ON THE RATE LIMITING STEP OF YEAST tRNA^{Phe} AMINOACYLATION

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1. Introduction

Phenylalanyl tRNA synthetase (PRS) from yeast is a tetrameric enzyme with an $\alpha_2\beta_2$ subunit structure [1–3]. Functionally it is a double dimer containing all substrate binding sites at least twice [3–7]. These sites, however, do not act independently but are coupled through subunit interactions. The synthetase exhibits negative cooperativity in substrate binding and half-of-the-sites reactivity in amino acid activation and tRNA aminoacylation [3–5,8,9]. As originally stated in [10], one of the advantages of half-of-the-sites reactivity in enzyme catalysis consists in the possibility of energetic coupling through interacting sites between successive steps of the reaction mechanism. In the case of PRS from yeast this has been demonstrated experimentally for phenylalanyl adenylate hydrolysis which was shown to be accelerated upon binding of phenylalanine and ATP to the second active site [8].

Recently, the transfer reaction has been identified as the rate limiting step of yeast tRNA^{Phe} aminoacylation by pre-steady state kinetic experiments using quenched flow methods [11]. Here, we report on quenched flow experiments which confirm this finding for high substrate concentrations but show that activation of phenylalanine becomes rate limiting at low concentrations of ATP and phenylalanine. These results are discussed as further support for intersubunit catalytic cooperativity in the reaction mechanism of PRS.

2. Materials and methods

The preparation of PRS and its characteristics were as in [12]. tRNA^{Phe} from yeast, purified according to [13], was a gift of R. Thiebe and W. Wintermeyer. [^{14}C]Phenylalanine was purchased from The Radiochemical Centre (Amersham), [^{32}P]Pyrophosphate from New England Nuclear (Boston). ATP was obtained from Sigma (München), all other chemicals were from Merck (Darmstadt).

All experiments were performed in a pH 7.0 buffer system containing 50 mM Tris–HCl, 120 mM KCl, 1 mM GSH, 1 mM potassium cacodylate, 0.5 mM $MgCl_2$, 0.15 mM EDTA, 1 mg/ml bovine serum albumin, and 2% glycerol. The quenched-flow kinetic experiments were conducted at 21°C, all other experiments at 25°C.

Aminoacylation of tRNA^{Phe} was measured as in [12]. For incubation times of 5–350 ms a quenched flow device [14] was used. The reaction was started by rapid mixing of enzyme and substrates (300 μl from each) and was quenched automatically by addition of an excess of ice-cold 5% trichloroacetic acid. [^{32}P]Pyrophosphate–ATP exchange was measured similarly as in [15].

3. Results and discussion

The reaction mechanism of PRS was investigated at two different concentrations of ATP and phenylalanine. At low concentrations one should be able to analyze the activity of only one catalytic center of the synthetase while at high concentrations the influence of the second active site upon the first one may be studied. According to the known K_m-values for ATP (K_m (I): 65–70 μM; K_m (II): 400–700 μM) and phenylalanine (K_m (I): 2–6 μM; K_m (II): 30–60 μM) [3,8], the first set of experiments was carried out at 100 μM ATP and 50 μM phenylalanine whereas ATP and phenylalanine were 5 mM and 0.5 mM, respectively, in the second group of experiments.

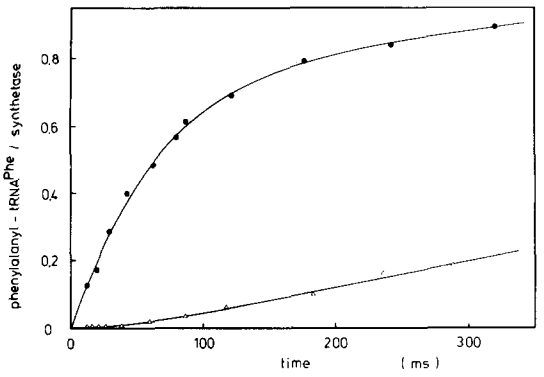

Fig.1. At low concentrations of ATP and phenylalanine a lag phase exists in the pre-steady state time curve of aminoacylation when free synthetase is combined with the substrates (△) while a burst of Phe-tRNA^{Phe} formation occurs when the reaction is started with a preformed synthetase adenylate complex (•). In the first experiment, PRS (2.5 μM) was combined in the quenched flow apparatus with an equal volume of tRNA^{Phe} (40 μM), ATP (200 μM), $MgCl_2$ (10 mM) and [^{14}C]phenylalanine (100 μM). In the second experiment, PRS (2.5 μM) was preincubated for 10 min with ATP (100 μM), $MgCl_2$ (5 mM), and [^{14}C]phenylalanine (50 μM) and then mixed with an equal volume of tRNA^{Phe} (40 μM), ATP (100 μM), $MgCl_2$ (5 mM), and [^{14}C]phenylalanine (50 μM).

When the synthetase was combined in the quenched flow apparatus with a mixture of tRNA^{Phe} and low concentrations of ATP and phenylalanine, measurable amounts of charged tRNA^{Phe} appeared only after a lag phase of ~20 ms (fig.1). The rate of aminoacylation then increased until, after ~100 ms, the steady state rate (0.9 s^{-1}, table 1) was reached. On the other hand, an initial burst in phenylalanyl tRNA^{Phe} formation was observed when PRS was incubated with ATP and phenylalanine prior to the combination with tRNA^{Phe} (fig.1). Since in the latter experiment the synthetase was allowed to activate the amino acid during the preincubation period, the appearance of a burst indicates that the transfer of preactivated phenylalanine onto tRNA^{Phe} proceeds considerably faster than the overall aminoacylation reaction. This is in agreement with the lag phase observed in the former experiment which shows that the rate limiting step of tRNA^{Phe} aminoacylation at low concentrations of ATP and phenylalanine is located within a reaction preceding the transfer step. Quenched flow experiments with synthetase–substrate complexes formed by preincubation of PRS with only one substrate (ATP, phenylalanine or tRNA^{Phe}) yielded no significant change in the length of the lag period (not shown) and therefore argue against slow substrate association processes. Consequently, the results suggest that activation of phenylalanine is the rate determining step. In accordance with that, pyrophosphate–ATP exchange experiments at the same substrate concentrations gave an exchange rate which is only slightly higher than the overall aminoacylation rate (table 1).

In order to obtain values for the reaction rates of the activation and the transfer step, a curve fitting procedure [16] was applied to the data of fig.1. From the results (table 2) it is seen that the transfer rate is >10-times higher than the overall aminoacylation rate. The amplitude value of ~0.75 mol activated phenylalanine transferred onto tRNA^{Phe}/mol synthe-

Table 1
Rates of pyrophosphate-ATP exchange and tRNA^{Phe} aminoacylation at different concentrations of ATP and phenylalanine

Substrate concentrations	Exchange rates (s^{-1})		Aminoacylation rate (s^{-1})
	–tRNA^{Phe}	+tRNA^{Phe}	
0.1 mM ATP 5.0 mM $MgCl_2$ 50.0 μM Phe	2.0 ± 0.5	1.2 ± 0.3	0.9 ± 0.2
5.0 mM ATP 10.0 mM $MgCl_2$ 0.5 mM Phe	48 ± 7	39 ± 5	10 ± 2

The exchange reaction mixture contained 2 mM [^{32}P] pyrophosphate/1 mM $MgCl_2$ and, where indicated, 20 μM tRNA^{Phe}. The same tRNA^{Phe} concentration was also employed in the aminoacylation tests. Mean values of three independent experiments are given

Table 2
Rate constants for the activation (k_{act}) and the transfer (k_{tr}) reaction as obtained by quantitative evaluation of the quenched flow experiments of fig.1

Substrate concentrations	Preincubation mixture	Amplitude k_{act}		k_{tr} (s^{-1})
		%	(s^{-1})	
0.1 mM ATP 5.0 mM $MgCl_2$ 50.0 μM Phe 20.0 μM $tRNA^{Phe}$	Free enzyme (E) E, ATP, Phe	75 75 ± 10	1.2 ± 0.4 –	16 16 ± 2
5.0 mM ATP 10.0 mM $MgCl_2$ 0.5 mM Phe 20.0 μM $tRNA^{Phe}$	E E, ATP, Phe	100 120 ± 10	80 ± 15 –	16 16 ± 2

The high substrate experiments are described in detail in [17]. The amplitudes of charged $tRNA^{Phe}$ formation, which were determined directly in the transfer experiments, are given in percent of the synthetase concentration [12]. Mean values of at least two independent experiments are listed

tase indicates that at the low concentrations of ATP and phenylalanine employed one active site of PRS is yet largely complexed with substrates.

Analogous experiments were also carried out at high concentrations of ATP and phenylalanine. While our experiments were in progress similar ones were described [11]. The experimental conditions in [11] were somewhat different (pH 7.8, in the presence of pyrophosphatase) from ours and the results differed slightly. In order to compare kinetic data obtained under identical conditions we report also our results at high substrate concentrations, but only in the form of the final data (tables 1,2). We confirm the finding [11] that at high substrate concentrations the transfer reaction is the rate limiting step of $tRNA^{Phe}$ aminoacylation; the activation rate is several times higher than the overall aminoacylation rate.

As shown above, at low concentrations of ATP and phenylalanine the rate limiting step is not the transfer reaction (and also not the association of substrates) but the activation step. The reason for that lies probably in the negative cooperativity of substrate binding by PRS. At any one time only one catalytic center is occupied by ATP and phenylalanine. As proposed in [18], the second site does not become accessible to these substrates until the activation reaction has occurred at the first center. At high substrate concentrations, however, ATP and phenylalanine may associate at the second site before activation has taken place at the first center. With that, the activation reaction is considerably accelerated. The aminoacylation rate is also increased but less than the rate of the activation reaction since it is now limited by the transfer rate. According to this interpretation, subunit interactions exist at high substrate concentrations in yeast PRS by which the binding of substrates at one catalytic center stimulates the activation of phenylalanine at the other center. With the help of these interactions the catalytic efficiency of PRS is increased much more than by a factor of 2 when substrate concentrations are raised from a level, where on an average one catalytic center of the synthetase is active, to conditions under which both centers act simultaneously.

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