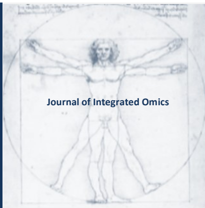

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Thiochrome activates DNA polymerase

V. E. Yakimenko, O. K. Budnyak, S. S. Chernadchuk, A. V. Zaharov, S. A. Petrov*

Department of Biochemistry, Odessa I.I. Mechnikov National University, Odessa, Ukraine .

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Abstract

We studied the effect of thiochrome on the activity of DNA polymerase isolated from the liver of white rats. This article shows the possibility of the influence of the most important thiamine metabolite – thiochrome on DNA polymerase activity. Roles of carboxyl groups of the enzyme and hydrophobic and hydrogen bonds in it in the realization of the activating action of thiochrome on the enzyme have been studied. The influence of thiochrome on the activity of DNA polymerase was studied. It was found that thiochrome is capable to activate this enzyme through interaction with the enzyme.

Аннотация

Мы изучали влияние тиохрома на активность ДНК-полимеразы, выделенной из печени белых крыс. В данной статье показана возможность влияния важнейшего метаболита тиамина – тиохрома на активность ДНК-полимеразы. Изучена роль карбоксильных групп фермента и гидрофобных и водородных связей в нем в реализации активирующего действия тиохрома на фермент. Исследовано влияние тиохрома на активность ДНК-полимеразы. Было установлено, что тиохром способен активировать этот фермент путем взаимодействия с ферментом.

Keywords: Thiochrome, DNA polymerase, enzyme

1. Introduction

The most of investigations in the field of biochemistry of thiamine are devoted to its coenzyme form – thiamine pyrophosphate and diseases associated with the deficiency of this vitamin in the body. Studies on non-coenzyme functions of both thiamine and its metabolites have been published during last three years. In particular, the specific role of thiamine triphosphate has been shownin the nervous system [1]. The regulatory role of thiochrome (thiamine catabolite) in the activity of a number of NAD-dependent enzymes [2-5], pyruvate dehydrogenase complex [4] and some proteolytic enzymes were demonstrated in our previous studies [5].

According to the recent findings, thiamine itself and the products of its oxidation and decomposition in the body can affect the activity of many enzymes. So, it is known that thiamine inhibits human saliva amylase at millimolar concentrations[6]. The carbohydrate absorption process depends on the thiazole moiety of thiamine. Thus, in the study of the effect of thiamine and its phosphates on the activity of the purified preparation of succinate dehydrogenase, it was established that thiamine, TMP, and TPF activate this enzyme [4]. In our laboratory the regulatory role of thiamine and its metabolites in the regulation of the activity of tissue and purified alcohol dehydrogenase and lactate dehydrogenase was investigated. In these experiments, it was shown that only thiochrome among all thiamine metabolites, is able to inhibit the activity of both studied enzymes effectively [4, 7]. It is important to note that the cytoplasmic arrangement of these enzymes coincides with that for thiochrome, and its effective

***Corresponding author:** Sergiy Anatoliyovich Petrov, doctor of biological sciences, professor of Odesa National Mechnykov I. I. University, Department of Biochemistry, 2, Dvoryanska Str., Odesa, 65082, Ukraine.

concentrations are actually present in the cytoplasm after loading with thiamine. These results gave us reason to believe that the thiamine metabolite, thiochrome, can act as an inhibitor of some NAD-dependent dehydrogenases [4]. The study of the binding of thiamine and its metabolites with the purified alcohol dehydrogenase preparation in the presence of various concentrations of NAD showed that thiochrome binds to the enzyme 4-5 times stronger than other metabolites of thiamine. In the presence of NAD in an environment of equal or even superior doses to thiamine and its metabolites, the binding of thiochrome remained fairly high. In these studies, it was shown that thiochrome is able to inhibit the activity of alcohol dehydrogenase even at concentrations three times lower than that of NAD, and only a six-fold excess of the concentration of NAD compared to thiochrome removed the inhibitory effect of the latter. The results suggest that inhibition of thiochrome alcohol dehydrogenase is competitive with NAD. Since thiochrome is a compound with pronounced hydrophobic properties, it can be assumed that when it joins the “hydrophobic pocket” of the active center of ADH, it prevents the addition of NAD. This assumption is supported by the same type of shifts to the shortwave region of the NAD and thiochrome absorption maxima when they are added to alcohol dehydrogenase, which we found in special studies [4]. Data on the participation of thiamine in the regulation of RNA synthesis have been obtained [8]. Thanks to these studies, it was found that this effect can be achieved by binding vitamin B1 to certain motifs of DNA, which affects DNA-dependent RNA polymerase [9]. The direct involvement of thiamine in the synthesis of RNA in tumor cells is also shown. In this case, this vitamin should have a potentiating effect on the transcription process [4].

The stimulating role of thiochrome in the reproduction of a number of invertebrate organisms [10] and its effect on the level of DNA and RNA in white rat tissues [7] are shown in our previous studies. The purpose of this work was to study the possibility of the thiochrome effect on the activity of DNA polymerase.

2. Material and Methods

2.1 Method of DNA polymerase isolation

Isolation of DNA polymerase was carried out using the method of isolation of Taq-polymerase [11].

The liver of a rat was homogenized and precipitated by centrifugation at 6000g per 30 minutes. The pellet was resuspended in 100 ml TE-buffer (10 mm Tris-HCl, pH 8.0, 1 mM EDTA), centrifuged for 6000g per 10 minutes. The resulting precipitate was stored at -20°C. To destroy the cells, 3g of the obtained liver pellet was resuspended in 30 ml Tris-HCl buffer with the addition of the necessary components (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1.25 mM PMSF, 2 mg/ml lysozyme). After 15 minutes incubation at 20°C, the cells were destroyed in the ultrasonic disintegrator UZDN-A ("sounding", 1 minute with interruptions) in theice bath. This procedure was carried out until the optical density was reduced by a factor of ten at a wavelength of 590 nm. To denature the proteins to the suspension with constant stirring and 4°C, ammonium sulfate was added to the crusts to a concentration of 0.2M.

The suspension of the destroyed cells was centrifuged for 10000g per 1 hour on a Beckman LS-75 centrifuge (T35, Beckman rotor, USA). To denature the proteins, the supernatant was heated at 75°C for 30 minutes. Then polyimine P was added to the solution with constant stirring to a concentration of 0.6% at 4°C. The solution was incubated for 2 hours on ice, and then centrifuged for 10000 gper 1 hour. The supernatant was applied to the column with 6 mL of phenyl sepharose, equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 0.2 M ammonium sulfate. The column was washed with bufferA containing 20% glycerol (wt/vol.). The bound proteins elution was carried out with 100 ml of a linear gradient of urea concentration (0 – 4 M) on buffer A at a rate of 10 ml/hr, 5 mlfractions were collected. The optical elution profile was determined from the absorbance at 280 nm on a spectrophotometer. Fractions containing the DNA polymerase were pooled and dialyzed overnight against buffer B (100 mM KCl, 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.2% Tween 20). The dialyzed preparation was applied to the column with heparin sepharose equilibrated with buffer B. Elution was carried out with 60 ml of a gradient of 100-700 mM KC1 in buffer B at a rate of 10 ml/hr. Fractions (3 ml)were collected. Fractions with DNA polymerase were pooled and dialyzed against buffer for preservation (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2% Tween 20, 50% glycerol). Dialysis preparation stored at -20°C.

2.2 Determination of the DNA polymerase activity

Determination of DNA-dependent DNA polymerase activity was carried out in A_1 and A_2 systems.

System A_1: 3 mM $MgCl_2$, 0.07 M KCl, 0.05 M tris-HCl buffer (pH = 8.5), 2mM dithiothreitol, 40 mg of bovine serum albumin, 10 μg of active DNA and 100 μM of each of the four deoxyribonucleotides (dNTP).

System A_2: 8 mM $MgCl_2$, 0.12 M KCl, 0.05 M Tris-HCl buffer (pH = 8.5), 2 mM dithiothreitol, 40 μg bovine serum albumin, 10 μg of active DNA, and 100 μM of each of the four deoxyribonucleotides (dNTP).

0.1 ml of DNA polymerase was added to each system, the control groups were incubated for 30 minutes at 37°C. During the pre-incubation tests, thiochrome was added with concentrations: 1 nM, 10 nM, 100 nM, 1 μM.

Then, the reaction was stopped by the addition of 40 μl of the mixture consisting of 0.2 M EDTA and a saturated solution of $Na_4P_2O_7$ (1:1). 1 ml of 30% TCA was added to each system in which 1/10 volume of $Na_4P_2O_7$ was dissolved. It was centrifuged for 3000g per 10 minutes. The

supernatant and sediment were separated.

The pellet was resuspended in the same volume as the supernatant, centrifuged for 3000g per 10 minutes, and the precipitates were determinedfor the DNA content

2.3 Determination of the binding rate of thiochrome to DNA polymerase

Determination of the thiochrome binding rate to DNA polymerase was carried out in two systems.

System A_1: 3 mM $MgCl_2$, 0.07 M KCl, 0.05 M Tris-HCl buffer (pH = 8.5), 2 mM dithiothreitol, 40 mg of bovine serum albumin, and 100 mM each of the four deoxyribonucleotides (dNTP).

System A_2: 8 mM $MgCl_2$, 0.12 M KCl, 0.05 M Tris-HCl-buffer (pH = 8.5), 2 mM dithiothreitol, 40 μg bovine serum albumin, and 100 μM each of four deoxyribonucleotides (dNTP).

DNA polymerase was added in a concentration of 100 μg/ml and thiochrome in a concentration of 1 μm. Then inhibitors were introduced in the test samples: ether, urea, formaldehyde, lead chloride in concentrations of 1, 2, 5, 10 μm ($PbCl_2$ for blocking sulfhydryl groups, formaldehyde for blocking amino groups, diethyl ether for blocking the carboxylgroups, urea for partial destruction of hydrophobic and hydrogen bonds). The samples were incubated in a thermostat at 30°C for 30 minutes, then the samples were centrifuged at 20000g per 30 minutes, the supernatants were merged. The precipitates were washed twice with incubation medium and the thiochrome content was determined by the fluorimetric method.

All obtained data were processed statistically. All data were analyzed by statistical data processing using the non-parametric parameter Mann-Whitney [12]. Statistical significance was considered to be $p \leq 0.05$[13].

The bulk of used reagents meet the high purity requirements (Reanal, Fluka, Sigma, Orion, Beloris), others are Reahim production, with a characteristic of not less than chemically pure, which, if necessary, are further refined.

3. Results

In our work, DNA polymerase of rat Rattus norvegicus was used.

Inour previous studies [5, 14, 15] it was shown that thiochrome is a thiamine metabolite that responsible for activation of the process of reproduction in the mammals and some invertebrates tissues. To find out the mechanism of this action of thiochrome we studied the effect of this metabolite on the activity of DNA polymerase isolated from the liver of white rats. The results of these studies showed (Figure 1) that thiochrome even at a concentration of 1 nm is able to activate DNA polymerase in the A_2 system, and at a concentration of 10 nm it activates the enzyme in both systems. The greatest effect was observed at a concentration of 1 μm.t

Next it was necessary to establish which groups in the DNA polymerase molecule participate in interaction with thiochrome and are responsible for the activation of this enzyme. To that case we used the inhibitor analysis method, in which the following compounds were added to pre-incubation medium.To block the sulfhydryl groups of the enzyme, we used $PbCl_2$, formaldehyde blocked the amino groups, diethyl ether blocked the carboxyl groups, urea partially destroyed the hydrophobic and hydrogen bonds.

These inhibitors have been used in micromolar concentrations in order to avoid a nonspecific effect on the enzyme (Table 1).

The data in Table 1 indicate that when the $PbCl_2$ inhibitor was introduced into the incubation medium, the activity of the DNA polymerase decreased significantly in the A_1 system. At the same time, the introduction of only

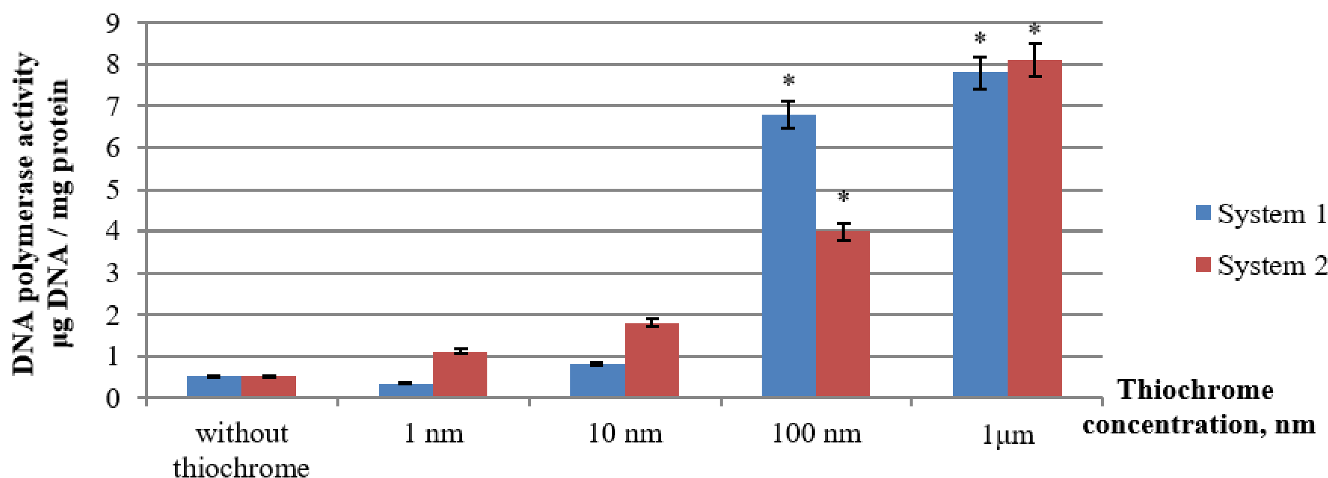

Figure 1 | The DNA polymerase activity in the presence of various thiochrome concentrations (μg DNA / mg protein)(n=8) *p<0.05 in comparison with control. ДНК-полимеразная активность в присутствии различных концентраций тиохрома (мкг ДНК / мг белка) (n = 8) * p<0,05 по сравнению с контролем

Table 1 | The activity of DNA polymerase in the presence of thiochrome and functional groups blockers (μgt hiochrome /mg protein) (n=8).* - p=0.05 in comparison with control. ** - p=0.05 in comparison with thiochrome. Активность ДНК-полимеразы в присутствии тиохрома и функциональных групп (мкг тиохрома / мг белка) (n = 8) * - p = 0,05 по сравнению с контролем. ** - p = 0,05 по сравнению с тиохромом.

$PbCl_2$							
	Control group	$PbCl_2$ 1 μm	Thiochrome 1 μm	$PbCl_2$+thiochrome			
				1 μm $PbCl_2$	2 μm $PbCl_2$	5 μm $PbCl_2$	10 μm $PbCl_2$
SystemA_1	0,065	$0,038^{*}$	$0,115^{*}$	$0,100^{*}$	$0,110^{*}$	$0,096^{*}$	$0,110^{*}$
SystemA_2	0,070	0,041	$0,110^{*}$	$0,095^{*,**}$	$0,105^{*}$	$0,100^{*}$	$0,110^{*}$
Formaldehyde							
	Control group	Formaldehyde 1 μm	Thiochrome 1 μm	Formaldehyde +thiochrome			
				1 μm formaldehyde	2 μm formaldehyde	5 μm formaldehyde	10 μm formaldehyde
SystemA_1	0,065	0,085	$0,115^{*}$	$0,110^{*}$	$0,100^{*}$	$0,111^{*}$	$0,118^{*}$
SystemA_2	0,070	$0,041^{*}$	$0,110^{*}$	$0,120^{*}$	$0,116^{*}$	0,128	$0,118^{*}$
Ether							
	Control group	Either 1 μm	Thiochrome 1 μm	Ether +thiochrome			
				1 μm ether	2 μm ether	5 μm ether	10 μm ether
SystemA_1	0,065	$0,030^{*}$	$0,115^{*}$	$0,048^{*,**}$	$0,042^{*,**}$	$0,033^{*,**}$	$0,030^{*,**}$
SystemA_2	0,070	$0,032^{*}$	$0,110^{*}$	$0,05^{**}$	$0,044^{*,**}$	$0,039^{**}$	$0,039^{**}$
Urea							
	Control group	Urea 1 μm	Thiochrome 1 μm	Urea +thiochrome			
				1 μm urea	2 μm urea	5 μm urea	10 μm urea
SystemA_1	0,065	$0,040^{*}$	$0,115^{*}$	$0,055^{*,**}$	$0,050^{*,**}$	$0,048^{*,**}$	$0,033^{*,**}$
SystemA_2	0,070	0,041	$0,110^{*}$	$0,058^{*,**}$	$0,054^{*,**}$	$0,049^{*,**}$	$0,032^{*,**}$

thiochrome led to the activation of this enzyme in both systems. The most interesting results were obtained when $PbCl_2$ was introduced into the incubation medium together with thiochrome. This inhibitor in concentrations from 1 μm to 10 μm did not reduce the activating effect of thiochrome, except at the concentration of 1 μm in system A_2.

When using formaldehyde, it was found that this inhibitor reduced the activity of DNA polymerase only in the A_2 system. The introduction of only thiochrome into the incubation medium increased the activity of the enzyme in both systems. The combined use of formaldehyde and thiochrome did not reduce the activating effect of thiochrome.

The addition of diethyl ether to the incubation medium as well as in the previous cases led to the decrease in activity of the enzyme, and the addition of thiochrome led to its activation. However, unlike previous inhibitors, diethyl ether inhibited the activity of DNA polymerase even in the presence of thiochrome.

A similar pattern was observed when using urea, which reduced the activity of the enzyme under study. The introduction of thiochrome into the incubation medium did not prevent this effect.

Proceeding from the data given in the table, it should be noted that both $PbCl_2$ and formaldehyde at concentrations from 1 to 10 μm did not decrease the activating effect of thiochrome on DNA polymerase. Diethyl ether and urea already at a concentration of 1 μm removed the activation effect of thiochrome on DNA polymerase. Higher concentrations of these two compounds exerted a greater inhibitory effect. Thus, these data indicate the role of carboxyl groups of the enzyme, hydrophobic and hydrogen bonds in the realization of the activating effect of thiochrome on this enzyme. To clarify the obtained data, we studied the binding of thiochrome to DNA polymerase in the presence of the above inhibitors at the same concentrations.

So, from Table 2 it can be seen that diethyl ether caused a tendency to decrease for the registered parameters already at its introduction in the concentration of 1 and 2 μm in comparison with the control. Higher concentrations, such as 5 and 10 μm, caused a significant decrease in these parameters by an average of 40.1% in the A_1 and A_2 systems at 5 μm, and at 10 μm this average was lower by 49.8% in both systems compared with the control option.

It is noted that when added to the medium, urea at all concentrations is capable of reliable reduction of the binding of thiochrome to the DNA polymerase. Thus, from Table 2 it can be seen that even with the introduction of urea at a concentration of 1 μm, the recorded value on average in the two systems was reduced by 39.2% with respect to the control, and at urea concentration of 2 μm, the value was on average 53.4% lower than in the control version. Introduction of the investigated blocker into the medium at a concentration of 5 μm led to the decrease in the indices in

Table 2 | Thiochrome binding to DNA polymerase in the presence of different functional groups blockers (μg thiochrome/mg protein) (n=8). * - p=0.05 in comparison with control. Связывание тиохрома с ДНК-полимеразой в присутствии блокаторов различных функциональных групп (мкг тиохрома / мг белка) (n = 8). * - p = 0,05 по сравнению с контролем).

System	Control	Ether			
		1 μm	2 μm	5 μm	10 μm
A_1	0,90	0,70	0,65	0,58*	0,32*
A_2	0,94	0,74	0,69	0,52*	0,61*
System	Control	Urea			
		1 μm	2 μm	5 μm	10 μm
A_1	0,90	0,54*	0,44*	0,32*	0,21*
A_2	0,94	0,58*	0,42*	0,29*	0,19*
System	Control	$PbCl_2$			
		1 μm	2 μm	5 μm	10 μm
A_1	0,90	0,99	0,85	0,86	0,78
A_2	0,94	0,93	0,87	0,83	0,85
System	Control	Formaldehyde			
		1 μm	2 μm	5 μm	10 μm
A_1	0,90	1,11	0,94	0,88	0,83
A_2	0,94	0,92	0,99	1,02	0,95

systems A1 and A2 below the control by 66.8% on average. When urea was introduced at a concentration of 10 μm, a similar pattern was observed. So, in systems A_1 and A_2, on average, the binding intensity decreased by 78.3% in relation to the control.

Table 2 shows the data on the binding of thiochrome to DNA polymerase. After the analysis of data, it was determined that their concentrations fully correspond to the content of thiochrome in animal tissues [4].

4. Concluding Remarks

It can be assumed that the binding of thiochrome to certain sites of DNA polymerase is carried out using hydrophobic interactions.

In this way, probably, thiochrome is capable of activating the DNA polymerase through interaction with specific sites of the enzyme containing amino acids with hydrophobic radicals.

Заключение

Можно предположить, что связывание тиохрома с определёнными сайтами ДНК-полимеразы осуществляется с использованием гидрофобных взаимодействий.

Таким образом, вероятно, тиохром способен активировать ДНК-полимеразу путем взаимодействия с конкретными сайтами фермента, содержащего аминокислоты с гидрофобными радикалами.

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