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Research Article

Triosephosphate isomerase from baker's yeast – ribozyme versus protein

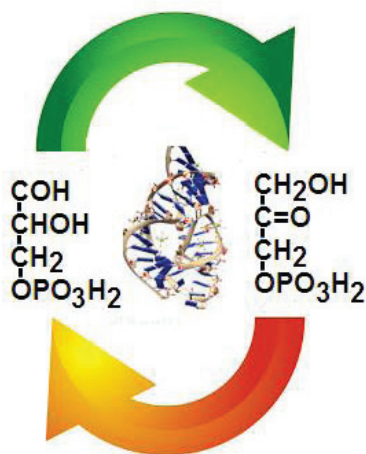
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Abstract

It was previously shown that in baker's yeast *Saccharomyces cerevisiae*, transketolase can exist not only free, but in complex with RNA. The complex does not possess transketolase activity [N.K. Tikhomirova, G.A. Kochetov, A new method of isolation and a new form of transketolase from baker's yeast, *Biokhimiia* 56 (1991) 1123-1130].

We discovered that this RNA is a ribozyme which catalyzes the interconversion of glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone 3-phosphate (DHAP), i.e. acting as triosephosphate isomerase (TPI). It also catalyzes an unusual reaction of ribose 5-phosphate (R5P) decomposition to G3P and erythrose. TPI-ribozyme was found in baker's yeast not only in complex with transketolase, but also in free form. Transketolase-RNA complex was easily isolated on an immunoaffinity column with antibodies to transketolase. TPI-ribozyme consists of 87 nucleotides and has a molecular weight of 26.6 kDa. The optimum of pH-activity is 7.5 for DHAP, 6.7 for R5P and 9.0 for G3P. K_m and V_{max} are accordingly 0.29 mM and 2.6 U/mg for DHAP, 22 mM and 0.65 U/mg for R5P, 0.05 mM and 4.3 U/mg at pH 7.6 and 0.11 mM and 16 U/mg at pH 9.0 for G3P. These kinetic characteristics are the same for free RNA and in the complex with transketolase. K_i for RNA binding to transketolase was 1.0 μ M. Accordingly, the TPI-ribozyme performs a dual function – it shows TPI activity and blocks the work of transketolase, thereby switching the metabolic process to glycolysis. The location of the TPI-ribozyme gene is determined. Blocking the activity of transketolase by ribozyme may be of practical importance in medicine, particularly, in cancer therapy.





Abbreviations

TK: Transketolase; ThDP: Thiamine Diphosphate; TPI: Triosephosphate Isomerase; GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase; GPDH: Glycerol 3-Phosphate Dehydrogenase; ACN: Acetonitrile; DHAP: Dihydroxyacetone 3-Phosphate; G3P: Glyceraldehyde 3-Phosphate; R5P: Ribose 5-Phosphate

Introduction

Ribozymes were first reported in 1981 [1-3]. They catalyze a variety of reactions, along with protein enzymes. The ribozymes involved in the synthesis and cleavage of RNA [4-7] and protein [8] are well studied. RNA ligase ribozyme (DSL) [9,10], Tetrahymena ribozyme [11,12], RNA polymerase ribozyme [13] and others are involved in the cleavage of RNA. In biosynthesis of amino acids is involved glmS ribozyme [14-16]. There are ribozymes that catalyze amino acids transfer reactions [17].

Ribozymes with peroxidase activity [18] and urea synthase activity [19] were detected. Self-cleaving ribozymes are also detected [20-23]. In addition, the ribosomes function as ribozymes [24-26], the RNase works in a protein complex with a ribozyme [27,28]. Among the ribozymes that convert carbohydrate substrates are the Diels-Alderase ribozyme, which is involved in the formation of C-C bonds [29,30], and the ribozyme that catalyzes the synthesis of dipeptides [31]. About 100 reviews have been written about these ribozymes. The review [32], contains a table of RNA classes, their sizes and functions. The review [33], provides a detailed description of the chemical properties of ribozymes, the role of metals and the characteristics of individual ribozymes, as well as the methods used in ribozyme studies. Self-cleaving ribozymes are described in review [23], riboswitches in reviews [34,35]. The latest review of RNase was published in 2009 [28].

Since 2003, 5 articles have been published on ribozymes that are not involved in the biosynthesis of RNA or protein. They are involved in the reactions of alcoholic fermentation - pyruvate decarboxylase ribozyme [36] and alcohol dehydrogenase ribozyme [37,38] and in glycolysis - aldolase ribozyme [39,40]. Interestingly, free thiamine is used in the pyruvate decarboxylase ribozyme, while thiamine diphosphate is used in the pyruvate decarboxylase protein [36]. Alcohol dehydrogenase ribozyme oxidizes alcohol only in the presence of NAD⁺ and Zn²⁺ [37]. Like alcohol dehydrogenase, a protein, it is able to catalyze a reverse reaction in the presence of NADH and Zn²⁺ [38]. Aldolases (protein enzymes) utilize either an enamine mechanism or a Zn²⁺ cofactor. Aldolase ribozyme is a Zn²⁺-dependent. Ribozyme, unlike the protein enzyme, can also catalyze a transfer of a biotinylated benzaldehyde derivative to the aldol donor substrate [39]. All were obtained by *in vitro* selection using an RNA library.

In this paper, we first show the existence of another glycolytic ribozyme, Triosephosphate Isomerase (TPI)-ribozyme, which was found in baker's yeast, both free and associated with Transketolase (TK), performing, respectively,

two functions - the work of the glycolytic enzyme and blocking the enzyme of the pentose phosphate pathway and accordingly the switching of metabolism to glycolysis. The activity of TPI-ribozyme is the same as a part of the complex and in free form. It was previously shown that TK can be isolated from yeast in the form of a complex with RNA [41,42]. G3P, which is formed from DHAP upon catalysis by TPI (enzyme and ribozyme), is one of the substrates of transketolase. Nevertheless, it is not clear why the shutdown of TK is carried out precisely by TPI-ribozyme.

We suggest that in perspective for most enzymes, ribozymes with a similar function will be found. It is possible that other ribozymes will also be associated with proteins and so will be easy to isolate. The presence of a complex with nucleic acids is known for many enzymes, including those not involved directly in the biosynthesis of nucleic acids and proteins [43-45]. For glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the interaction with the hepatitis delta virus RNA was shown [46,47].

Materials and methods

Materials

Thiamine Diphosphate (ThDP), dithiothreitol, glycyglycine, MES were from Serva, NAD⁺, NADH, CaCl₂, GAPDH, glycerol 3-phosphate dehydrogenase (GPDH), RNase A, Dihydroxyacetone Phosphate (DHAP), Ribose 5-Phosphate (R5P), erythrose, CHES, acetonitrile (ACN), perchloric acid, ammonium sulfate, protamine sulfate from Sigma, HEPES from Fluka, human albumin, pepsin and lysozyme from Reanal, other commercially available chemicals were of the highest grade.

Transketolase purification

TK (free enzyme and in complex with RNA) was isolated from baker's yeast *Saccharomyces cerevisiae* on a column with immobilized antibodies to TK isolated from the serum of an immunized rabbit according to the method described previously [42]. Extraction was carried out by cytolysis of dry yeast with 0.5 M NH₄OH. During the elution of TK from the column, the pH was maintained at 7.6 by adding 4M HCl to the eluate. The eluted protein was concentrated in Amicon® 50,000 MWCO Ultra Centrifugal Filters, then aliquoted and stored frozen. Before use, the TK solution was passed through a Sephadex G-50 column equilibrated with 50 mM glycyglycine, pH 7.6.

Measuring protein and RNA concentrations

The concentration of free TK was determined spectrophotometrically using the absorption coefficient A 1% 1-cm of 14.5 at 280 nm [48]. The concentration of TK in TK-RNA complex was determined according to the Bradford method [49], using the free TK for calibration. The amount of nucleotides was calculated by [50], in which the optical density at 260 nm equals 1.0 for the 40 µg/mL solution of RNA, for a 1-cm pathlength. The molar concentration of nucleotides was calculated by taking an average nucleotide mass of 345 Da.

Purification of the TK-RNA complex

The TK isolated on the immune column (mixture of free TK and TK-RNA complex) was passed through a Sephadex G-50 column in 10 mM potassium phosphate buffer, pH 7.6. Then, 2 mL of TK + TK-RNA was passed through the IRA-400 column (2.4 mL), previously washed with 100 mL of 0.1 M KOH and with water until neutral pH and eluted with the same buffer. Free enzyme was absorbed onto the column.

Obtaining free RNA from the TK-RNA complex

ACN in a 2:1 ratio was added to the TK-RNA complex, stirred, and frozen for 1-2 h at -12°C. The upper layer of ACN, which did not contain RNA, was removed, in the lower layer of water, the denatured protein was removed by centrifugation. The RNA obtained in this way was precipitated with 96% ethanol (3:1) and dissolved in water. Determination of protein by Bradford showed its absence.

Obtaining free TK from the TK-RNA complex

ApoTK (TK without ThDP and RNA) was obtained by keeping TK (2mg/mL) in 1.6M ammonium sulfate at pH 7.6 for 24h at 4°C. Then the enzyme was precipitated with 4 M ammonium sulfate, the precipitate was dissolved in 50 mM glycylglycine or potassium phosphate buffer, pH 7.6, and passed through a Sephadex G-50 column equilibrated with the same buffer.

Determination of RNA molecular mass

Initially, the molecular mass of RNA was determined by gel chromatography on a Sephadex G-100 column in 6 M urea. A column of 15 x 1 mL was applied to 1 mL of RNA. Human albumin 66.44 kDa, pepsin 34.5 kDa, and lysozyme 14.5 kDa were taken as markers. All compounds were kept for 2 h in urea before being applied to the column.

Then, the molecular mass of RNA was determined using MALDI-TOF MS analysis, which was performed on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with an Nd laser by detection of MH⁺ molecular ions.

Determination of thiamine diphosphate concentration

The concentration of ThDP was determined spectrophotometrically by measuring optical density at 272.5 nm (molar extinction coefficient 7500 M⁻¹ · cm⁻¹) [51] and in TK-RNA - enzymatically, after boiling or incubation with RNase A [52].

Measuring of transketolase activity

The catalytic activity of TK was measured at room temperature using spectrophotometer *Ultrospec 110 pro* by the rate of NAD⁺ reduction in a coupled system with GAPDH [53]. The reaction mixture in final volume 1 mL contained 50 mM glycylglycine pH 7.6, 2.5mM CaCl₂, 1 mM sodium arsenate, 3 mM dithiothreitol, 0.1 mM ThDP, 1.6 mM NAD⁺, 3 units of GAPDH and 3.5 mg/mL potassium salt of the phosphopentose mixture that was used as substrate. The reaction was initiated

by the addition of TK. Phosphopentose mixture was obtained from R5P [54].

Determination of the affinity of RNA to TK

50 µg/mL of TK were incubated without RNA and with fixed RNA concentrations of 0.55, 1.0, and 2.65 µM and varying ThDP concentrations of 0.6-3 µM for 40 min without adding cations to the medium. Under these conditions, Ca²⁺ is located in only one active site of the dimeric TK molecule. Therefore, ThDP can be bound with only one of the two active sites [55,56]. The results were processed in reverse coordinates. K_i was calculated using the formula $K_m [I] / (K_m app - K_m)$.

Also, 0.5 mg/mL TK (3.4 µM) was incubated for 40 min with 6 µM ThDP without the addition of cations (condition of ThDP binding in one of two active sites). Measuring the activity without the addition of cofactors, we were convinced that it is 50% of the maximum, i.e. ThDP was bound only in the first active site. 6 µM RNA was then added and incubated for 20 min and the activity of TK was measured in the presence of 0.1 mM Ca²⁺ and 0.1 mM ThDP.

Measuring of RNA and TPI (protein) activity

The catalytic activity of free RNA and in the complex with TK, as well as the activity of the TPI - protein enzyme was measured by the method [57].

When the activity was measured using DHAP, the reaction mixture in the final volume of 1 mL contained 50 mM glycylglycine, 1.6 mM NAD⁺, 1 mM DHAP, 3 units GAPDH, 6 mM sodium arsenate, 3 mM dithiothreitol, 0.5-2.5 µg/mL RNA, pH 7.6.

When the activity was measured using glyceraldehyde 3-phosphate (G3P), the reaction mixture in the final volume of 1 mL contained 50 mM glycylglycine, 0.3 mM NADH, 1 mM G3P, 3 units of GPDH, 0.5-2.5 µg/mL RNA, pH 7.6.

When the activity was measured using DHAP or G3P, their freshly prepared solutions were used. The reaction was started by adding TK-RNA or RNA, after waiting for a zero background.

When the activity was measured using R5P, the reaction mixture in the final volume of 1 mL contained 50 mM glycylglycine, 0.3 mM NADH, 3 mM R5P, 3 units of GPDH, 0.5-2.5 µg/mL RNA, pH 7.6.

K_m for substrates was measured in the concentration range of DHAP 0.03-2.2 mM, G3P 0.015-0.3 mM, R5P 2.5-120 mM. Experimental data were analyzed using the Michaelis equation

$$v = V \cdot [S] / ([S] + K_m)$$

When measuring the pH optimum, 50 mM buffers were used - MES at pH range 5.0-7.0, HEPES at pH range 7.0-8.5 and CHES at pH range 8.5-10.5.

Erythrose assay

After the total conversion of R5P by RNA in the presence of GPDH and NADH, the reaction mixture was treated with

perchloric acid. The precipitated protein was removed by centrifugation, and the supernatant was assayed for the amount of erythrose in the cysteine - H_2SO_4 reaction [58].

Isolation of total RNA from yeast extract

Extraction was carried out from dry yeast with 0.5 M ammonia, as in the isolation of TK. Half of the extract was passed through an anti-TK immunoaffinity column to bind and thus remove the TK and the TK-RNA complex. ACN 3:1 was added to both extracts to remove proteins.

Partial purification of TPI - protein enzyme

After removing the nucleic acids from the yeast extract by treatment with protamine sulfate and removing the TK-RNA by passing through an immunoaffinity column with antibodies to TK, dry ammonium sulfate was added to 70% saturation. The precipitate was collected, dissolved in the same volume, and the protein fraction was collected with saturation of 60-70% ammonium sulfate. The absence of GAPDH activity was verified [59].

RNA sequence

RNA was extracted from RNA-protein complex with PureLink RNA Micro Kit (Invitrogen) according to manufacture instruction. NGS Illumina library was made from RNA with NEBNextNEBNext® Ultra™II RNA Library Prep Kit for Illumina® (NEB) according to manual. Sequencing was performed on HiSeq1500 with 50 bp read length. 13 millions of reads were generated for each sample and mapped to reference genome *Saccharomyces cerevisiae*. Read counts to every transcripts was calculated.

Results and discussion

Isolation of TK-RNA on a column with IRA-400

To obtain the TK-RNA complex without free TK, the TK isolated on the immunoaffinity column was passed through the column IRA-400. TK-RNA was eluted with 10 mM potassium phosphate buffer at pH 7.6. Free TK remained bound to the ion exchanger even when passing 500 mM buffer through the column (Figure 1). The TK-RNA complex did not possess transketolase activity, which was also shown earlier [41,42]. ThDP in the complex was absent (It was measured enzymatically). RNA can be separated from TK-RNA by ammonium sulfate and therefore is associated non-covalently.

Absorption spectra of TK-RNA and RNA

Figure 2 shows the absorption spectra of TK-RNA (curve 1), RNA obtained from TK-RNA by protein precipitation using ACN (curve 2), free TK (curve 3). In all samples, the concentration of TK was 0.1 mg/mL. The protein concentration in TK-RNA complex was determined according to Bradford [49]. The correctness of the determination was confirmed by the fact that in the difference spectrum (TK-RNA - TK) (curve 4) a spectrum was obtained that almost completely coincided with the spectrum of RNA (curve 2).

Measuring the activity of TK-RNA in the usual for TK transferase reaction in the presence of cofactors showed its complete absence. This means that RNA was associated with both active sites of TK, that is, two RNA molecules are linked to one dimeric TK molecule. Comparing the concentration of TK and nucleotides in the TK-RNA complex, we calculated that one RNA molecule contains 87 nucleotides, i.e. the mass of RNA is about 30 kDa. The molar extinction coefficients calculated from the absorption spectra at different wavelengths are shown in Table 1. A_{280} for TK bound to RNA is 6.03, while for free TK this value is 1.45 [48].

Determination of the RNA molecular mass

The molecular mass of RNA determined by gel chromatography on Sephadex G-100 under denaturing conditions was 26.6 kDa (Figure 3A). Then, the molecular mass of RNA was determined using MALDI-TOF MS analysis. The maximum mass value of 13.766 ± 2 kDa was determined (Figure 3B). So, it can be assumed that the RNA is double-stranded. These data require further research.

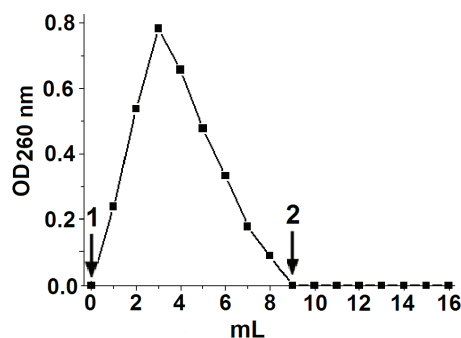


Figure 1: Elution of TK-RNA on an IRA-400 column. 2 mL of TK (a mixture of free enzyme and its complex with RNA) was applied to the column. 1 - 10 mM potassium phosphate buffer pH 7.6 was passed through the column, 2 - 500 mM potassium phosphate buffer pH 7.6 was passed through the column.

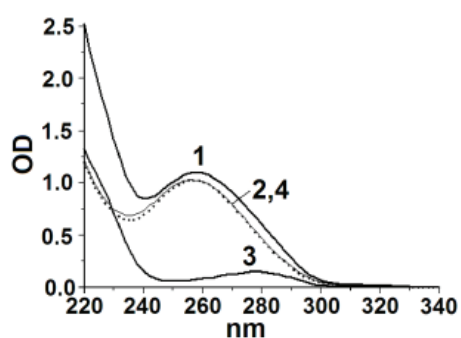


Figure 2: Absorption spectra of TK-RNA (1), RNA (2), free TK (3) in 50 mM potassium phosphate buffer, pH 7.6. (4) - difference spectrum (1) - (3). The concentration of TK 0.1 mg/mL.

Table 1: The absorption of TK-RNA, TK and RNA per 1 mg/mL TK at different wavelengths.

	220 nm	260nm	280nm	290nm	280/260	260/280	260/290	220/280
Free TK	13.2	0.81	1.45	0.91	1.8	0.56	0.89	9.1
TK-RNA	10.07	10.93	6.03	2.76	0.55	1.81	3.95	1.67
RNA	12.1	10.13	4.7	1.97	0.46	2.15	5.14	2.57

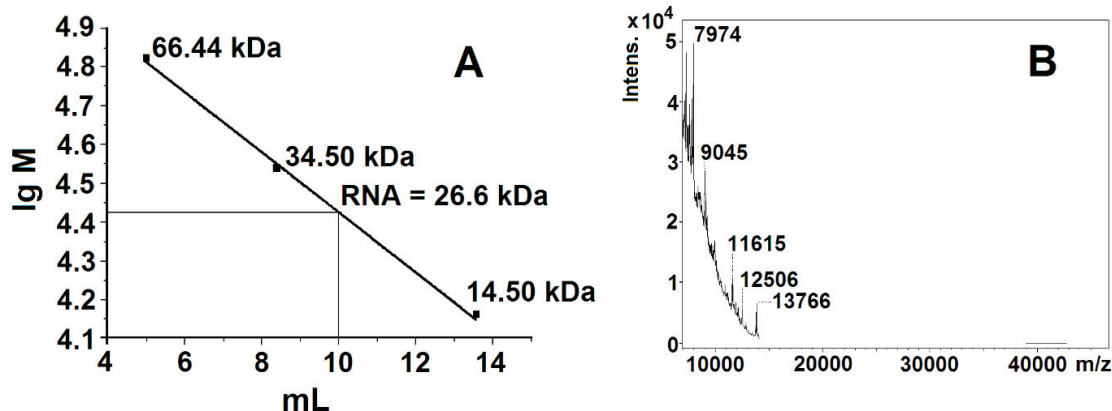


Figure 3: Determination of the molecular mass of RNA on Sephadex G-100 column in the presence of 6 M urea; mass calibrants: human albumin 66.44 kDa, pepsin 34.5 kDa, lysozyme 14.5 kDa (A) and using MALDI-TOF MS analysis (B).

Determination of RNA affinity for TK

ApoTK was incubated with varying concentrations of ThDP in the presence of fixed concentrations of RNA and in its absence to determine the type of inhibition and K_i upon binding of RNA to TK. Binding was performed without the addition of cations in the incubation samples and then the TK activity was measured without the addition of cofactors. As it was shown earlier [51,52] in the absence of added cations from the outside, Ca^{2+} is bound in one of two active sites of TK and, accordingly, ThDP can only be linked in the first active site. In Figure 4 it is shown that in this case competitive binding of RNA and ThDP occurs. This means that RNA binds to or near the active site of the TK. Under these conditions, binding of RNA can only be seen in the first active site, according to the decrease in TK activity. K_i for RNA binding was $1.0 \pm 0.2 \mu M$, K_d for ThDP - $2.4 \pm 0.2 \mu M$.

In the second active site, which does not contain a cation, RNA binding also occurs. This was shown as follows. In the absence of cations in the medium, a small excess of ThDP was added to apoTK, with the result that ThDP was bound only in the first active site, and, accordingly, the enzyme activity was 50% of the activity measured in the presence of Ca^{2+} and ThDP. Then an excess of RNA was added to this enzyme. Subsequent measurement of TK activity with the addition of Ca^{2+} and ThDP showed that it was still 50% of the maximum. This means that RNA was bound to half of the active sites and that no cation is needed for its binding.

If the binding of RNA was performed in the presence of Ca^{2+} in the medium, it did not occur. Thus, RNA does not bind to TK in the presence of Ca^{2+} in the medium but it binds in the presence of Ca^{2+} in one active site. Besides, RNA does not displaced by Ca^{2+} and ThDP from the TK-RNA complex (data not shown).

Thus, RNA binds to TK in the region of its active sites competitively with respect to ThDP. Cations are not needed for RNA binding. After RNA treatment with RNase A, ThDP was not detected in it (data not shown). Previously, it was shown that the acceptor substrates bind to holoTK through the residue

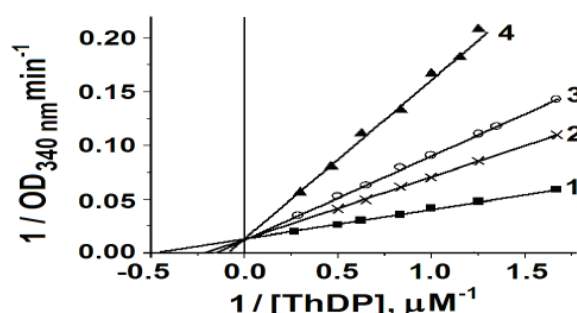


Figure 4: Dependence of TK activity on ThDP concentration in the reciprocal coordinates. 50 $\mu g/mL$ of TK were incubated for 40 min with 0.6 – 3 μM ThDP in the absence of RNA (1) and in the presence of 0.55 μM (2), 1 μM (3) and 2.65 μM (4) of RNA. Then the activity of TK was measured without the addition of ThDP.

Asp^{477} , which forms a hydrogen bond with the C2-hydroxyl group of the acceptor substrate [60]. Therefore, it can be assumed that RNA also binds directly to TK (perhaps also with the residue Asp^{477}) through its R5P.

Reactions catalyzed by RNA. RNA is TPI-ribozyme

RNA associated with TK catalyzes the interconversion reaction of G3P and DHAP, like the protein enzyme TPI, as well as the unusual reaction of converting R5P to DHAP and erythrose. So, it is a ribozyme.

In Figure 5 it is shown the dependence of the rate of these reactions on the concentration of RNA. The reaction rate and kinetic characteristics were the same for free RNA and RNA in a complex with TK. The values of K_m and V_{max} for the reactions with these three substrates are shown in Table 2.

In the reaction of RNA with a five-carbon R5P, a three-carbon G3P is formed. Consequently, it should be expected that glycolaldehyde will be the second reaction product. However, the second product of the reaction was erythrose, which may be formed as a result of the condensation of two glycolaldehyde molecules. Assuming that this is the case, two molecules of RNA-converted R5P would yield one molecule of erythrose, as was confirmed experimentally. Table 3 shows that the amount of erythrose formed corresponds to the calculated data.



In Scheme 1 are shown the schemes of reactions with RNA and of the one-substrate reaction with TK. It is interesting that in one-substrate reaction of free TK with xylulose 5-phosphate, G3P is released in the medium and erythrulose is formed from the two molecules of glycolaldehyde [61], (Scheme 1C). For RNA, when R5P is used, two molecules of DHAP are sequentially released in the medium, and erythrulose is formed from the two molecules of glycolaldehyde (Scheme 1B). So, when the ketose xylulose 5-phosphate is converted, the aldose G3P and the ketose erythrulose are formed. When the aldose R5P is converted, the ketose DHAP and the aldose erythrulose are formed. It is interesting that two molecules of glycolaldehyde can form both ketose and aldose. Since the formation of erythrulose on RNA occurs stoichiometrically, the first molecule of glycolaldehyde formed is not released into the medium, but remains bound to RNA, similarly to the same process in the case of a one-substrate reaction with TK.

The optimum pH in the reactions of RNA with G3P, DHAP and R5P

In Figure 6 are shown the results of the measuring the optimum pH activity in the reactions of RNA with substrates. The optimum pH in the reaction of RNA with DHAP is 7.5 (curve 1), with G3P 9.0 (curve 2), with R5P 6.7 (curve 3).

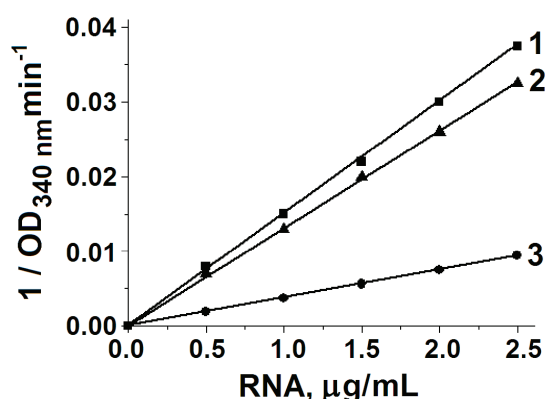


Figure 5: Dependence of the reaction rate on the RNA concentration. 1- activity with DHAP, 2 - activity with G3P, 3 - activity with R5P. Reaction mixture in final volume 1 mL contained 50 mM glycylglycine, pH 7.6, 0.5-2.5 µg/mL RNA and (1) 1.5 mM DHAP, 1.6 mM NAD⁺, 3 units GAPDH, 6 mM sodium arsenate, 3 mM dithiothreitol; (2) 0.5 mM G3P, 0.3 mM NADH, 3 units GPDH; (3) 110 mM R5P, 0.3 mM NADH, 3 units GPDH. Reaction was initiated by RNA (free or in complex with TK).

Table 2: Determination of kinetic parameters for RNA.

Substrate	pH	Enzyme	K _m , mM	V _{max} , U/mg
DHAP	7.6	TK-RNA	0.29±0.02	2.77±0.3
		RNA	0.29±0.02	2.6±0.3
		TPI (enzyme)	0.72±0.05	20±1
		TPI (enzyme) [62]	0.6 ± 0.02	490
G3P	7.6	TK-RNA	0.05±0.003	4.51±0.4
		RNA	0.05±0.003	4.3±0.4
		TPI (enzyme)	2.2±0.1	20±1.5
		TPI-extract [62]		41
G3P	9.0	TPI (enzyme) [62]	1.27±0.06	10000
		TK-RNA	0.11±0.006	16±2
		RNA	0.11±0.006	16±2
R5P	7.6	TK-RNA	22±2	0.67±0.04
		RNA	22±2	0.65±0.04

Table 3: Consumption of R5P and formation of erythrulose in the reaction catalysed by RNA. RNA concentration 30 µg/mL.

Consumption of R5P, µM	Amount of erythrulose formed		
	Theoretically expected, µM	Actually formed, µM	% of the expected
10	5	4.7	94
20	10	9.2	92

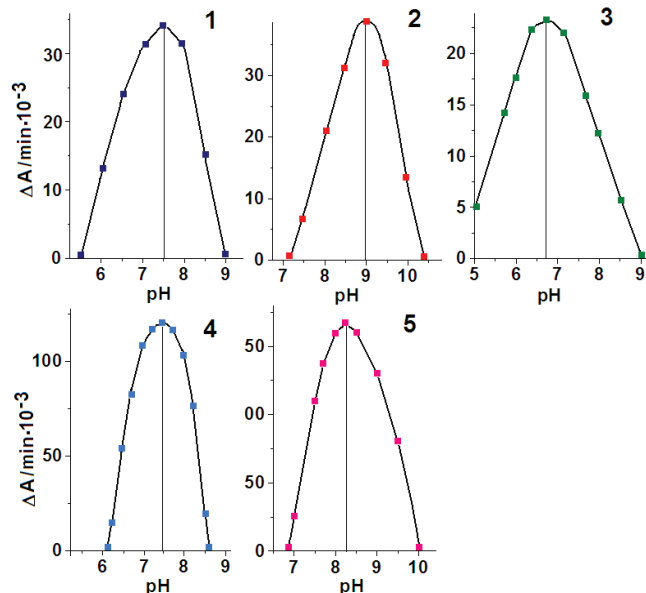
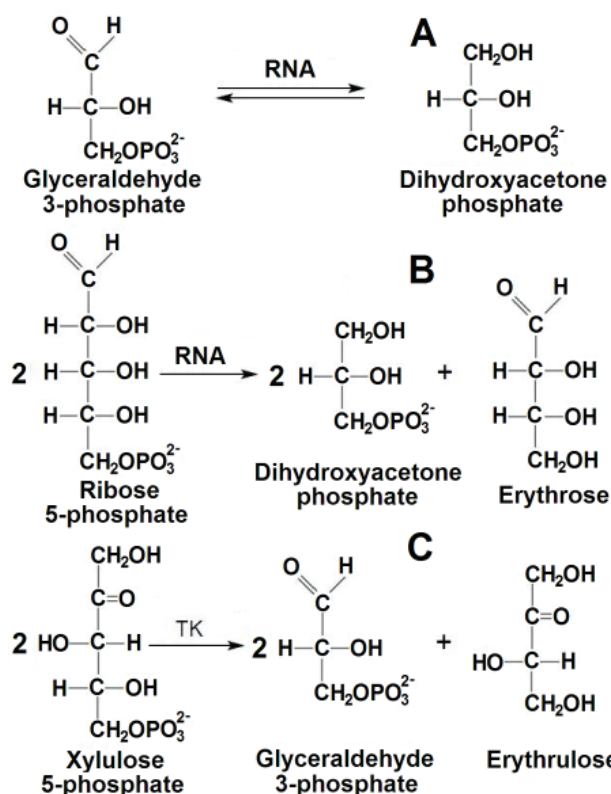


Figure 6: Optimum pH activity of RNA with DHAP (1), G3P (2) and R5P (3) and of TPI (enzyme) with DHAP (4) and G3P (5). Activity was measured, as indicated in the captions to Figure 5. Instead of glycylglycine were used 50 mM buffers - MES at pH range 5.0-7.0, HEPES at pH range 7.0-8.5 and CHES at pH range 8.5-10.5. RNA concentration was 2.1 µg/mL (1), 0.35 µg/mL (2) and 5.7 µg/mL (3).



Scheme 1: Scheme of RNA catalysis in the TPI reaction (A) and in the reaction with R5P (B) and of TK catalysis in one-substrate reaction (C).

Comparison of the properties of TPI-ribozyme – free and in complex with TK and TPI-protein in yeast extract

Total RNA was isolated from yeast extract. A portion of the RNA was passed through an immunoaffinity column to remove the TK-RNA complex. Before and after removal of TK-RNA from the extract, there was RNA activity with G3P, DHAP and R5P. Consequently, TPI-ribozyme is in the extract not only in the form of a complex with TK, but also in a free form.

An ammonium sulfate fraction containing TPI (protein) was also obtained from the yeast extract and the kinetic constants were measured. The ratio of direct and reverse K_m reactions is different for RNA and protein: for RNA, the affinity for G3P is 6 times higher than for DHAP, and for protein, the affinity for DHAP is 3 times higher than for G3P. At the same time, the affinity for G3P is 40 times higher in RNA than in protein (Table 2). Only RNA has the ability to catalyze the reaction with R5P. V_{max} of the salt fraction containing TPI was 20 U/mg. Previously, it was shown that the specific activity of TPI from brewer's yeast was 41 U/mg in crude homogenate and 10,000 U/mg in the final purification stage [62]. K_m for TPI (enzyme) of two types of yeast is almost the same for both substrates (Table 2).

The optimum pH of the reactions for RNA and TPI (enzyme) is approximately the same – 7.5 in the reaction with DHAP (Figure 7, curves 1 and 4) and 9.0 and 8.3 in the reaction with G3P for RNA and protein, respectively (Figure 7, curves 2 and 5).

RNA sequence

An RNA sequence was performed. The results are presented in Table 4 and Scheme 2. The largest and with the same number of readings falls on the genes of the ribosomal RNA RDN37-1 and RDN37-2, located in the 12th chromosome and each 5947 nucleotides in length. Several genes that are located within the genes RDN37-1 and RDN37-2 are also sequenced. These are genes RDN25-1 and RDN25-2 of 3396 nucleotides each and RDN18-1 and RDN18-2 of 1600 nucleotides each, as well as several short genes. It can be assumed that TPI-ribozyme is formed by splicing of some exons and is represented by two forms – TPI-ribozyme1 and TPI-ribozyme2, each about 87 nucleotides in length.

Conclusion

Previously was detected inactive TK from baker's yeast [41,42]. TK activity was absent as a result of it's binding to RNA and was restored when the TK-RNA complex dissociated. So, RNA binding is non-covalent. Due to the great interest in ribozymes, we tried to determine whether RNA associated with TK possesses enzymatic activity. As it turned out, this RNA is a TPI ribozyme and, in addition, unlike protein TPI, slowly cleaves R5P into DHAP and erythrose.

Currently, there are a lot of ribozymes with diverse functions. However, only ribozymes with peroxidase activity [18], urea synthase activity [19], pyruvate decarboxylase activity [36], alcohol dehydrogenase activity [37,38] and aldolase activity

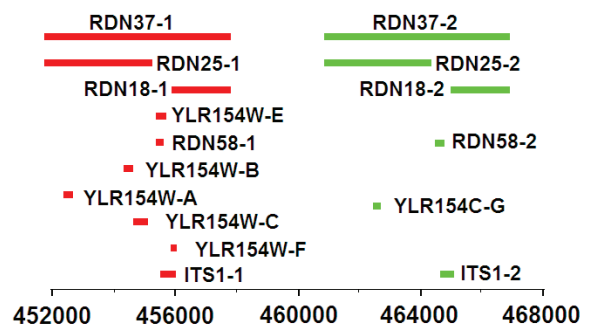
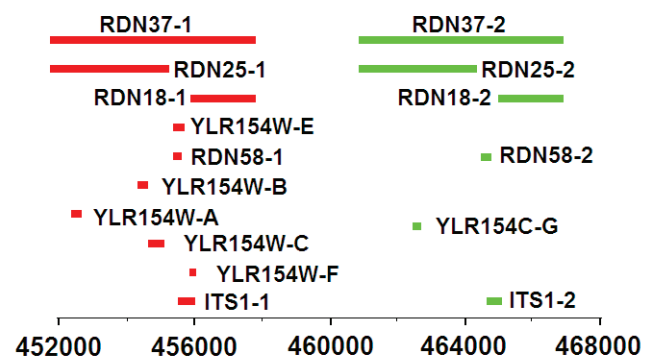


Figure 7: Results of RNA sequence.

Table 4: Results of RNA sequence.

	Chromosome Position	Length, Bp	Id	Name	Biotype	Count
1	XII:451786-457732	5947	RDN37-1	RDN37-1	rRNA	9572705
2	XII:460923-466869	5947	RDN37-2	RDN37-2	rRNA	9569749
3	XII:451786-455181	3396	RDN25-1	RDN25-1	rRNA	5719278
4	XII:460923-464318	3396	RDN25-2	RDN25-2	rRNA	5718568
5	XII:455933-457732	1800	RDN18-1	RDN18-1	rRNA	3081458
6	XII:465070-466869	1600	RDN18-2	RDN18-2	rRNA	3079401
7	XII:455433-455636	204	YLR154W-E	-	*	792119
8	XII:455414-455571	158	RDN58-1	RDN58-1	rRNA	776841
9	XII:464551-464708	158	RDN58-2	RDN58-2	rRNA	776653
10	XII:462522-462671	150	YLR154C-G	-	putative protein coding	579327
11	XII:454392-454556	165	YLR154W-B	-	putative protein coding	259589
12	XII:452438-452623	186	YLR154W-A	YLR154W-A	*	254822
13	XII:454696-455070	375	YLR154W-C	TAR1	protein coding	226668
14	XII:455883-456023	141	YLR154W-F	-	*	144282
15	XII:455572-455932	361	ITS1-1	-	rRNA	35927
16	XII:464709-465069	361	ITS1-2	-	rRNA	35714

*Dubious open reading frame unlikely to encode a protein



Scheme 2: Results of RNA sequence.

[39,40] which exhibit activity similar to enzymes, were found. There is no doubt that such ribozymes are actually much more.



In the case of TK, TPI-ribozyme not only duplicates the work of the protein enzyme, but also blocks the work of TK, thus switching yeast metabolism from the pentose phosphate pathway to glycolysis. The lack of transketolase activity was seasonal. Maximum of complex TK-RNA was in December. The reason for this switch remains to be found. But this fact indicates that turning off the functioning of TK by forming its complex with RNA is a real mechanism for switching the pentose phosphate pathway and glycolysis in baker's yeast.

We were unable to determine the nucleotide sequence of the RNA ribozyme. According to preliminary data, this RNA is formed from the loci of two genes - RDN37-1 and RDN37-2 which have the same number of nucleotides (Scheme 2 and Table 4). The fact that the mass of RNA determined on the mass spectrometer is 2 times less than that determined with using gel chromatography (13.283 and 26.6 kDa consequently), indicates the possibility that our RNA is double-stranded. Perhaps these are two ribozymes with the same function.

It is known that to suppress the growth of cancer cells, inhibition of TK is performed [63,64]. Blocking of TK activity could be carried out by forming its complex with RNA.

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