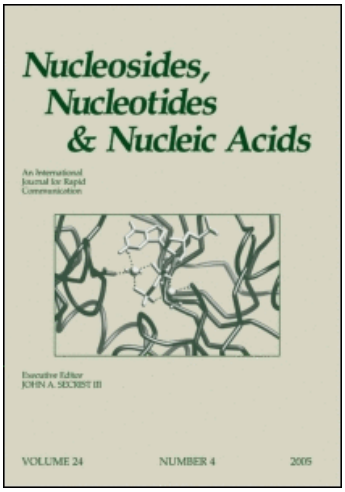

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REVERSE ACTION OF RIBONUCLEASE T1 VARIANTS IN ICE

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Abstract. Synthesis of guanylyl(3'→5')cytidine catalysed by RNase T1 variants (Tyr42Trp, Tyr24Trp and Glu58Ala) was studied in frozen aqueous systems at -10 °C and in solution at 0°C. Freezing the reaction mixture resulted in significantly enhanced dinucleoside monophosphate yields independently of the effect of mutation on substrate binding and catalytic mechanism.We assume that the protonation state of the catalytic residues is influenced by freezing, possibly due to conformational changes of the enzyme proteins.

Introduction. During the last years, synthetic reactions catalysed by proteases and other hydrolytic enzymes in frozen aqueous systems have been studied extensively (for recent reviews, see [1,2]). In enzyme-catalysed peptide synthesis, a substantial increase in peptide yield could be achieved by freezing the reaction mixture due to the suppression of competitive hydrolytic reactions. This effect has been mainly attributed to the concentration of the reactants in the remaining unfrozen liquid phase of the partially frozen reaction mixture [3,4]. However, specific properties of these reaction systems such as changes in specificity of proteases observed under frozen state conditions strongly indicate that, besides the freeze-concentration effect, there are other factors involved in the yield-increasing effect of freezing [1].

Recently, we reported on the reverse action of ribonuclease T1 (RNase T1) in frozen aqueous systems [5]. In the synthesis of guanylyl(3'→5')cytidine (GpC) from 2'3'-cylic guanosine monophosphate (2'3'cGMP) and cytidine (C), freezing resulted in increased dinucleoside monophosphate yields. In contrast to syntheses catalysed by other hydrolytic

enzymes (proteases [1], glycosidases [2] and ribonuclease A [2]), the most important yield-limiting factor was not a competitive hydrolysis reaction but transesterification of the newly formed (3'→5')phosphodiester bond which was suppressed by freezing the reaction mixture [5].

Starting from these results, in this paper we report on studies of the catalytic behaviour of RNase T1 variants modified in amino acid positions which are engaged in substrate binding and catalysis, respectively, in frozen aqueous systems.

Tyr42 is involved in substrate binding by stacking interactions. Substitution of this position by tryptophan resulted in a decreased relative activity (19%) for GpC transesterification, probably caused by changes in the active site geometry [6]. Nevertheless, the Tyr42Trp variant was reported to be a more efficient catalyst in GpC synthesis in solution than the wild type enzyme [7].

In contrast, Tyr24 is not involved in catalysis and substrate binding and although its substitution by Trp was expected to have no effect on catalytic activity, the transesterification activity of this variant was reported to be only 64 % compared to the wild type enzyme [6]. In 2'3'cGMP hydrolysis to 3'GMP, the Tyr24Trp variant exhibited an enhanced activity (160 %) compared to the wild type [7]. Therefore, in GpC synthesis studies, competitive hydrolysis of the cyclic donor has to be taken into account.

Furthermore, a variant with a different catalytic mechanism was chosen for GpC synthesis experiments. It was proposed that in the wild type enzyme Glu58 and His92 perform general base and acid catalysis, respectively [8,9]. If Glu58 is substituted by Ala the resulting variant still exhibited a GpC transesterification activity of about 5 % [10,11]. Steyaert et al. [11] postulated that the role of the base catalyst in the Glu58Ala variant has to be attributed to His40 and that this mutant is mechanistically similar to RNase A in that two histidine residues act as base and acid catalysts [12]. It was of special interest to compare the catalytic behaviour of this variant under frozen state conditions with that of RNase A we reported recently [2].

Material and Methods. Materials: RNase T1 variants were constructed according to Landt et al. [13]. The corresponding proteins were isolated as described [14]. Enzyme concentrations were determined spectrophotometrically using an extinction coefficient of

$\varepsilon_{278\ nm}$=17300 M^{-1} cm^{-1} for wild type RNase T1 and for the variant Glu58Ala [15], and of $\varepsilon_{278\ nm}$= 23789 M^{-1} cm^{-1} for the variants Tyr24Trp and Tyr42Trp [7]. The concentrations of 2'3'cGMP and of 3'GMP were determined using ε_{252nm}= 13400 M^{-1} cm^{-1} [7]. GpC was from Pharma Waldhof (Duesseldorf, Germany), 2'3'cGMP, 3'GMP and cytidine were obtained from Sigma (Deisenhofen, Germany).

Dinucleoside monophosphate synthesis: GpC synthesis reactions were performed in total volumes of 50 µl (-10°C) and 100 µl (0 °C), respectively, in 1.5 ml polypropylene tubes. 2'3'cGMP (2.75 mM) and cytidine (0.275 M) were dissolved in 0.1 M Tris / HCl buffer (pH 7.0) and cooled to 0 °C. The enzyme solution in the same buffer was added, the tube rapidly shaken and shock frozen in liquid nitrogen for 20s. After transfer into a cryostat, the samples were incubated at -10 °C. After distinct incubation times, reactions were stopped by the addition of 50 µl 0.4 M $ZnSO_4$ and analyzed by HPLC immediately. Synthesis reactions in solution at 0 °C were carried out in an ice bath without freezing.

Reaction times required to obtain maximal dinucleoside monophosphate yields varied from 2 to 48 h (0 °C) or 24 to 96 h (-10 °C). Control reactions were performed without addition of enzyme.

HPLC analysis: HPLC analyses were carried out using a Shimadzu LC-10A system with a Compaq personal computer and Shimadzu LC-10 software. A Lichrospher RP-18 column (5µm, 250 x 4 mm, Merck, Germany) was used. GpC, 2'3'cGMP and cytidine were eluted using a gradient system (0.01 M potassium dihydrogen phosphate, pH 4.15, and methanol) and detected at 252 nm. GpC was quantified from calibration curves obtained with the pure standard.

Results and Discussion. In GpC syntheses catalysed by the wild type enzyme, we found a strong influence of the enzyme concentration on the dinucleoside monophosphate yield [5]. Therefore, we studied the influence of the concentration of the variants Tyr42Trp, Tyr24Trp and Glu58Ala on GpC yield in solution at 0 °C and in frozen aqueous reaction mixtures at -10 °C (FIG. 1 A-C). For comparison, the behaviour of the wild type enzyme is shown in FIG. 1D (taken from [5]). Variants Tyr42Trp and Tyr24Trp were investigated in the same concentration range as has been reported for the wild type enzyme [5] whereas Glu58Ala, due to its low residual transesterification activity [10,11], was required in higher concentrations.

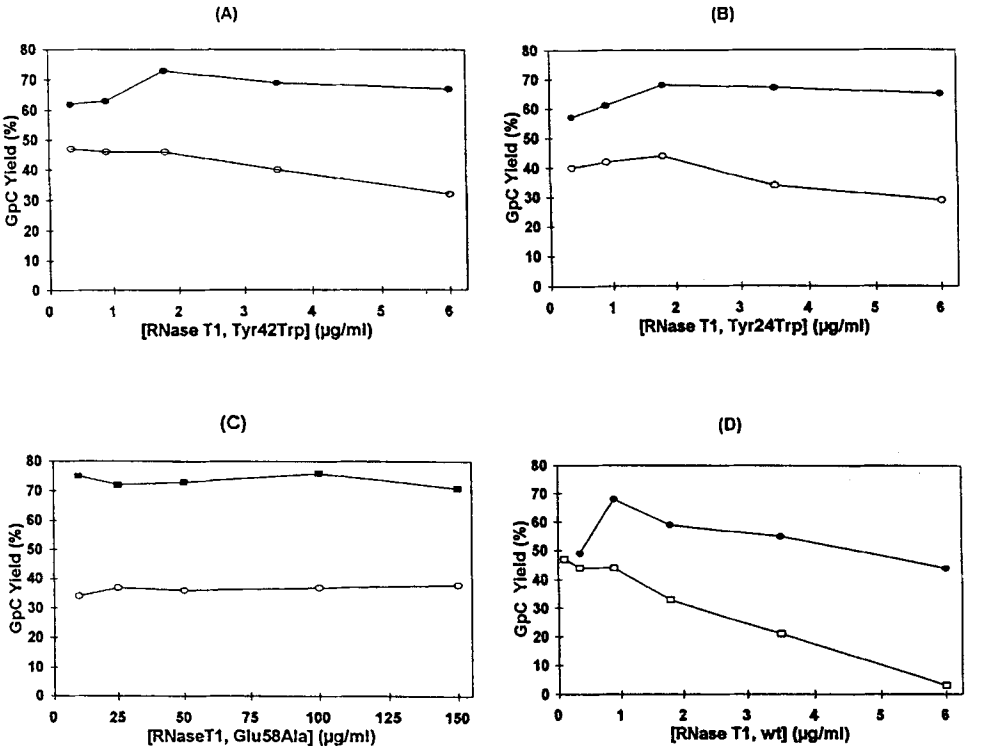


FIG.1: Dependence of GpC synthesis on the enzyme concentrations of wt RNase T1 (D) and the variants Tyr42Trp (A), Tyr24Trp (B) and Glu58Ala(C).

● -10 °C; □ 0 °C; D: from [5]

All variants studied were able to form phosphodiester bonds and freezing the reaction mixtures resulted in significantly enhanced GpC yields. The dependence of GpC yield on enzyme concentration proved to be markedly less distinct than observed in wild type RNase T1-catalysed synthesis [5].

Altered substrate binding in the Tyr42Trp variant had no significant influence on dinucleoside monophosphate synthesis. If one considers GpC yields obtained at the optimal enzyme concentrations (FIG.1A and D) the Tyr42Trp variant is not superior to the wild type in solution at 0 °C as well as in the frozen state. Backmann et al. [7] reported that Tyr42Trp performed dinucleoside monophosphate synthesis better than the wild type protein but they did not vary the enzyme concentration. Therefore, GpC yield of 128%

compared to the wild type enzyme they obtained using Tyr42Trp might have been due to an unfavourable concentration of wild type RNase T1.

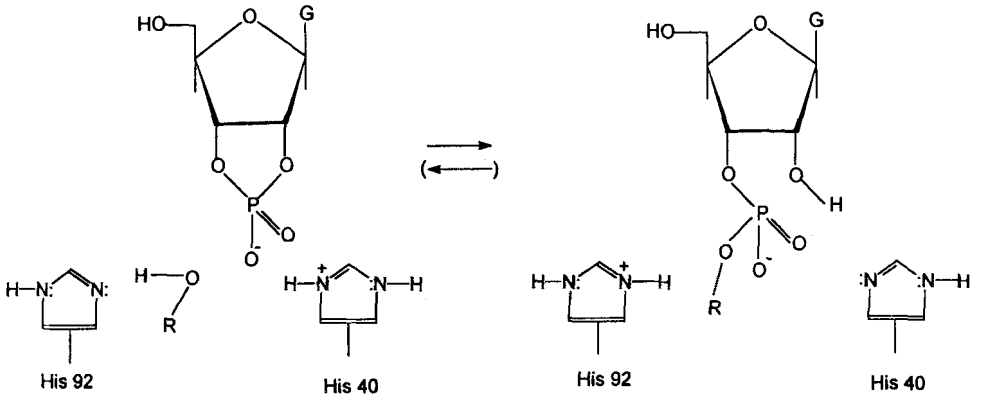
Enhanced hydrolytic activity of the Tyr24Trp variant towards the 2'3'-cyclic donor did not influence GpC synthesis reactions both in solution and in the frozen aqueous system (FIG. 1B).

Surprisingly, the Glu58Ala variant which exhibits a changed mechanism, similar to that of RNase A, gave the highest freezing-induced yield-enhancement observed in this study. No competitive hydrolysis of 2'3'cGMP was observed. In contrast, in RNase A-catalysed synthesis experiments we performed recently [2], significant hydrolysis of the cyclic donor (2'3'-cyclic cytosine monophosphate) was observed. This competitive reaction could only partially been overcome by freezing the reaction mixture.

Although mechanistically similar to RNase A, concerning phosphodiester bond formation, Glu58Ala exhibits a completely different behaviour. Glu58Ala-catalysed GpC synthesis rather takes place in the same way as synthesis catalysed by the wild type enzyme.

Recently, to explain increased GpC yields in wild type-catalysed reactions in ice, we assumed that the His92/Glu58 protonation equilibrium proposed by Backmann et al. [7] is influenced by freezing the reaction mixture[5]. Steyaert et al. [11] established that, whereas protonation of His92 and His40 is essential for maximal activity of the wild type enzyme, His40 is required to be in the deprotonated form in the Glu58Ala variant to act as the general base (right part of SCHEME 1). In (3'→5')phosphodiester bond formation catalysed by Glu58Ala, however, His40 instead of Glu58 has to be protonated (left part of SCHEME 1). Therefore, the results of Glu58Ala-catalysed GpC synthesis reactions strongly indicate a change in the protonation state of His92/His40 in frozen reaction media similar to that proposed for the wild type enzyme (His92/Glu58, [5]).

As for the wild type [5], this assumption is supported by the results of GpC cleavage experiments. Besides GpC synthesis, protonation of His40 should also favour irreversible hydrolysis of the cyclic donor to give 3'GMP (left part of SCHEME 1). Whereas in solution at 0 °C no 3'GMP was obtained until complete transesterification of GpC to 2'3'cGMP (FIG. 2A), under frozen state conditions transesterification of GpC and irreversible hydrolysis of 2'3'cGMP took place simultaneously (FIG. 2B). In GpC



SCHEME 1: Proposed catalytic mechanism of the RNase T1 variant Glu58Ala-catalysed conversion of 2'3'cGMP (derived from Heinemann and Saenger [8] and Steyaert et al. [11]).
Irreversible hydrolysis to 3'GMP: R = H; synthesis of GpC: R-OH = C.

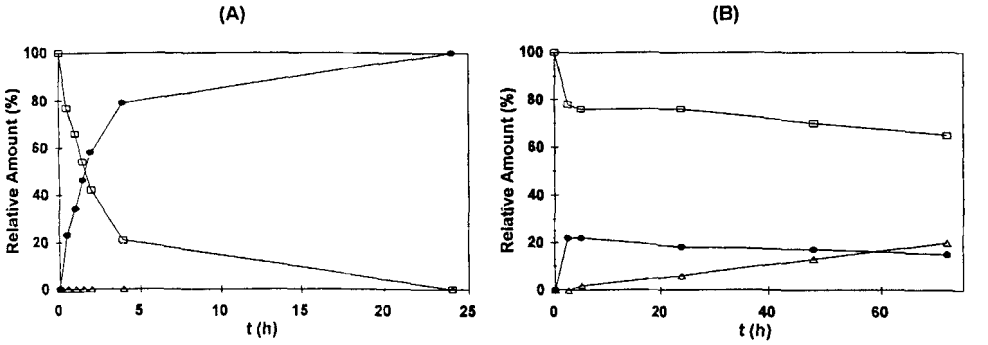


FIG. 2: RNase T1(Glu58Ala)-catalysed cleavage of GpC at 0 °C (A) and at -10 °C (B).
□ GpC, • 2'3'cGMP, Δ 3'GMP; GpC] = 2.75 mM, 0.1 M Tris / HCl pH 7.0, enzyme concentration: 1 μg/ml (0 °C), 50 μg/ml (-10 °C)

synthesis experiments, the excess of cytidine used prevented hydrolysis of the cyclic donor.

In conclusion, we have established that freezing the reaction mixture can substantially increase dinucleoside monophosphate yields in reactions catalysed by RNase T1 variants differing from the wild type enzyme in substrate binding and in the catalytic mechanism. We assume that the protonation state of the catalytic residues is influenced by freezing, possibly due to conformational changes under frozen state conditions as also described recently by Strambini and Gabillieri for Lys25-RNase T1[16].

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