss of the activity of the modified enzyme in the reactions of tRNA^{Trp} aminoacylation and ATP–[^{32}P]pyrophosphate exchange [10]. Possibly, the hindered release of pyrophosphate from the active centre may account for this phenomenon. This retaining of pyrophosphate formed would interfere with the binding of radioactive pyrophosphate and its participation in pyrophosphorolysis of tryptophanyl adenylate with the formation of [^{32}P]ATP. At the same time, the tRNA^{Trp} binding would impaire because of the ping–pong mechanism of the substrates binding [13,14]. The experiment in fig.2 shows that addition of inorganic pyrophosphatase partially restored the aminoacylation activity of the enzyme entirely inactivated with mesitoyl-AMP. Theoretically, it is possible to restore the activity up to 50%. Although we could not achieve this value, it is obvious that the restoration of the activity is due to functioning of the non-modified subunit after removal of pyrophosphate from the active centre.

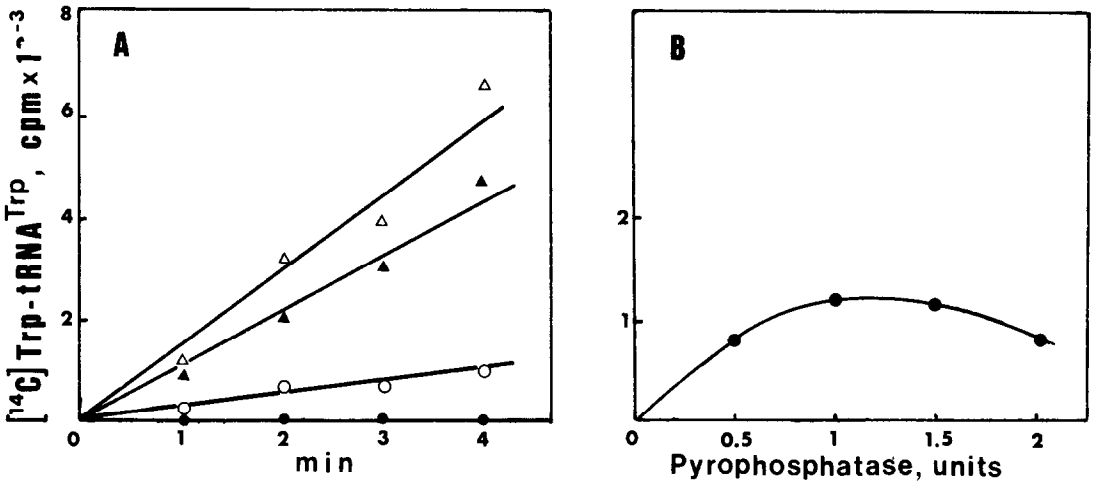

Fig.2. Effect of inorganic pyrophosphatase on the activity of native and modified tryptophanyl-tRNA synthetase in the reaction of $tRNA^{Trp}$ aminoacylation. (A) Kinetics of aminoacylation. The native (△,▲) and modified (○,●) enzymes in the absence (▲,●) and in the presence (△,○) of pyrophosphatase (1 unit/tube). (B) Effect of pyrophosphatase concentration on the rate of aminoacylation reaction catalysed by modified tryptophanyl-tRNA synthetase.

4. Discussion

The covalent blocking of the enzyme functional group follows the mechanism of phosphorylation as was shown by incorporation of the [^{14}C]AMP moiety from mesitoyl-AMP. We observed the phenomenon of 'half-of-the-sites' reactivity. Most likely, the active centre of the second subunit cannot be acylated by the non-radioactive mesitoyl moiety of the analog molecule; this was supported by model experiments [9] and by the fact that the second subunit entirely retains the capacity to synthesize tryptophanyl adenylate. Artifacts connected with potential non-homogeneity of the enzyme preparation are also excluded by the experiments on active site titration before and after modification (fig.1).

The 'half-site' stoichiometry was observed also when the enzyme was modified with *N*-chloroambucilyl-tryptophanyl-$tRNA^{Trp}$ [5], in complex formation with tryptophanyl adenylate [4], and when the tryptophanylated enzyme was formed [7]. Besides the two active centres displayed non-equivalence in binding tryptophan [15] and tryptophanyl-$tRNA^{Trp}$ [5]. These properties of tryptophanyl-tRNA synthetase reflect apparently strong negative co-operativity between the active centres.

However, both the centres of tryptophanyl-tRNA synthetase undergo modification under the action of tryptophanyl chloromethyl ketone [4] and γ-(*p*-azidoanilide)-ATP [5]. Such a difference in the reactivity of one half of the subunits while using different modifying agents is characteristic of enzymes with a strong negative co-operativity [16–18]. This difference reflects the specificity in transfer of conformational changes which occur in one of the subunits, to other subunit(s) depending on the nature of a modifying agent.

Binding of the AMP moiety of the inhibitor to one half of identical subunits [3] switched off both occupied and free centres in exchange and acylation reactions [10]; addition of inorganic pyrophosphatase partially restores the activity. The pyrophosphate inhibition of reactions catalysed by aminoacyl-tRNA synthetases and their stimulation by inorganic pyrophosphatase observed earlier were interpreted in terms of pyrophosphorolysis of aminoacyl adenylate [11, 19,20]. The observations made here show clearly that, at least in the case of bovine tryptophanyl-tRNA synthetase, the role of pyrophosphate is not limited to its participation in the reverse reaction. The pyrophosphate binding sites play probably the crucial role in mutual influence of the subunits. Indeed, the conformational change transmitted from the modified subunit to the neighbouring free one affects the binding site for pyrophosphate interfering with its release from the enzyme. In turn, the hindrance of pyrophosphate release prevents the tRNA binding due to a ping–pong mechanism. Therefore, the observed

behaviour of the enzyme is in accordance with the flip–flop mechanism proposed [21] for enzymes consisting of identical subunits and adopted for tryptophanyl-tRNA synthetase (see [3]).

In conclusion, the stoichiometrically reacting affinity analog of aminoacyl adenylate allowed us to reveal some intrinsic features of anti-cooperative interactions between the subunits of tryptophanyl-tRNA synthetase. Obviously, the use of mesitoyl-AMP with other synthetases may contribute to our knowledge of this still intriguing enzyme.

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