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EXTENDED KINETIC ANALYSIS OF RIBONUCLEASE T1 VARIANTS LEADS TO AN IMPROVED SCHEME FOR THE REACTION MECHANISM

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Recombinant ribonuclease (RNase) T1 variants were characterized kinetically taking into account the different reactions catalized by this enzyme. In addition to established assays, monitoring the transesterification activity, a photometric assay for fast screening of RNase T1 and variants thereof for ester hydrolysis activity is described, which is based on the application of phenol red as pH indicator. Moreover we established an HPLC assay to evaluate RNase T1 variants by their ability to carry out the transesterification towards an internucleotide diphosphoester (reverse or synthetic activity). In this way we found that the transesterification and hydrolyzing activities of variants change in various directions though in all reactions the same active site and the same transition state are involved. The variant where Tyr42 has been replaced by Trp performes RNA synthesis better than the wild type protein. The scheme of the hypothetic RNase T1 mechanism had to be improved to take into account the non processive character of the reaction.

Ribonuclease (RNase) T1, originally isolated from the mould fungus *Aspergillus oryzae*, is a small (MW 11084) and stable enzyme which cleaves single stranded RNA specifically after guanosine via terminal 2':3'-cyclophosphates to 3'-monophosphates. The X-ray structure of the wild type (wt) enzyme and of several variants has been solved to high resolution (for review see [1]). In 1983 Heinemann and Saenger [2] proposed a catalytic mechanism for this enzyme (Fig.1.) based on biochemical findings, an earlier suggested general mechanism [3] and on their data from X-ray structure analysis.

As the amino acid sequence of RNase T1 can be changed using recombinant DNA technology, the enzyme is an appropriate object for the investigation of structure-function relationships [1]. The

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Abbreviations:
2'3'cGMP - 2':3'cyclic guanosine monophosphate, 2'GMP - 2'guanosine monophosphate, 3'GMP - 3' guanosine monophosphate, GpC - guanylyl (3'→5') cytidine, GpN - guanylyl (3'→5') nucleoside, wt - wild type.

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aim of such studies is the characterization of the catalytic activity of RNase T1 variants and its correlation with structural properties. So far, variants have been analyzed kinetically only regarding their transesterification ability (cleavage of GpN; for instance see: [4-6] and RNA degrading activity according to Oshima *et al.* [7]).

We additionally investigated the effects of amino acid exchanges on the cyclophosphate hydrolysis and the reverse transesterification reaction. Earlier measurements of the cyclophosphate hydrolyzing and reverse activity were carried out with the wild type protein (for instance see [8,9]), but the corresponding data of the different reactions for variants have never been compared yet. By measuring the activities of the variants we were also looking for recombinant proteins which might be able to catalyze the reverse "synthetic" reaction better than the wt.

MATERIALS AND METHODS

Materials. RNase T1 variants were constructed according to Landt *et al.* [10] and the corresponding proteins were isolated as described [11]. The pH-indicator phenol red (phenosulfonphthalein, sodium salt) and the 2'3'cGMP (sodium salt) were purchased from Sigma (St. Louis). For spectrophotometric measurements we used a Hewlett Packard HP8452A and a Shimadzu UV-106A spectrophotometer. The enzyme concentrations were determined using an extinction coefficient of ε_{278nm} = 17 300 $M^{-1}cm^{-1}$ for wt RNase T1 [12] and the double variants Tyr42Trp/Trp59Tyr, Tyr45Trp/Trp59Tyr, Tyr24Trp/Trp59Tyr consisting of the same number of tyrosines and tryptophans. The value ε_{278nm} = 23 789 $M^{-1}cm^{-1}$ was chosen for the variants Tyr24Trp, Tyr42Trp, and Tyr45Trp, ε_{278nm} =13 120 $M^{-1}cm^{-1}$ for Trp59Tyr. These values were calculated from the extinction coefficient difference of tyrosine and tryptophan. The 2'3'cGMP concentration was determined using the value for 3'GMP (ε_{252nm} = 13 400 $M^{-1}cm^{-1}$ [13]).

Dinucleoside phosphate transesterification. The measurements of the dinucleoside phosphate transesterification activity were carried out as described [6]

Cyclophosphate hydrolysis. The hydrolysis of 2'3'cGMP was followed in a mixture consisting of deionized water with 1 mM phenol red, 500 µM substrate and about 0.2 µM enzyme for wt protein. Enzyme concentrations were adjusted between 0.1 µM and 3 µM, so that the change of absorption within four minutes was approximately a linear function of time. The measurements were performed at 25°C. The reaction mixtures were prepared from stock solutions of 2 mM phenol red, substrate and protein. A fresh substrate solution in phenol red buffer had to be prepared for every test. 10-mm cuvettes with a capacity of 1 ml were used for the test.

Before starting the reaction, appropriate amounts of substrate, indicator and water were mixed so that the total volume gave 1 ml after adding the protein solution with a resulting substrate concentration of 500 µM. The cuvettes with the reaction mixtures were preincubated in the photometer for four minutes in order to monitor autolysis and absorption shifts caused by mixing the components. The protein concentration in the stock solution has been chosen so that the volume of the added enzyme solution was less than 10 µl to minimize mixing effects. The measurements were carried out at 600 nm wave length.

High-performance liquid chromatography (HPLC) kinetics. The HPLC equipment consisted of a Shimadzu HPLC system, a reversed-phase column (5 mm; Euromer Oligo 100-5 C18; 120 x 4 mm ID; precolumn 5 x 4 mm ID) and a Shimadzu photodiode array UV-VIS detector SPD-M6A. A 72.5/12.5/15 (v/v) mixture of 50 mM $NH_4H_2PO_4$, 770 mM tetrabutylammonium hydroxide and methanol (HPLC-grade) was used as a 90:10 (v/v) mixture of 50 mM $NH_4H_2PO_4$ and methanol (HPLC-grade) for GpC synthesis experiments. The pH value of the mobile phase was 4.7, the flow rate was 0.75 ml/min, and the pressure varied between 80 and 90 bar.

The GpC synthesis experiments were carried out at room temperature or at 2°C in 100 mM Tris-HCl, pH 7.0. The initial concentrations were 2.75 mM for 2'3'cGMP and 0.35 M for C. For each measurement, aliquots of the reaction mixture were taken every 10 minutes and analyzed by HPLC immediately.

Thin-layer chromatography. Thin-layer chromatography was carried out on cellulose glas sheets. Saturated $(NH_4)_2SO_4$ solution in water/100 mM 2-[N-morpholino]ethanesulfonic acid buffer (pH 6.0)/2-propanol: 79/19/2 was used as mobile phase. The R_f values were 0.1 for GpC, 0.35 for 2'GMP, 0.25 for 3'GMP, 0.45 for C, and 0.14 for 2'3'cGMP.

RESULTS AND DISCUSSION

The purity of the kinetically analyzed wt protein and variants was tested by electron spray mass spectroscopy by P. Franke (Freie Universität Berlin). The spectra did not give any indication of serious impurities or amino acid sequence defects.

Indicator assay. The UV-spectra of 2'3'cGMP and 3'GMP do not differ sufficiently [14] and therefore the hydrolysis of 2'3'cGMP cannot be followed by usual absorbance spectroscopy. In former studies the cyclophosphate hydrolyzing activity of RNase T1 was measured by chromatographic or electrophoretic methods, or by using a pH-stat. These approaches appear tedious in comparison with a 4-minutes spectrometric assay. We therefore decided to apply the pH-indicator phenol red for the detection of cyclophosphate hydrolysis. This indicator has been proved to be suitable for biophysical measurements [15-20].

The shortcomings of such a method are the limited pH range of any indicator and the inaccuracy of the data from indicator tests. The advantage of an indicator assay in comparison to an electrode measurement is its sensitivity, the ease of measurement and the fast response of an indicator. Indicator methods differ in their kind of buffering the reaction system. In some assays the system is buffered by a separate buffer [15] and in others the indicator itself is used as a buffer [18], as in our case the phenol red. Consequently a high indicator concentration (1mM) had to be used because the indicator takes the part of the buffer and so the absorption change could not be measured in the maximum of the red spectrum (558 nm) in consequence of the photometer working range. In preliminary HPLC experiments, we proved that the indicator does not inhibit the activity of RNase T1 towards guanosine cyclophosphate.

The activity determined in the phenol red test is a value related to a second order rate constant i.e. to the enzyme kinetic parameter k_{cat}/K_M under the given conditions (low substrate concentration in comparison with the K_M = 3.0 mM [21]). Obviously this value is proportional to the expression $(A/t)_0 \cdot (1/\varepsilon) \cdot (1/[E]) \cdot (1/[S])$, where A stands for absorption, ε for the extinction coefficient of the deprotonated form of phenol red, [E] for enzyme concentration, [S] for substrate concentration and the index "0" for "initial value". As the pK_a of the phosphate group in guanosine cyclophosphate is about 1 [8] and the counter ion is sodium, there was not any buffer effect resulting from the substrate. The pK_a of N7 is near 3, of N1 near 10, and of the phosphate group of 3'GMP - the product of the reaction - 6.66 [22]. Within the short period of time required to measure the initial velocities, only a small fraction of the substrate has been converted and so the buffering capacity of the product can be neglected. Hence in the initial phase, the conversion of a substrate molecule causes the protonation of phenol red buffer molecules with high efficiency. We measured the absorption at 600 nm, because the high concentration of phenol red does not allow the detection in the maximum of the alkali spectrum (red form) and because the yellow form of phenol red does not absorb at this wavelength.

According to Irie [8] the RNase T1 activity for cyclophosphate hydrolysis is highest near pH 7.2. We could confirme this by HPLC analysis at different pH values. This result is remarkable because the activity maximum for the transesterification for the wt protein is near pH 6 [6,23]. During the measurements the absorption usually changed between 0.42 and 0.32 optical units. After inserting these values into the equation $pH=pK_a+\log(A/Amax-A)$ it turns out that the pH varies between 7.28 and 7.14 in the reaction mixtures, e.g. the reaction was carried out near the activity maximum of the wt enzyme for cyclophosphate hydrolysis.

Cyclic phosphate hydrolysis and dinucleoside phosphate cleavage. The data for the 2'3'cGMP hydrolysis activity of wt RNase T1 and the variants are shown in Table I. The sensivity of the test for less active variants is low, since a small pH-shift is observed after adding an inactive protein, and this pH-shift is comparable to the effect of the cyclophosphate hydrolysis, which is brought about by a variant with 50% wt activity. Therefore we wrote <50% for not exactly detectable low values. The standard deviation of the values for enzymes with an activity above 80% lies at about 20%. The absolute velocity value for the wt enzyme was determined by HPLC measurements. If one knows this k_{cat}/K_M value, it is possible to rate absolute activity values for the variants with the aid of relative values. The absolute value of k_{cat}/K_M for the cyclophosphate hydrolysis by the wt enzyme is 900 $M^{-1}s^{-1}$.

In Table I the results of the dinucleoside phosphate cleavage experiments are also shown. The absolute and relative activities are listed. It seemed to be more suitable to compare the velocities at the corresponding pH-optima of the two reactions. The feature of the table would not have been changed significantly if we would have taken the k_{cat}/K_M values for the transesterification reaction at pH 6 as determined by Grunert *et al.* [6]. The velocity of the GpC cleavage is much higher than of the 2'3'cGMP hydrolysis, which coincides with the results of other authors [14,21,24].

We verified by thin-layer chromatography that all variants we have tested here do not produce any other substances than the wt after degrading the dinucleoside phosphate GpC (data not shown).

Table I. Kinetic data of GpC cleavage and 2'3'cGMP hydrolysis by RNase T1 and variants

Variant	GpC cleavage k_{cat}/K_M #		2'3'cGMP hydrolysis (second order constant) *	
	$[s^{-1}\mu M^{-1}]$	[%]	$[s^{-1}\mu M^{-1}]$	[%]
wt	1630 ± 143	100	900	100
Tyr24Trp	1228 ± 186	64	1400	160
Tyr24Trp/Trp59Tyr	1360 ± 210	70	3474	389
Tyr42Trp	366 ± 44	19	<450	<50
Tyr42Trp/Trp59Tyr	618 ± 71	32	~450	~50
Tyr45Trp	2739 ± 434	142	1260	140
Tyr45Trp/Trp59Tyr	3146 ± 533	163	2916	324
Trp59Tyr	3007 ± 213	156	2655	295
Trp59Tyr/His92Ala	6.9 ± 0.4	0.4	<450	<50

These measurements were carried out at pH 6.0, the optimum of the GpN cleavage.
* The deviations of these values are discussed in RESULTS AND DISCUSSION; *The indicator assay*. The measurements were carried out in a small pH range near the optimum - see text.

When we started our measurements we expected - corresponding to the earlier proposed mechanisms [25] - that in each RNase T1 variant the change of activity for both reactions would have the same tendency, that means, if the protein catalyzes better the transesterification, it would also better do the hydrolysis. Contrary to our expectation we found some variants with different tendencies in activity changes: Tyr24Trp and Tyr24Trp/Trp59Tyr, which have a higher cyclophosphate hydrolyzing activity than the wt enzyme though their GpC cleaving activity is lower.

Dinucleoside phosphate synthesis. The amounts of products were recorded as the area of the HPLC peaks in O.D. · min which were converted into concentration units with the aid of a calibration function. Our results for the wt protein correspond to the data of other authors [9]. The dependence of reaction kinetics is also the same as described by them: at low temperature (0°C) the maximum concentration of dinucleoside phosphate is higher but this maximum is achieved by far later.

The feature of the experimental curves (not shown) is that the concentration course for the Tyr42Trp variant differ essentially from that of wt protein and the Tyr24Trp variant. The highest achieved concentration for the Tyr42Trp variant would be 128 % compared to the wt.

We chose for our model C as phosphate acceptor and 2'3'cGMP as the phosphate donor because 2'3'cGMP is the simplest donor and C the best incorporated acceptor. As the price of the cyclophosphate is much higher than that of the nucleoside we decided to carry out the experiments under conditions of maximum guanosine incorporation proposed by Rowe and Smith [9]. The 130-fold excess of the acceptor and low enzyme concentration are the features of this reaction.

Structure function relationships. As we decided to accomplish complex kinetic characterization of RNase T1 variants we expected that the changes in the different activities, transesterification and hydrolysis, will have the same tendency (increase or decrease) and the first variant of this series we tested (Tyr45Trp/Trp59Tyr), appeared to corroborate this. This expectation was founded on the following suppositions: the Heinemann-Saenger mechanism [25] and the fact that all variants generate the same products. In order to explain the big difference between the velocities of dinucleoside phosphate cleavage and cyclophosphate hydrolysis one has to consider that the consecutive lapse of the two steps within the same enzyme substrate complex is improbable. So the active site of the enzyme has to return into the starting-point (His92 protonated and Glu58 deprotonated) for the next transesterification without the proton transfer caused by the cyclophosphate hydrolysis step. One possibility is that the proton could immediately be donated by the carboxylic group of Glu58 to His92. This is in agreement with molecular dynamics calculations which show that both groups can draw near to each other to the necessary distance (F. Cordes, personal communication). So the protonation of these groups could be changed via direct contact or probabely by coordinated water.

As we could show for instance for the Tyr42Trp variant, the two RNase T1 catalyzed steps can be effected in differing directions and as the Heinemann-Saenger mechanism (Fig. 1) cannot explain activity changes of different tendencies we propose to improve the known scheme as shown in Fig. 2. Our new mechanism explains, that the activity changes can have different tendencies, but does not explain, why. As we know from X-ray structure analyses [26] the variants which show special activity changes do not have remarkable differences in their three dimensional structures in comparison to the wt protein. The proposed mechanism extends also the protonation schemes of RNase T1 as described by several authors [5,23,27,28], who discussed only the changes of the total

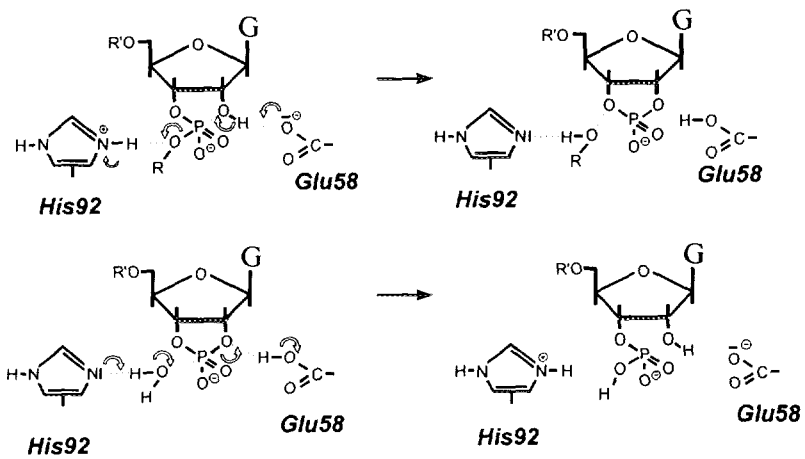

Fig. 1. Catalytic mechanism of RNase T1 as proposed by Heinemann and Saenger [2].

protonation but not different intramolecular protonations with the same all over number of protons. The fact that the velocity of GpC (the best substrate for this type of reaction) cleavage is higher than that of cyclophosphate hydrolysis can be explained by the position of the protonation equilibrium (central part of Fig. 2) which is probably shifted to the left. Another reason is the inability of

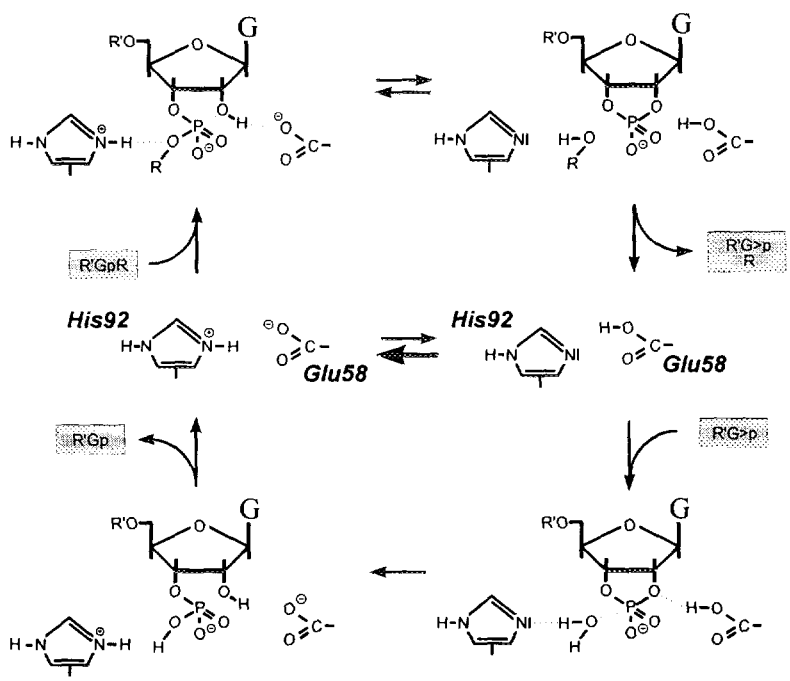

Fig. 2. Proposed catalytic mechanism of RNA degradation by RNase T1. In the central equilibrium the protonation of Glu58 and His92 is changed via a direct contact or coordinated water. The reverse activity (RNA synthesis) is neglected in this scheme to simplify the figure.

2'3'cGMP for subsite interactions. This mechanism however might be not valid for distinct RNase T1 variants, e. g. those where Glu58 has been replaced by Ala, Asp or Gln and which still remain active [5,29].

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