THE pK_a AND DIMINISHED RELATIVE REACTIVITY OF THE α-AMINO GROUP OF PHENYLALANYL-tRNA FROM *ESCHERICHIA COLI*

Barend KRAAL and Guido W. M. SWART
Department of Biochemistry, State University of Leiden, PO Box 9505, 2300 RA Leiden, The Netherlands

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1. Introduction

Immense progress has been made in the characterization of the tRNA structure and its function of carrying a particular amino acid to the growing peptide chain (reviewed [1]). Peptide bond formation is a condensation reaction in which the α-amino group of an aminoacyl-tRNA is brought into contact with the active ester linkage between the preceding tRNA and the nascent peptide. The reaction takes place close to the 3′- and 5′-ends of the tRNA, which have a special role in it [2].

Until now no data have been available concerning the pK_a and reactivity of the α-amino group of aminoacyl-tRNA. The major problem for conventional physical techniques is the instability of the macromolecule in the physiological pH range and the resulting contamination of the sample with hydrolysis products. We solved this problem by application of the technique of competitive labelling as used for our studies of the elongation factor Tu [3]. The essential feature of the method is that single-hit trace-labelling is performed under non-denaturing conditions. This is realized here with pmole amounts of sample and by the use of short labelling times (60 s).

Our results show that the pK_a (0°C) for the α-amino group of Phe-tRNA is ~8.1, a similar value as for the reference compound Phe–Gly. This indicates that the salt bridge between the protonated amino group and the 5′-phosphate of the tRNA moiety suggested in [7,8] does not exist under physiological conditions. The relative reactivity of the α-amino group, however, is somewhat lower than expected.

Abbreviations: AcPhe, *N*-acetylphenylalanine; AcAla, *N*-acetylalanine

2. Materials and methods

2.1. *Essentials of the method of competitive labelling*

The main points of the method follow (details in [4]). The labelling reaction takes place in mixtures containing:

- Native macromolecules with various groups RX;
- Reference molecules as internal standard of reactivity with groups RS;
- Very low amounts of modification reagent.

Concentrations are such that at most 1 group/macromolecule is modified. This means that if this event would be followed by denaturation, the chance of an interfering second hit is negligible. During the reaction, competition for reagent takes place between reactive groups RX and RS. The labelling results for each depend on factors such as pK_a, nucleophilicity and microenvironment. This can be expressed by the following formula:

$$\frac{\text{Labelled fraction of group RX}}{\text{Labelled fraction of group RS}} = r.\ \frac{\alpha_x}{\alpha_s}$$

with α, the degree of dissociation (pK_a- and pH-dependent) and $r = {}^{k}x/{}^{k}s$, the ratio of velocity constants in the modification reaction of unprotonated RX and RS. The values measured for RX in a range of pH values can be evaluated by means of a Brønsted plot with a linear relationship between log r and pK_a for standard compounds.

2.2. *Competitive labelling of Phe-tRNA with acetic anhydride*

L-[2,4,6-^{3}H]Phenylalanine (71 Ci/mmol) and L-[2,3-^{3}H]alanine (35 Ci/mmol) were obtained from The Radiochemical Centre, Amersham. [^{3}H]Phe-tRNA was prepared using tRNA from *E. coli* MRE600

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(Boehringer, Mannheim) as in [5]. Labelling experiments were performed over pH 7.0–10.8 (0°C) using buffers containing 5 mM Mg^{2+}, 25 mM K^+, 80 mM Na^+ and either phosphate (pH < 8) or bicarbonate/borate anions. Reaction mixtures consisted of 150 μl buffer solution with 10 pmol [^{3}H]Phe-tRNA 10 pmol [^{3}H]Ala (as standard RS, compare section 2.1.) and 2–20 nmol non-radioactive acetic anhydride as the labelling agent. After a 60 s reaction time at 0°C, modification was stopped by acid precipitation with 0.18 M HCl in ethanol, followed by centrifugation. Pellet fractions contained Phe-tRNA and AcPhe-tRNA, whereas supernatant fractions contained Ala and AcAla together with traces (< 5%) of Phe and AcPhe as hydrolysis by-products from the original Phe-tRNA.

The degree of acetylation of Phe-tRNA and Ala was determined as follows. Pellet fractions were treated first with 1% (v/v) triethylamine to release the aminoacyl moieties by ester bond hydrolysis. All the samples with amino acids and their acetylated derivatives were subsequently analyzed by separation on Whatman 3MM paper using high-voltage electrophoresis at pH 6.5 and descending chromatography as in [5]. The ^{3}H-containing spots of Phe and AcPhe derived from pellet fractions and Ala and AcAla from supernatant fractions were cut out, combusted in a Packard Model 306 Tricarboxidizer, and finally counted at an efficiency of 40%. Direct calculations of the degree of labelling could then be made.

A number of control experiments was performed which yielded the following information. During the reaction time pH-values remained stable. Labelling conditions were such that the reaction followed pseudo first-order kinetics, with a linear dependence only on the concentration of acetic anhydride. This was checked to an extent of 7% acetylation, using various ratios of Ala and Phe and total amounts up to 700 pmol α-amino groups in 150 μl reaction vol. Aminoacyl ester bond cleavage of the original and the N-acetylated Phe-tRNA occurred to only negligible extents and could not interfere with the final calculations because of the fractionation procedure. Moreover, the deliberate use of Ala as the internal standard RS instead of the usual free phenylalanine [3,4,6] also prevented this sort of interference.

3. Results

A representative picture of the competitive labelling

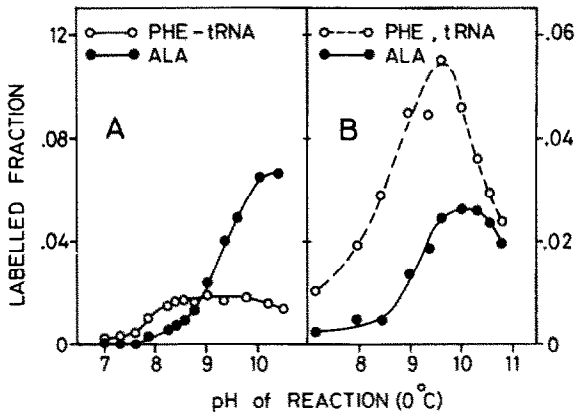

Fig.1. Competitive acetylation of [^{3}H]Phe-tRNA and [^{3}H]Ala (A) and of [^{3}H]Phe and [^{3}H]Ala as a control (B). In the latter case [^{3}H]Phe was added as pre-hydrolyzed [^{3}H]Phe-tRNA. Under these conditions the amount of tRNA remained the same as in (A), whereas upon termination of the labelling reaction only the supernatant fraction needed to be analyzed for the respective [^{3}H]compounds. For further details see section 2.

of Phe-tRNA and Ala over a range of pH values is shown in fig.1A. For comparison and control a similar picture is given in fig.1B with the labelling of Phe and Ala, Phe being added as pre-hydrolyzed Phe-tRNA. The curves for Phe and Ala have a comparable shape because of their close pK_a values, whereas that for the Phe-tRNA is quite different. Above pH 10 the increasing concentration of OH^- causes a decrease of the labelling values because of its progressive effect on the hydrolysis of acetic anhydride.

From the values plotted in fig.1A and 1B the ratios of the labelled fractions of Phe-tRNA as well as Phe versus those of the internal standard Ala were calculated, which corresponds to $r.\alpha_x/\alpha_s$ (section 2.1.). These labelling ratios are a function of the pH of reaction, but they are independent of experimental deviations in reaction time or concentration of reagent. The various forms of relationship between $r.\alpha_x/\alpha_s$ and pH, such as presented in fig.2, yield the following information. The degree of ionization for Ala, $\alpha_s = 1/(1 + [H^+]/K_s)$ can be calculated for each point from the measured pH and the known ionization constant K_s (pK_a 10.4 at 0°C [3]). Consequently, a value for $r.\alpha_x$ can be determined which can also be written as:

$$r.\alpha_x = \frac{r}{1 + [H^+]/K_x}$$

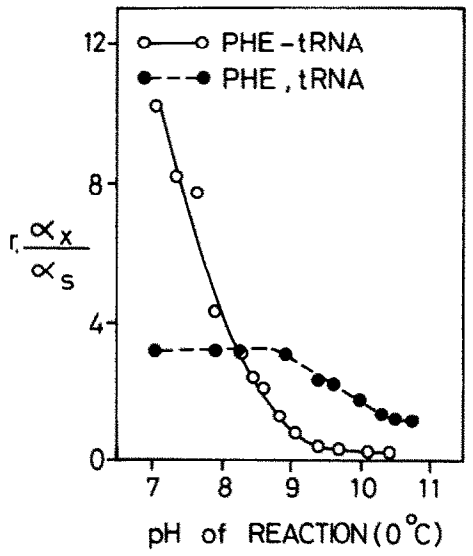

Fig.2. The ratio of labelled fractions of either Phe-tRNA or Phe versus those of Ala as a function on pH. Calculations were based on the data plotted in fig.1A,B. The curve for Phe (added as pre-hydrolyzed Phe-tRNA) is indicated with Phe, tRNA.

This can be converted to the formula:

$$r\alpha_x \cdot [H^+] = -K_x \cdot r\alpha_x + K_x r$$

a linear function of $r\alpha_x$, the slope of which yields the unknown ionization constant K_x. From the intercept a value for r, the ratio of velocity constants, can be determined.

Applied to the experimental data of fig.2, two curves were obtained as shown in fig.3. For non-bound Phe a linear plot was found with a slope corresponding to pK_a 9.8 (0°C) and an intercept leading to an r value of 0.81 with respect to the standard alanine. For Phe-tRNA a biphasic curve could be drawn with two linear sections. Section I, the major one at pH 7.0–9.4 (0°C), had a slope corresponding to pK_a 8.1 and an r 0.04. For section II, at pH 9.6–10.6 (0°C), the calculated values were pK_a 9.7 and r 0.11, respectively. This would point to a conformational change in the Phe-tRNA above pH 9.4 (0°C) which affects the α-amino group (see section 4).

All the calculated values were examined in a standard Brønsted plot as illustrated in fig.4. Since the r values in such a plot are usually based on the reactivity of phenylalanine instead of that of alanine, all our r values were first multiplied with a factor of

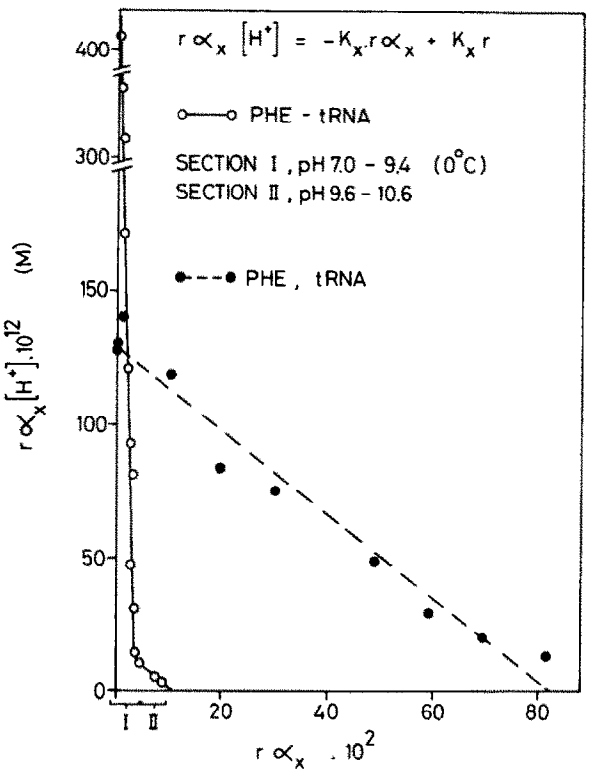

Fig.3. Plot of $r.\alpha_x \cdot [H^+]$ against $r.\alpha_x$ for the α-amino groups of Phe-tRNA and Phe (indicated with Phe, tRNA), respectively. Points were calculated from the data plotted in fig.2. The curve for Phe-tRNA is biphasic, sections indicated with I and II. This plot was used for the calculation of pK_a and reactivity values as described in the text.

$k_{Ala}/k_{Phe} = 1.25$ before entering the plot. For each amino group with a given pK_a the plot predicts a value for normal reactivity, r°_{Phe}. Steric hindrance for a buried amino group will be reflected in a point with an r_{Phe} below the plot. The magnitude of this and other effects (such as super-reactivity) can be expressed by the term relative reactivity, which is r_{Phe}/r°_{Phe}. The relative reactivities belonging to the experimental points in fig.4 are shown, together with the other parameters, in table 1.

It can be seen that Phe from pre-hydrolyzed [^{3}H]Phe-tRNA has values almost identical to the standard ones for Phe (as calculated with a fixed value for Ala). The pK_a for Phe-tRNA is ~8.1 at 0°C, a similar value as for the reference compound Phe–Gly. Its relative reactivity is a factor of 2 lower than expected, however. Above pH 9.4 (0°C) the pK_a changes to ~9.7, whereas the relative reactivity drops even further.

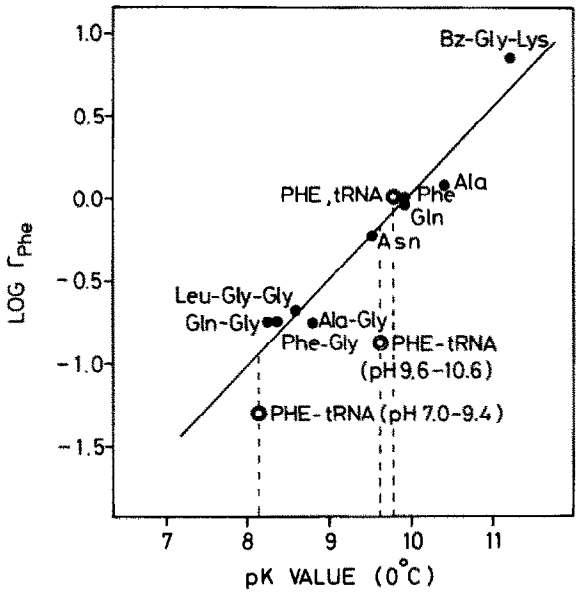

Fig.4. Brønsted plot for the modification of primary aliphatic amino groups at 0°C. Reference values of standard compounds were taken from [3] with r_{Phe} as the ratio of velocity constants for the labelling of an unprotonated amino group to that of Phe. On the basis of $r_{Phe} = 1.00$ for Phe, for Ala a value of $r_{Phe} = 1.25$ was taken for calculation of the parameters from fig.3 (see text).

4. Discussion

The subject of this contribution is a good illustration of the merits of the method of competitive labelling. Advanced physical techniques such as NMR have not yet sufficiently answered questions on structional properties of aminoacyl-tRNA under physiological conditions. The main problems there are the need of relatively long measuring times and of high concentrations of monodisperse material, which is hampered by the great instability of the aminoacyl ester linkage above pH 7. These problems have been overcome here with respect to the determination of pK_a and reactivity of the α-amino group. Due to our fractionation procedure only intact aminoacyl-tRNA was taken into account, whereas the extent of hydrolysis was small in the short labelling time used. Only picomole amounts of material were used because of the high specific activity (35–71 Ci/mmol) of the [^{3}H]amino acids employed. In this respect the present experimental approach is an order of magnitude more sensitive than previous reports dealing with non-radioactive amino acid sidechains labelled with tritiated acetic anhydride [4,6] or sodium borohydride [3].

Table 1
pK_a and reactivity of amino groups in Phe and Phe-tRNA with acetic anhydride at 0°C

Amino group	pK_a (0°C)	r_{Phe}	r°_{Phe}	Relative reactivity
Phe (theor. standard)	9.9	1.00		
Phe (from pre-hydrolyzed Phe-tRNA)	9.8	1.01	0.92	1.1
Phe-Gly (theor. ref)	8.4	0.18	0.16	1.1
Phe-tRNA (pH 7.0–9.4)	8.1	0.05	0.11	0.5
Phe-tRNA (pH 9.6–10.6)	9.7	0.14	0.75	0.2

r_{Phe} is the ratio of velocity constants for the acetylation of an unprotonated amino group to that of Phe r°_{Phe} is the ratio of velocity constants as predicted for a normal amino group from its pK_a in the Brønsted plot (fig.4). Relative reactivity is the quotient r_{Phe}/r°_{Phe}

From the results as summarized in table 1 can be concluded that our approach has worked satisfactorily. [^{3}H]Phe together with stripped tRNA displays the same pK_a and reactivity, within the limits of experimental error, as expected for the α-amino group of non-radioactive phenylalanine on its own. Phe-tRNA, on the other hand, has a much lower pK_a close to that of the reference compound Phe–Gly. The pK_a difference between the amino groups of Phe and Phe–Gly is due to the difference in position of their negatively charged carboxylate groups. Consequently, the presence of a salt bridge between the negatively charged 5′-phosphate of the tRNA and the protonated α-amino group of the aminoacyl moiety (see fig.5) as suggested by [7,8] can be excluded, as in the latter case a much higher pK_a value for Phe-tRNA should have been found. On the other hand, such a high value was indeed found at pH > 9.6 (0°C). Apparently, the intact Phe-tRNA molecule undergoes a conformational change under those alkaline conditions, by which a specific electrostatic interaction at the α-amino group seems to occur. Whether this phenomenon represents a functional change that could also be induced by interactions such as with the peptide elongation factor Tu yet remains an open question. The putative salt bridge is not indispensable for Tu · GTP · Phe-tRNA complex formation, since Phe-tRNA can have its α-amino group replaced by a hydroxyl group and still retain some affinity for Tu · GTP [9]. Furthermore, in [10] it was reported,

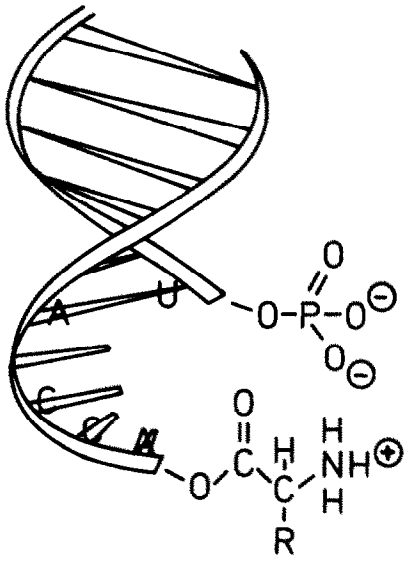

Fig.5. No salt bridge occurs between the α-amino group and the 5′-phosphate at the acceptor arm of Phe-tRNA in the physiological pH range.

contrary to [7], that the absence of the 5′-phosphate had no influence on the ternary complex formation either.

Finally, the diminished relative reactivity of the α-amino group in Phe-tRNA could point to a type of steric interaction. A limited accessibility after amino-acylation was reported [8] for the –C–C–A end to the binding of complementary oligonucleotides in comparison to free tRNA.

Acknowledgements

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