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TRYPTOPHANYL-tRNA SYNTHETASE IS FOUND CLOSELY ASSOCIATED WITH AND STIMULATES DNA POLYMERASE α-LIKE ACTIVITY FROM WHEAT EMBRYOS.

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SUMMARY: DNA polymerase α-like from wheat embryos is found to purify closely associated with a tryptophanyl-tRNA synthetase activity. No other aminoacyl-tRNA synthetases were present. A purified preparation of wheat tryptophanyl-tRNA synthetase free of polymerase activity was able to stimulate plant DNA polymerase of the α-like type, while the γ-like polymerase from wheat embryos was not affected by the enzyme. We have not been able to find a diadenosine 5', 5'''-P^1,P^4-tetraphosphate binding activity associated to the polymerase-synthetase complex. We have also observed a specific inhibition by beef $tRNA^{Trp}$ of DNA polymerase α-like activity, while other tRNAs will not change the enzyme activity.

INTRODUCTION: The presence of multiple DNA polymerases (DNA-directed DNA polymerase EC 2.7.7.7.) is a characteristic of both prokaryotic and eukaryotic cells (1, 2, 3). In the case of animal cells three DNA polymerases have been described: DNA polymerase α involved in nuclear DNA replication (4); DNA polymerase β most probably involved in DNA repair synthesis (5) and DNA polymerase γ, the enzyme that replicates mitochondrial DNA (6). In the case of other eukaryotes the presence of DNA polymerases of the α type has been described in lower eukaryotes, as well as in higher plants (7,8). No DNA polymerase of the β type has been found in higher plants, but a DNA polymerase very similar to the γ enzyme was found in wheat embryos soluble cytoplasm (9) and spinach chloroplasts (8).

Recently, it was shown that the high molecular weight form of HeLa cells DNA polymerase α contained tryptophanyl-tRNA synthetase activity, as well as a diadenosine 5', 5'''-P^1,P^4-tetraphosphate (Ap_4A) binding activity (10). Some aminoacyl-tRNA synthetases are able to synthesize this analog (11). Ap_4A has been found to be a positive growth signal in animal cell cultures (12).

We want to report in this article that one of the two forms of DNA polymerase α-like we have purified from wheat embryos (previously called B and C) (7) copurifies in close association with wheat embryo tryptophanyl-tRNA synthetase. The enzyme activity of both DNA polymerases α-like is strongly stimulated by the synthetase.

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THE NATURE AND REACTIVITY OF THE 'ESSENTIAL' THIOL IN RABBIT MUSCLE CREATINE KINASE III (EC 2.7.3.2)

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Summary: Rabbit muscle creatine kinase III (EC 2.7.3.2) can be reacted with 2-chloromercuri-4-nitrophenol and this results in the incorporation of two moles of mercurial per mole of enzyme subunit in a biphasic reaction. The second-order rate constant for the slow reaction is 475 ± 42 M^{-1} s^{-1}. S-Carboxamidomethyl-creatine kinase reacts with a single mole of mercurial per mole of subunit. The rate constant, 466 ± 57 M^{-1} s^{-1}, is almost identical to that for the slow reaction of the native enzyme. The reaction between 3-carboxy-4-nitrophenylthio-creatine kinase and 2-chloromercuri-4-nitrophenol has a second-order rate constant of 449 ± 56 M^{-1} s^{-1}. The results may be explained if the mercurial reacts very rapidly with that cysteine residue which reacts independently with iodoacetamide or 5,5'-dithiobis(2-nitrobenzoic acid). However, 2-chloromercuri-4-nitrophenol also reacts more slowly with a second cysteine residue. Definition of the essentiality of thiol groups in enzymes by reaction with *labile ligands*, here represented by organomercurials, clearly must be approached with caution.

The interpretation of experiments involving the reaction of rabbit muscle creatine kinase III (EC 2.7.3.2) with thiol-specific reagents has remained a problem for many years. Many inhibitors have been used, some apparently leading to complete inhibition (1-11), others to at least partial retention of activity (12-15).

There has been considerable uncertainty regarding the location of the substituent after reaction. In some experiments, a thiol distinct from the 'essential' thiol is reported to have been modified (14-16). Little attention has been paid to the possibility of exchange and/or displacement of modifying groups before and during assays for residual activity.

Der Terrossian and Kassab (12) postulated the presence of two thiol groups in such close proximity that modification of the first by anything bulkier than cyanide sterically prevented modification of the second thiol. In this Communication, we report results which discount this proposition and explain the reactions of two (presumably juxtaposed) thiols at the active site of the enzyme with a variety of thiol-specific reagents.

Experimental Section

Rabbit muscle creatine kinase III from two sources was used. Enzyme purchased from Boehringer Mannheim had a maximum specific activity of 1,934 μkat/g. Later, enzyme was isolated according to Noda *et al.* (17). The recrystallisation procedure was not used. Fresh enzyme had a specific activity of 2,100 μkat/g, but this decreased on storage. Enzyme of specific activity 1,800 – 2,000 μkat/g was used for the experiments below.

Enzyme activities were measured using a Radiometer pH Stat assembly at pH 8.8 and 30°C. The reaction mixture contained 40 mM creatine, 4 mM ATP, 5 mM magnesium acetate, 0.1 mM dithiothreitol and 0.05 mM EDTA. In situations where dithiothreitol would cause unwanted reactivation of the enzyme, 0.1 mM EDTA was used instead.

Spectrophotometric titration of fresh enzyme with DTNB indicates that the maximum specific activity for rabbit muscle creatine kinase III is ~2,300 μkat/g, a result consistent with a number of previous reports (3, 18-20).

Stock solutions of enzyme with fully reduced cysteine residues were prepared by dialysis against oxygen-free 0.1 M Bicine-NaOH buffer, pH 8.01 (1 mM each in EDTA and dithiothreitol). This was followed by exhaustive dialysis under oxygen-free conditions against the same buffer without dithiothreitol. The concentration of reactive thiols was determined by titration with DTNB.

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Table I. Characterization of wheat DNA polymerases.

Conditions	% Inhibition		
	Wheat DNA polymerase		
	α^1-like	α^2-like	γ-like
Aphidicoline (50 µg/ml)	87	93	4
ddTTP(ratio ddTTP/TTP=20)	2	0	78
N-ethylmaleimide (10 mM)	58	62	70
Ethidium bromide (10 µM)	15	10	71
Template poly A-dT_{12}	0§	0§	2534§

The concentration of inhibitors used are indicated in brackets. Incubation was carried out with activated DNA for 15 min at 37°C. The incubation with aphidicolin was done in the presence of 10 µM dCTP. More details in Methods.
§ = pmoles of TMP incorporated when the DNA polymerase γ assay was used.

The use of selective inhibitors of animal DNA polymerases have been found to be very important for the characterization of these enzymes (1, 3, 17). In Table I it can be seen that wheat DNA polymerases α^1 and α^2-like, strongly ressemble DNA polymerase α, as judged by the strong inhibition by aphidicolin and NEM, the lack of effect of ddTTP, the low level of inhibition by ethidium bromide and the lack of activity of these enzymes in the presence of a template poly A-oligo dT. DNA polymerase γ-like from wheat embryos is very similar to the animal γ polymerase in its resistance to aphidicolin, the strong inhibition by ddTTP and ethidium bromide, and the fact that poly A-oligo dT is by far the best template for this enzyme. It is interesting, however, to point out that the DNA polymerase purified from wheat mitochondria is not a γ-like DNA polymerase (18). It remains to be established whether the γ-like polymerase we have purified is related to the chloroplastic DNA polymerase described recently (8).

The search of aminoacyl-tRNA synthetases in partially purified preparations of DNA polymerase gave the results shown in Table II. The assay was done prior to DEAE-cellulose chromatography since the three DNA polymerases are still unresolved. As seen in this Table the most important activity corresponded to tryptophanyl-tRNA synthetase (Table II A). In Table II B are shown the tryptophanyl-tRNA synthetase activity of DNA polymerase α^1-like, α^2-like and γ-like after the DNA-cellulose chromatographic step. Only DNA polymerases α-like gave an important synthetase activity. Not shown are the negative results concerning the activities of aspartyl, seryl and valyl tRNA-synthetases that were completely absent in the highly purified preparations.

The strong association between DNA polymerase α^2-like and tryptophanyl-tRNA synthetase is shown in Figure 1. It can be seen that tryptophanyl-tRNA synthetase, freed of polymerase activity, elutes at a very different position than the complex between the polymerase and the synthetase. We have failed to

Table II. Aminoacyl-tRNA synthetase activity associated with wheat DNA polymerases.

A

aminoacid	Aminoacylation (pmoles/A^{260nm} unit of tRNA)	
	Crude aminoacyl-tRNA synthetases(wheat germ)	aminoacyl-tRNA synthetase associated to wheat DNA polymerases
Alanine	19	0
Arginine	51	0
Asparagine	104	0
Aspartic acid	46	7
Cysteine	57	0
Glutamine	39	0
Glutamic acid	39	0
Glycine	43	0
Histidine	30	0
Isoleucine	62	0
Leucine	118	0
Lysine	94	0
Methionine	65	0
Phenylalanine	52	0
Proline	36	4
Serine	91	0
Threonine	67	0
Tryptophan	45	45
Tyrosine	34	0
Valine	132	0

B

Wheat DNA polymerase	pmoles Trp/A^{260} unit of tRNA
α^1-like	9
α^2-like	43
γ-like	0

Table II A: Activity was assayed after the first phosphocellulose column. Incubation was carried out for 20 min at 37°C (plateau) using about 400 µg protein per assay.

Table II B: Incubation (5 min at 37°C) was carried out with each DNA polymerase after the DNA-cellulose step. About 10 µg of enzyme protein was used. Other details are described in Methods.

show the complex by Sephadex G-200 chromatography or sucrose gradient, because plant polymerases are extremely fragile to dilution, and a very low yield in activity was recovered after Sephadex filtration or ultracentrifugation on sucrose or glycerol gradients. Both enzyme activities can be partially separated on a phosphocellulose column. It is possible to elute a peak of synthetase activity completely free of polymerase and a peak of DNA polymerase α^2-like activity with very low levels of synthetase associated (not shown).

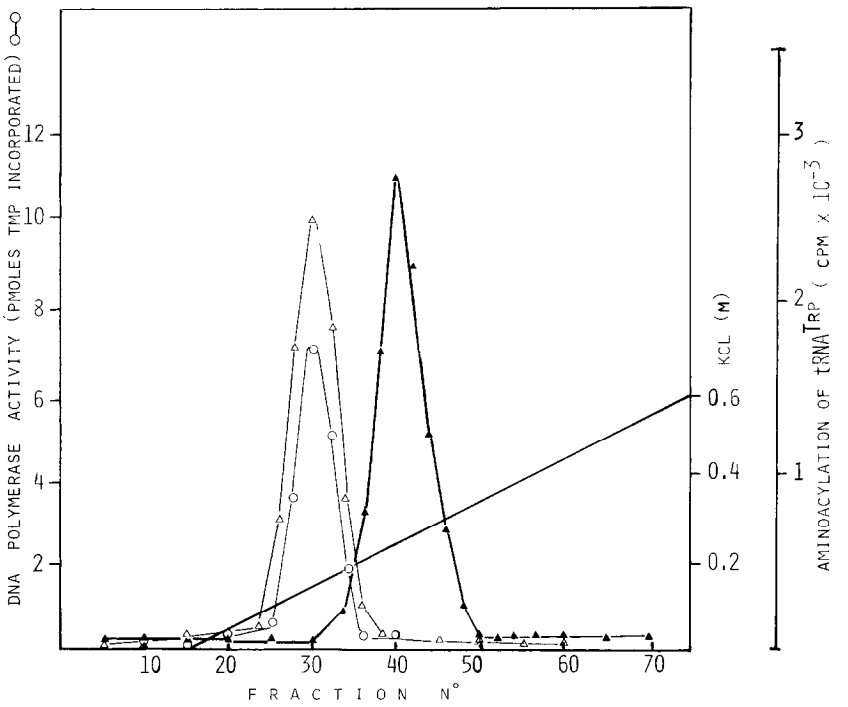

Figure 1. DNA-cellulose chromatography of tryptophanyl-tRNA synthetase and the complex DNA polymerase α^2-like - tryptophanyl-tRNA synthetase. Pooled fractions with DNA polymerase activity from the hydroxyapatite step were absorbed in a DNA-cellulose column and eluted with a gradient from 10 to 600 mM KCl. Incubation assays were done with 5 µl aliquots for 5 min at 37°C (synthetase assay) and for 15 min at 37°C (DNA polymerase assay). A phosphocellulose fraction of tryptophanyl-tRNA synthetase free of DNA polymerase activity was used. (○) DNA polymerase activity in the polymerase-synthetase complex. (△) Tryptophanyl-tRNA synthetase activity in the polymerase-synthetase complex. (▲) Tryptophanyl-tRNA synthetase activity in the polymerase-free synthetase fraction. The result shown in this Figure correspond to two separate columns, one with synthetase alone and the other with the polymerase-synthetase complex.

When highly purified wheat tryptophanyl-tRNA synthetase was added to DNA polymerase α^2-like, a very strong stimulation of DNA polymerase activity was obtained as seen in Figure 2 A. The activity of this polymerase was not affected by bovine serum albumin or phenyl-tRNA synthetase. On the contrary, the activity of DNA polymerase γ-like, as seen in Figure 2 B, is not changed by synthetases or bovine serum albumin. In Table III it can be seen that the stimulation of DNA polymerase α^2-like is also observed with a tryptophanyl-tRNA synthetase from beef pancreas, but the degree of stimulation is lower than with the wheat synthetase. An interesting result was observed with $tRNA^{Trp}$ which is shown to be a powerful inhibitor of the polymerase activity, while beef $tRNA^{Gly}$, $tRNA^{Val}$ or $tRNA^{Met}$ did not affect the activity of the α^2-like enzyme. As we have not been able to obtain a polymerase preparation absolutely free of tryptophanyl-tRNA synthetase we do not know at present if the inhibitory effect of $tRNA^{Trp}$ is related to the presence of a synthetase associated with the polymerase.

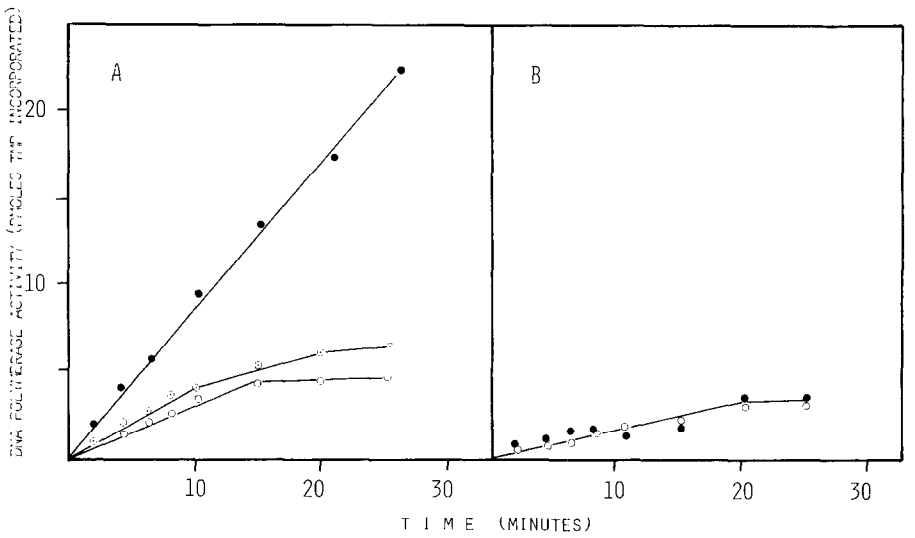

Figure 2. Effect of aminoacyl-tRNA synthetases on wheat DNA polymerase activity.
Figure 2 A: (○) DNA polymerase α^2-like (10 µg).
(●) Plus wheat tryptophanyl-tRNA synthetase (15 µg)
(⊙) Plus wheat phenylalanyl-tRNA synthetase (15 µg)
Figure 2 B: (○) DNA polymerase γ-like assay (10 µg)
(●) Plus wheat tryptophanyl-tRNA synthetase (15 µg)
The incubation at 37°C was as described under Methods. The tryptophanyl-tRNA synthetase used was as in Fig. 1; the DNA polymerase was a DNA-cellulose fraction and the phenylalanyl-tRNA synthetase was purified as described by Carias (16).

Our results clearly show that the strong affinity of DNA polymerase α and tryptophanyl-tRNA synthetase showed previously in animal cells (10) is not an isolated phenomenon and can be also found in a distant eukaryote, the wheat embryo. However, we have not been able to find an Ap_4A binding protein associated

Table III. DNA polymerase activity in the presence of aminoacyl-tRNA synthetases or tRNAs.

Conditions	*% of control activity*
DNA polymerase α^2-like alone	*100*
Plus TRS^{Wheat}	*650*
Plus TRS^{Bovine}	*327*
Plus $tRNA^{Trp}_{bovine}$	*22*
Plus $tRNA^{Val}_{bovine}$	*89*
Plus $tRNA^{Gly}_{bovine}$	*100*
Plus TRS^{Wheat} plus ATP (1 mM) plus Trp (50 µM)	*600*

The amount of DNA polymerase α^2-like used was 10 µg. Optimal stimulation was obtained with 15 µg of wheat tryptophanyl-tRNA synthetase and 2 µg of beef pancreas tryptophanyl-tRNA synthetase. The amount of tRNA used was 2 µM. Incubation was carried out with activated DNA for 30 min at 37°C.

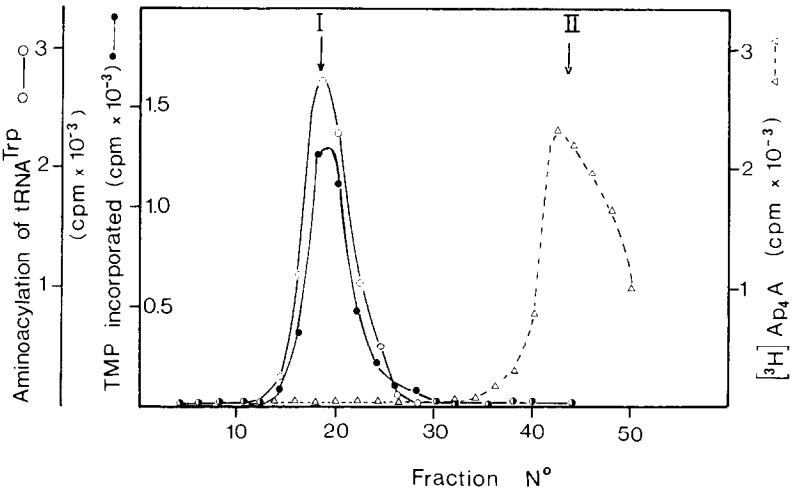

Figure 3. Search for an Ap_4A binding protein by gel filtration chromatography. A Sephadex G-75 column was equilibrated with a buffer 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM 2-mercaptoethanol and 20% glycerol. DNA polymerase α^2-like (about 50 µg dialyzed against the buffer described above) was submitted to gel filtration. DNA polymerase and tryptophanyl-tRNA synthetase were assayed as in Methods. 1 µM $[^3H]Ap_4A$ (specific activity = 450 cpm/pmole) was mixed with the polymerase or chromatographed alone. The radioactivity of $[^3H]Ap_4A$ was determined by blowing 50 µl of each fraction on glass fiber filters GF-C. The filters were dried and counted in a toluene, PPO, POPOP scintillation mixture. The arrow I indicates the void volume and arrow II indicates the elution of Ap_4A alone.

to the polymerase-synthetase complex. For this purpose we have used several techniques. As seen in Figure 3 no Ap_4A binding protein can be detected by gel chromatography. Similar negative results were obtained by using nitrocellulose binding, charcoal adsorption and glycerol gradient centrifugation (not shown). Moreover, plant DNA polymerases are unable to use Ap_4A as primer for DNA synthesis under the same conditions that animal DNA polymerase initiates DNA synthesis with this dinucleotide (19).

The presence of a protein able to interact specifically with tRNA in a nucleic acid biosynthesis system has been already described. In the case of the Qβ replicase the host protein synthesis elongation factors, EF-Tu and EF-Ts are subunits of the multimeric replicase (20). We postulated some time ago that the tRNA-like structure found at the 3' end of some plant viral RNA genomes (21) may be related to the presence of tRNA specific factors present in the plant viral replicase (22). Another example concerns the DNA polymerase from retrovirus (reverse transcriptase). This enzyme uses tRNA as primer for DNA synthesis over RNA templates (23). A thorough study of the interaction between an avian reverse transcriptase and its specific primer, $tRNA^{Trp}$, allowed us to describe the regions of primer tRNA recognized by the polymerase and the possible involvement of reverse transcriptase in positioning the primer on the viral genome (24, 25, 26). More recently, it has been found that E. coli alanyl-tRNA synthetase can

bind to a specific DNA sequence and regulates the transcription of its own gene (27). Similarly, it has been found that the transcription of Xenopus laevis $tRNA^{Leu}$ is controlled by split promoters located in the DNA sequence in the regions coding for the TΨ and D regions of tRNA (28). Thus, it is possible that protein factors able to recognize some tRNA structural features are involved in the transcription complex. More experimental evidences are needed to confirm a general role played by tRNA and (or) tRNA-specific proteins in the process of nucleic acid biosynthesis.

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