THERMODYNAMICS AND KINETICS OF THE INTERACTION OF PHENYLALANINE-SPECIFIC tRNA FROM YEAST WITH ITS COGNATE SYNTHETASE AS STUDIED BY THE FLUORESCENCE OF THE Y-BASE

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1. Introduction

Most of the physicochemical studies of the various steps of the enzymatic acylation of tRNA [1–7] have been based on the fluorescence emitted by the Try-residues of some aminoacyl-tRNA synthetases or by fluorescent labels attached to the enzyme or the tRNA. The present paper reports changes of the fluorescence of the naturally occurring Y-base of $tRNA^{Phe}$ (yeast) upon binding to the cognate synthetase. This result implies an influence of the synthetase on the anti-codon region of the bound tRNA. Furthermore the kinetic parameters for the interaction of PRS and $tRNA^{Phe}$ are reported, as derived from stopped flow experiments monitoring the fluorescence of the Y-base. The association is almost diffusion controlled. The dissociation of the phenylalanyl-$tRNA^{Phe}$–PRS complex is not rate limiting for the steady state turnover of the acylation reaction.

Abbreviations:
PRS, phenylalanyl-tRNA synthetase; SRS, seryl-tRNA synthetase; DTE, dithioerythrol; EDTA, ethylenediaminetetraacetic acid; K_{ass}, association constant; k_R, rate constant for association; k_D, rate constant for dissociation.

2. Materials and methods

PRS was purified from commercial baker's yeast by a method similar to that of Fasiolo et al. [8]. The enzyme was homogeneous as judged by gel electrophoresis. The specific activity was the same as in the preparation of Fasiolo et al. [8].

In agreement with [8,9], the molecular weight as determined by equilibrium sedimentation was 230,000.

Purified Ser-tRNA synthetase (SRS) was kindly supplied by A. Pingoud.

Purified $tRNA^{Phe}$ from baker's yeast was purchased from Boehringer (Mannheim) and dialyzed against double distilled water. Concentrations of $tRNA^{Phe}$ were determined by absorbance measurements: 1.6×10^{-6} M $tRNA^{Phe}$ correspond to 1 $A_{1\,cm}^{260\,nm}$ unit. $tRNA^{Ala}$ (yeast) was prepared as described in [10]. Experiments were performed in 0.03 M potassium-phosphate buffer with 5×10^{-4} M 1,4-dithioerythrol, 10^{-4} M EDTA and varying amounts of $MgCl_2$ and KCl pH 7.2. Ultracentrifugation experiments were carried out at 12° in a Spinco model E analytical ultracentrifuge equipped with a photelectric scanner. Fluorescence titrations were performed in a Schoeffel RRS 1000 spectrofluorometer. The spectral bandwidth of

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Fig. 1. Sedimentation velocity experiment of 1.3×10^{-6} M $tRNA^{Phe}$ with 0.4×10^{-6} M PRS; 48 000 rpm, 12 mm cell; concentration profile measured at 265 nm (——); base line (----).

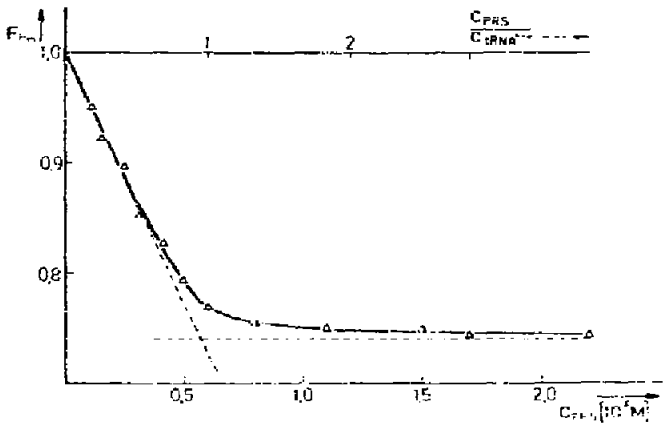

Fig. 2. Quenching of the fluorescence of the Y-base of 6×10^{-7} M of $tRNA^{Phe}$ induced by addition of PRS; 10^{-2} M Mg^{2+}, present 20°: Excitation at 313 nm; emission at 450 nm. Relative fluorescence of the Y-base (△—△—△) after correction for fluorescence of added PRS.

excitation and emission was 6 nm. When the titration was followed by monitoring the fluorescence of the Y-base of $tRNA^{Phe}$, the fluorescence of the added PRS was subtracted from the measured values.

Kinetic parameters were determined from stopped-flow measurements as described in the accompanying paper [7]. In all kinetic experiments the temperature was 10° and 10^{-2} M $MgCl_2$ and 0.1 M KCl were present.

3. Results

3.1. *Sedimentation experiments*

The stoichiometry of the $tRNA^{Phe}$–PRS complex was determined using the sedimentation boundary technique under conditions where the $tRNA^{Phe}$ was in excess of PRS. The concentration profile of a typical experiment is shown in fig. 1. The $S^0_{20,w}$- values of the slow and fast boundary were 3.9 S and 11.6 S, respectively. The enzyme alone sedimented with a value of $S^0_{20,w} = 8.7$ S (experimental result not shown). Therefore it is evident that the slow boundary represents the sedimentation of the free $tRNA^{Phe}$, whereas the fast boundary can be ascribed to that of the $tRNA^{Phe}$–PRS complex. Since the concentrations were more than one order of magnitude higher than the dissociation constant, virtually all enzyme was bound in the complex. Then, the concentration of the bound enzyme is identical to the total enzyme concentration. After subtraction of the absorbance of the enzyme from the total absorbance in the faster boundary the concentration of bound $tRNA^{Phe}$ can be evaluated from the relative heights of the two boundaries. The absorbances of both boundaries were corrected for dilution effects due to the sector shape of the cell.

In the experiment of fig. 1 0.4×10^{-6} M PRS are bound to 0.35×10^{-6} M $tRNA^{Phe}$. This result is interpreted as indicating a 1:1 complex between $tRNA^{Phe}$ and its cognate enzyme. Earlier (unpublished) results from our laboratory suggesting a 2:1 enzyme–tRNA stoichiometry were probably due to impurities in previous enzyme preparations.

Nonspecific binding of $tRNA^{Ala}$ to PRS could also be detected by sedimentation experiments. However, it is about two orders of magnitude weaker than the specific binding of $tRNA^{Phe}$ to PRS.

3.2. *Fluorescence equilibrium measurements*

In the presence of 10^{-2} M Mg^{2+}, PRS quenches the fluorescence of the Y-base of $tRNA^{Phe}$. A typical titration experiment is shown in fig. 2 from which the stoichiometry of the complex is seen to be 1:1. Titrations at lower concentrations of $tRNA^{Phe}$ [11] yielded a binding constant of about 8×10^7 M^{-1} and 7×10^6 M^{-1}, without and with 0.1 M KCl added, respectively. The binding constants were not affected by temperature in the range between 10 and 20°.

Purified SRS even at a 6-fold excess over $tRNA^{Phe}$ did not affect the fluorescence of the Y-base. Similarly, $tRNA^{Ala}$ at 10-fold excess did not compete with $tRNA^{Phe}$ for the tRNA binding site of PRS. The binding equilibrium of the $tRNA^{Phe}$–PRS interaction is not influenced by the presence of either 10^{-3} M ATP

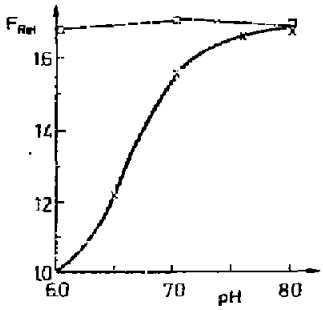

Fig. 3. pH-Dependence of the fluorescence of complex-bound $tRNA^{Phe}$ and free $tRNA^{Phe}$. 0.1 M KCl, present 5×10^{-4} M DTE, 20°. Excitation at 313 nm; emission at 450 nm; $tRNA^{Phe}$ saturated with PRS (○--○--○); free $tRNA^{Phe}$ (×—×—×).

or 10^{-3} M phenylalanine. The influence of PRS on the fluorescence of $tRNA^{Phe}$ strongly depends on the presence of Mg^{2+}. In the absence of Mg^{2+} and at low salt concentrations (c.f. fig. 3) PRS enhances the fluorescence of the Y-base in $tRNA^{Phe}$. Under these conditions the fluorescence of the tRNA bound to PRS (fig. 3) and of the Mg^{2+}–tRNA complex (not shown in the figure) is independent of pH in contrast to free $tRNA^{Phe}$

The tryptophane fluorescence of PRS evaluated as in [7] is quenched by about 5% when $tRNA^{Phe}$ is added. $tRNA^{Ala}$ did not affect the fluorescence of PRS.

3.3. *Stopped-flow measurements*

A typical oscillogram of a binding experiment is shown in fig. 4a. All measured curves could be described in terms of a simple bimolecular mechanism (cf. formula 1 in [7]) where the amplitudes (corrected for dead time) were consistent with the equilibrium measurements. The association rate constant was found to be $k_R = (1.6 \pm 0.3) \times 10^8\ M^{-1}\ sec^{-1}$. The concentrations of both reactants have been varied by about a factor of four ($0.25–1.0 \times 10^{-6}$ M) and the same rate constant was found. This demonstrates the validity of a bimolecular reaction mechanism.

Dissociation experiments were performed as described in the accompanying paper [7]. The dissociation rate constant k_D was determined to 27 sec^{-1}, which is in good agreement with the value calculated from K_R and K_{ass}.

The association rate constants in the presence of 10^{-3} M ATP or 10^{-3} M Phe are lower by less than a factor of two, and by less than a factor of 5 if both are present. In the latter case it was seen from the stopped-flow amplitude, that K_{ass}, which could not be determined by titrations, is not altered markedly by the simultaneous presence of ATP and Phe. Under these turnover conditions the binding process is seen in the lower trace of fig. 4b. After the binding, aminoacylation of the tRNA occurs and the release of part of the acylated tRNA is observed as the slow fluorescence increase (upper trace of fig. 4b) characterized by a rate constant of 2.5 sec^{-1}.

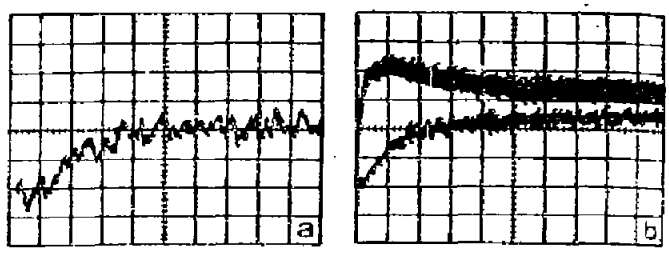

Fig. 4. Stopped-flow oscillograms after mixing of $tRNA^{Phe}$ with a) PRS: Total concentrations after mixing: 5×10^{-7} M PRS, 5×10^{-7} M $tRNA^{Phe}$. Oscilloscope settings: 5 msec/unit, 5 mV/unit. Final signal: 320 mV. b) PRS, ATP and Phe. Total concentrations after mixing: 5×10^{-7} M PRS, 5×10^{-7} M $tRNA^{Phe}$, 10^{-3} M ATP, 10^{-3} M Phe. Oscilloscope settings: 20 mV/unit; lower trace: 20 msec/unit; upper trace: 200 msec/unit. Final signal: 460 mV. The base lines of the two beams are different by 1.8 units. Common conditions of a) and b): Band filter (Schott & Gen. UG 11) at the excitation monochromator; cutt-off-filter (KV 408) for emitted light: 1 msec rise time; increasing fluorescence corresponds to negative deflection.

If the tRNA was preincubated for 15 min at 37° with a catalytic concentration of synthetase in the presence of 2×10^{-3} M ATP and 2×10^{-3} M Phe and after cooling to 10° mixed rapidly with an equal molarity of synthetase, it was possible to follow the binding of acylated tRNA (80% charged) to the synthetase. The lower amplitude observed in this experiment compared to the binding of unacylated tRNA is due to the lower binding constant of the acylated tRNA (since the saturation value of fluorescence quenching is the same). This difference is consistent with the amplitude of the slow fluorescence change in the turnover experiment. The binding constant of acylated tRNA was estimated from these amplitudes to be about $2 \times 10^6\ M^{-1}$, k_R was found to be $8 \times 10^7\ M^{-1}\ sec^{-1}$ and k_D calculated from K_{ass} and k_R to 40 sec^{-1}.

4. Discussion

The binding of PRS to $tRNA^{Phe}$ in the absence of Mg^{2+} causes an enhancement of the fluorescence of the Y-base. A similar effect is observed upon binding of Mg^{2+} to $tRNA^{Phe}$ [12]. This phenomenon has been interpreted as a shielding of the Y-base against the quenching effect of water molecules. The analogy between the effect of Mg^{2+} and PRS on the fluorescence of the Y-base is further supported by the fact that PRS and Mg^{2+} abolish the pH-dependence of the Y-base as observed in the free $tRNA^{Phe}$. This analogy would furthermore suggest, that the fluorescence changes upon binding of Mg^{2+} [12] and PRS are caused by a local influence of PRS on the anticodon region. PRS is less effective in enhancing the Y-base fluorescence than is Mg^{2+}. In a $tRNA^{Phe}$ solution containing both Mg^{2+} and PRS, the enzyme reduces the shielding effect of the Mg^{2+}-ions. This is demonstrated in the experiment depicted in fig. 2, where in the presence of Mg^{2+}, PRS quenches the fluorescence of the Y-base of $tRNA^{Phe}$.

The thermodynamic parameters derived from the binding studies agree with earlier studies on the same and on analogous systems. The stoichiometry is 1:1 [13], and the binding is specific, i.e. unspecific interactions are weaker by at least two orders of magnitude [1,6,7,14]. There is no influence of the small substrates phenylalanine and ATP on the binding equilibrium between PRS and $tRNA^{Phe}$ [15, 16].

The association rate constant, $k_R = 1.6 \times 10^8\ M^{-1}\ sec^{-1}$, is close to diffusion controlled. According to Alberty and Hammes [17] the diffusion controlled rate constant, k_R^{diff}, is estimated to

$$k_R^{diff} = \frac{2\pi}{1000} N_L\, r\,(D_{tRNA} + D_{PRS}) = 4 \times 10^8\ M^{-1}\ sec^{-1}.$$

D_{tRNA} and D_{PRS} are the corresponding diffusion coefficients [11] and r is a reaction radius estimated to 10^{-7} cm [17]. Electrostatic interactions, however, are not taken into account. Alberty and Hammes reported as a rough guess that the rate constant can increase by a factor of five due to electrostatic attraction. With these uncertainties in mind, we conclude that the experimental rate is not more than one order of magnitude slower than diffusion controlled. Furthermore, the binding under a variety of concentrations could be interpreted as a single step reaction. Consecutive rearrangements of the complex slower than 10^{-2} sec could be excluded because of the agreement between the dissociation rate constant which was measured directly and that which was calculated from k_R and K_{ass}.

Furthermore it is interesting to note the result that the rate limiting step in the entire process of aminoacylation occurs before the release of the acylated tRNA from the enzyme. Since the dissociation rate of the acylated $tRNA^{Phe}$ had been determined separately, it could be shown that this step is more than an order of magnitude faster than the rate limiting process which was observed as a slow fluorecence increase under conditions of aminoacylation (cf. fig. 4b). The turnover number evaluated from this experiment agreed well with that found in an aminoacylation assay. These findings are different from that reported by Yarus and Berg [15] in the Ile-system of *E. coli* using the nitrocellulose-filter technique. They found slower association rate constants and the dissociation of the acylated tRNA to be rate limiting for the whole aminoacylation process. In addition they found a 5-fold increase of the rate constants in the presence of the amino acid, whereas a minor decrease of the diffusion controlled rate constants is reported in this paper.

We feel, that the differences discussed above, are more likely due to different experimental conditions than to different systems, because in the Ser-system [7] and the Phe-system investigated under similar conditions comparable results are obtained.

Acknowledgements

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