COMPARISON OF THE ATP–[^{32}P]PYROPHOSPHATE EXCHANGE REACTIONS CATALYSED BY NATIVE (TWO-SITE) AND CHEMICALLY MODIFIED (ONE-SITE) TRYPTOPHANYL-tRNA SYNTHETASE

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1. Introduction

Most of aminoacyl-tRNA synthetases are functional dimers and the functioning of both active sites of the enzyme seems to be mutually dependent [1–4]. In particular, the data on the kinetics of the formation of tryptophanyl-tRNA can be best interpreted in terms of the trigger reaction mechanism involving coordinated alternate participation of the two active sites of the enzyme in catalytic transformation of substrates [5,6]. To gain better insight into the mode of interaction between the two active sites of tryptophanyl-tRNA synthetase, it is of interest to study the mechanism and detailed kinetics of the enzyme possessing only one active site since this permits characterization of its functioning and comparison to the native two-site enzyme [8,9]. Such an enzyme was prepared by affinity modification of tryptophanyl-tRNA synthetase using *N*-chloroambucilyl-tryptophanyl-tRNA because the reaction involves only one of the two catalytic sites [7].

The substrates were found to be bound to the 'one-site' enzyme in the same order as to the 'two-site' one: first ATP, and second tryptophan. As tRNA is being fixed at one of the two sites, the equilibrium of the reaction is shifted toward the formation of aminoacyl adenylate. The parameters of the productive reaction steps were found to be somewhat 'improved' whereas the opposite was true for the dead-end complexes. The results thus obtained are interpreted in terms of the active role of aminoacyl-tRNA in the reaction of amino acid activation.

2. Materials and methods

The isolation of tryptophanyl-tRNA synthetase (EC 6.1.1.2) from the beef pancreas has been described [10]. The enzyme form E_2 (α_2, mol. wt 120 000) homogeneous in polyacrylamide gel electrophoresis under denaturing conditions was used throughout the experiments. The preparation of [^{14}C]tryptophanyl-tRNA and *N*-chloroambucilyl-[^{14}C]tryptophanyl-tRNA as well as the modification of the enzyme by the alkylating analogue of tryptophanyl-tRNA were conducted according to [7].

The reaction of ATP–[^{32}P]pyrophosphate exchange was carried out at 25°C as in [8] with slight modification. The concentration of the alkylated enzyme in the reaction mixture was 1.1×10^{-8} M; bovine serum albumin was substituted by gelatine. Other reagents and materials were the same as in our previous work [6–8].

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3. Results and discussion

After affinity modification of tryptophanyl-tRNA synthetase by *N*-chloroambucilyl-tryptophanyl-tRNA the formation of aminoacyl-tRNA is entirely blocked, although the rate of ATP–[^{32}P]pyrophosphate exchange is still about 50% of the original [7]. The modified enzyme has been shown to contain one

Table 1
Reaction rates for ATP–[^{32}P]pyrophosphate exchange catalysed by tryptophanyl-tRNA synthetase after covalent binding of 1 mole of R-Trp-tRNA to the enzyme protein

Substrate or analog			Concentration number (M)			
			1	2	3	4
ATP			0.2 × 10^{-3}	0.4 × 10^{-3}	0.8 × 10^{-3}	4 × 10^{-3}
Pyrophosphate, (PP_i)			0.05 × 10^{-3}	0.1 × 10^{-3}	0.2 × 10^{-3}	
Tryptophan[a] (Trp)			0.176 × 10^{-6}	0.376 × 10^{-6}	0.676 × 10^{-6}	8.076 × 10^{-6}
Tryptamine (Tra)			0.0	2 × 10^{-6}	6 × 10^{-6}	20 × 10^{-6}
Concentration number			Tra concentration number			
Trp	ATP	PP_i	1	2	3	4
1	1	1	0.56	0.24	0.14	0.11
		2	0.67	0.50	0.25	0.23
		3	0.57*	0.32	0.36	0.18
	2	1	0.52	0.73	0.33	0.20*
		2	0.84	0.84	0.39	0.24
		3	0.88	1.00	0.42	0.17
	3	1	0.82	0.62	0.32	0.17
		2	1.34	0.81	0.42	0.18
		3	0.79	0.84	0.33*	0.18
	4	1	0.64	0.72	0.23	0.12
		2	1.10	0.71	0.23	0.12
		3	1.70	1.06	0.27	0.12
2	1	1	0.62	0.47	0.45	0.31
		2	0.80	0.82	0.55	0.34
		3	1.04	0.82	1.02	0.65
	2	1	1.19	1.24	0.61*	0.50
		2	0.94	0.99	0.82	0.22
		3	1.51*	1.51	0.83	0.40
	3	1	1.31	0.87	0.48	0.34
		2	1.26	0.93	0.78	0.32
		3	1.18	1.37	0.73	0.42
	4	1	0.75	0.88	0.32	0.20
		2	1.31	0.75	0.67	0.29
		3	1.80	2.14*	0.79	0.28*

Table 1 (continued)

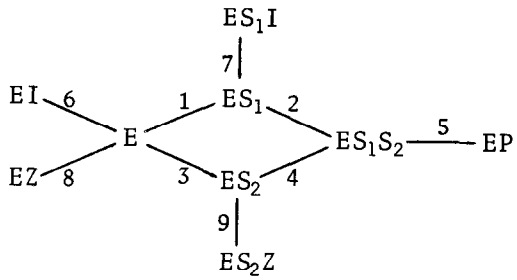
Concentration number			Tra concentration number							
Trp	ATP	PP_i	1		2		3		4	
		1		0.55		0.83		0.58		0.48
	1	2		1.03		0.86		0.58		0.41
		3		0.97*		0.95		0.78*		0.52
		1	0.98		1.29		1.07		0.66	
	2	2	1.51		1.29		1.27		0.53	
		3	1.72		1.82*		1.14		0.48	
3										
		1		1.39		1.01		0.99		0.42
	3	2		2.11		1.05		1.01*		0.35
		3		2.72		1.78		1.42		0.74
		1	0.96		0.90		0.58		0.25	
	4	2	2.00		1.58		1.01		0.58	
		3	1.81		1.81		0.98*		0.39	
		1		0.94		0.69		0.70		0.54
	1	2		1.11		0.78		0.86		0.42
		3		1.07		1.06		0.78		0.27
		1	1.18		1.39		1.15		0.96	
	2	2	1.68		1.41*		1.07		0.95	
		3	1.54		2.15		1.72		1.02	
4										
		1		1.27		1.32		0.95		0.80
	3	2		1.56		1.88		1.44*		1.19
		3		2.25		1.94		1.35		1.18
		1	1.15*		0.98		0.54		0.50	
	4	2	–		–		1.07		0.63*	
		3	6.05		7.41		3.57		3.58	

[a]The amount of endogenous tryptophan in the enzyme preparation was taken into consideration in determining the tryptophan concentration in the reaction mixtures [10]

*Experiments used for calculation of the initial dispersion matrix are marked by an asterisk

covalently bound tRNA molecule per enzyme dimer; alkylation does not cause the enzyme to dissociate into subunits. We used such an enzyme preparation to determine the rate of ATP–[^{32}P]pyrophosphate exchange. Table 1 shows the number of moles of [^{32}P]ATP formed per second per mole of enzyme at different substrate and inhibitor concentrations. These values were taken as measures of the isotope exchange rates since all measurements were conducted within the linear region of the kinetic curves.

Mechanisms involved in the action of various aminoacyl-tRNA synthetases are not the same (see [11,12]) and we do not know yet the cause of this diversity. Therefore, one cannot predict a priori how chemical modification in the vicinity of the active site would affect the behaviour of the enzyme. That is why, as was done earlier for the native enzyme, we considered three main kinetic mechanisms for the ATP–[^{32}P]pyrophosphate exchange reaction [8] which can be seen in following scheme I.



Abbreviations: E, enzyme; S_1, ATP; S_2, tryptophan; Z, pyrophosphate; P, aminoacyl adenylate; I, tryptamine.

Mechanism 1 involves all of the stages except 4 and 9 (ATP is bound first, and tryptophan, second). Mechanism 2 involves all stages but 2 and 7 (tryptophan binds first, and ATP, second). Mechanism 3 involves all the stages (random order of binding).

Kinetic equations for possible mechanisms of isotope exchange were compared with experimentally found dependences of the rate of [^{32}P]ATP formation on the concentrations of ATP, tryptophan, pyrophosphate and an inhibitor of the reaction, tryptamine. Statistical treatment of the results was made as described elsewhere [8]. It can be seen (table 2) that, by all criteria considered, mechanism 1 fits in best with the experimental data. The calculation of relative probabilities of the hypotheses has shown that the probability of mechanism 1 approximates to unity, whereas the probabilities of other mechanisms are close to zero. Therefore, modification of the enzyme by *N*-chloroambucilyl-tryptophanyl-tRNA did not change the kinetic mechanism involved in

Table 2
The sum of squares of differences of the measured and calculated values (S), mean relative errors (ϵ%) and relative probabilities (P) for the given mechanisms

Mechanism	S	ϵ%	P
I	10.4	20.9	1.0
II	12.2	21.7	6×10^{-11}
III	13.4	23.1	10^{-20}

ATP–[^{32}P]pyrophosphate exchange so that the first substrate to be bound by tryptophanyl-tRNA synthetase, in the formation of tryptophanyl adenylate, is ATP, and the second, tryptophan. Since the exchange mechanisms catalysed by native and modified tryptophanyl-tRNA synthetases were found to be the same, it would be relevant to compare reaction parameters for the two enzyme preparations.

Kinetic parameters for the reaction of ATP–[^{32}P] pyrophosphate exchange catalysed by the modified enzyme have been estimated in the same manner as for native tryptophanyl-tRNA synthetase [9], and are presented in table 3.

Reactions given in table 3 can be subdivided into two groups: productive and non-productive. The productive stages 1, 2 and 5 constitute the route for the formation of aminoacyl adenylate. The remaining stages correspond to the formation of dead-end complexes. Comparison of the characteristics of the route stages shows that modification of the enzyme results in an increase of the dissociation constant for pyrophosphate from the triple complex ES_1S_2 (stage 5).

Table 3
Dissociation constants of enzyme-substrate complexes for native and modified tryptophanyl-tRNA synthetase

No. of stage[a]	Type of stage	Reaction	K_{diss} (M)	
			Modified enzyme	Native enzyme [9]
1	Productive	$ES_1 \rightleftharpoons E + S_1$	$3.4 \times 10^{-4} \pm 0.2 \times 10^{-4}$	$6.3 \times 10^{-4} \pm 3.2 \times 10^{-4}$
2	Productive	$ES_1S_2 \rightleftharpoons ES_1 + S_2$	$2.8 \times 10^{-7} \pm 0.7 \times 10^{-7}$	$1.8 \times 10^{-7} \pm 0.8 \times 10^{-7}$
5	Productive	$ES_1S_2 \rightleftharpoons EP + Z$	$5.5 \times 10^{-5} \pm 0.04 \times 10^{-5}$	$1.4 \times 10^{-5} \pm 0.8 \times 10^{-5}$
3	Non-productive	$ES_2 \rightleftharpoons E + S_2$	$13 \times 10^{-7} \pm 1.0 \times 10^{-7}$	$1.2 \times 10^{-7} \pm 0.5 \times 10^{-7}$
6	Non-productive	$EI \rightleftharpoons E + I$	$5 \times 10^{-4} \pm 4.7 \times 10^{-4}$	$2.1 \times 10^{-6} \pm 1.3 \times 10^{-6}$
7	Non-productive	$ES_1I \rightleftharpoons ES_1 + I$	$13 \times 10^{-7} \pm 0.4 \times 10^{-7}$	$3.2 \times 10^{-7} \pm 0.6 \times 10^{-7}$
8	Non-productive	$EZ \rightleftharpoons E + Z$	7.7×10^{-4}	$1.6 \times 10^{-4} \pm 0.5 \times 10^{-4}$

[a]Stage number is taken from the reaction scheme (Scheme I) presented in the text. The constants were calculated as described earlier [9]

Judging by the equilibrium constants for the tryptophanyl adenylate synthesis catalysed by the modified or native enzyme, modification of the enzyme shifts the equilibrium towards formation of the complex between the enzyme and aminoacyl adenylate (5.8×10^5 M^{-1} as compared to 1.2×10^5 M^{-1} for the native enzyme).

Dead-end complexes are characterized by weaker interactions between different forms of the modified enzyme (E) with tryptamine (I), pyrophosphate (Z) and tryptophan (S_2), and of ES_1 with tryptamine, which is expressed in a considerable increase of the dissociation constants for these stages as compared to the native enzyme. Note that the increase in the dissociation constant of the non-productive complex ES_2 is in contrast with a low value of K_{diss} for the productive stage.

The trigger model for the functioning of aminoacyl-tRNA synthetases assumes the states of the two active sites of the enzyme to be reciprocal in the course of catalysis of the aminoacyl-tRNA formation [5,6,13]. While aminoacyl adenylate is being formed at one site, the other one is occupied with tRNA or aminoacyl-tRNA. Thus, the 'tRNA' phase and the aminoacyl adenylate phases alternate at each site. Apparently, the covalent binding of *N*-chloroambucilyl-tryptophanyl-tRNA to the enzyme causes the phases of both sites to 'freeze'; as a result, the functional dimer becomes a one-site enzyme capable of catalysing only the formation of aminoacyl adenylate at one of the two sites.

Further studies will show how precisely the modified enzyme models the behaviour of the native enzyme at the first phase of catalysis. Nevertheless, it might be relevant to note that the modified synthetase catalyses the ATP-pyrophosphate exchange even 'better' by a number of characteristics. First, the equilibrium constant for the formation of tryptophanyl adenylate is shifted, in the case of the tRNA-bound enzyme, toward the formation of the enzyme–tryptophanyl adenylate complex. Second, the formation of dead-end complexes is weakened, so that the free enzyme binds predominantly ATP, the first of the substrates in the order of their addition in the productive route of the reaction. Consequently, the presence of aminoacyl-tRNA on the enzyme molecule is essential for the amino acid activation and favours the productive reaction stages to proceed.

The almost twofold decrease in the maxium rate of the exchange reaction in the modified enzyme shows that one of the active sites is switched off when it is blocked by a $tRNA^{Trp}$ molecule, but does not suggest that the catalytic properties of the enzyme are impaired.

tRNA (apart from being a substrate) not only ensures the trigger character of functioning of the two enzyme active sites but also increases the effectiveness of the stages yielding aminoacyl adenylate.

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