The reactivity of sulfhydryl groups of yeast DNA dependent RNA polymerase I

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ABSTRACT. The number of reactive cysteine residues of yeast RNA polymerase I was determined and their function was studied using parachloromercury benzoate (pCMB), dithiobisnitrobenzoate (DTNB) and N-ethyl-maleimide (NEM) as modifying agents. By treatment with DTNB about 45 sulfhydryl groups react in the presence of 8M urea. Under non-denaturing conditions only 20 sulfhydryl groups are reactive with pCMB and DTNB. Both reagents completely inactivate the enzyme and this effect can be reversed by reducing agents. The sedimentation coefficient and the subunit composition are not affected when the enzyme is inactivated. Two of the most reactive sulfhydryl groups are necessary for activity. The modification of these groups is partially protected by substrates and DNA, suggesting that they are involved either in catalysis or in the maintenance of the conformation of the active site. Experiments with ^{14}C -NEM indicate that the most reactive groups are located in subunits of 185,000, 137,000 and 41,000 daltons.

INTRODUCTION

Eucaryotic RNA polymerases have been purified from a number of tissues and proved to be complex multimeric enzymes. However, very little is known about the nature of the amino acid residues involved in the action of the enzymes and their role in the various steps of the transcription process. The identification of these residues is therefore of great interest. We have purified yeast RNA polymerase I to homogeneity and determined its subunit composition (1). In addition, we have shown that this enzyme is rapidly inhibited by pyridoxal 5'-phosphate through the formation of a Schiff base with 3-4 lysyl amino groups presumably located at the active site (2). These lysyl residues were later found to be located mainly in the two largest subunits of the enzyme (3).

The availability of pure yeast RNA polymerase I allows us to study the function of other groups. Throughout the purification steps we have noticed that the stability and activity of the enzyme are strongly dependent on the presence of reducing agents such as dithiothreitol or 2-mercaptoethanol. Therefore we decided to study the function of cysteine

residues using the specific modifying agents parachloromercuribenzoate (pCMB) (4,5), dithiobisnitrobenzoate (DTNB) (6,7) and N-ethyl-maleimide (NEM) (5), which react rapidly and stoichiometrically under mild conditions with SH groups. Cysteine residues may exhibit a considerable variation in their reactivity which can be attributed to their location in the tridimensional structure of the protein. We report here that yeast RNA polymerase I has 45 SH groups, 20 of which react under non-denaturing conditions. We have determined that two of the most reactive SH groups are essential for enzymatic activity and are presumably located at the active site.

EXPERIMENTAL PROCEDURES

Materials

^3H -UTP (20 Ci/mmol) was obtained from New England Nuclear, Boston, Mass., N-ethyl (2,3- ^{14}C) maleimide (15 mCi/mmol), from Amersham-U.K. and ^{14}C -pCMB (10.4 Ci/mol) from Schwarz/Mann, Orangeburg, N.Y. Calf thymus DNA, bovine serum albumin, sodium dodecyl sulfate, 2-mercaptoethanol and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co., St. Louis, Missouri. Dithiothreitol and pCMB were from Calbiochem, La Jolla, Ca and DTNB from Aldrich Chemical Co., Milwaukee, Wis. Nucleotides were from P-L Biochemicals, Milwaukee, Wis. Nitrocellulose filters HAWP 24 (0.45 μ) were from Millipore Corp, Bedford, Mass. *S. cerevisiae* yeast cells were the kind gift of Lefersa, Santiago, Chile.

Enzyme Purification and Assay

DNA-dependent RNA polymerase I from yeast was purified by the method of Valenzuela et al. (1). The purification was carried out through the step of sucrose gradient. The enzyme preparation has a specific activity of 300 units per mg of protein using calf thymus DNA as a template. Units are defined as indicated previously (1). The enzyme is homogeneous as judged by gel electrophoresis in 0.1% SDS. Protein concentration was determined after precipitation with 10% trichloroacetic acid by the method of Lowry et al. (8). RNA polymerase was assayed as described previously (1-3) by measuring the incorporation of labeled nucleoside triphosphates into RNA. One unit of activity corresponds to the incorporation of 1 nmol of UMP into RNA.

Chemical Modification of the Enzyme

Chemical modification reactions were carried out with enzyme which had been dialyzed or filtered through a Bio-Gel P-10 column to eliminate 2-mercaptoethanol present in the storage buffer. This was done immediately before use because the enzyme is very unstable in the absence of reducing agents.

Modifying agents were dissolved in 50mM Tris-HCl, pH 8, filtered through Millipore filters and the concentration adjusted spectrophotometrically at at 325 nm ($18,000 \text{ M}^{-1}\text{cm}^{-1}$) (7), at 232 nm for pCMB ($E_{232} 16,900 \text{ M}^{-1}\text{cm}^{-1}$) (5) and at 305 nm for NEM ($E_{305} 620 \text{ M}^{-1}\text{cm}^{-1}$) (9). Solutions were freshly prepared each time or kept frozen in the dark.

Spectrophotometric determinations were carried out in a Varian Techtron 635 spectrophotometer using one ml cuvettes, in 50mM Tris-HCl, pH 8, 4% glycerol, 0.5mM EDTA, 0.1M KCl (buffer A). The sample cuvette contained enzyme and modifying agent at an appropriate concentration, and the reference cuvette, the agent alone. The reaction with DTNB was followed at 412 nm and with pCMB at 250 nm. A molecular weight of 650,000 (1) for yeast RNA polymerase I and $\Delta E_{412} 13,600 \text{ M}^{-1}\text{cm}^{-1}$ (6) or $\Delta E_{250} 7,600 \text{ M}^{-1}\text{cm}^{-1}$ (5) were used for calculations. Binding of ^{14}C -pCMB or ^{14}C -NEM to the enzyme was based on the quantitative retention of RNA polymerase on nitrocellulose filters (10). Other details are indicated in the figure legends. Other buffers used were: buffer B (50mM Tris-HCl, pH 8, 10% glycerol, 0.5mM EDTA, 0.1M KCl), buffer C (50mM Tris-HCl, pH 8, 0.5mM EDTA, 0.1M KCl) and buffer D (50mM Tris-HCl, pH 8, 0.5mM EDTA, 0.5M KCl, 25% glycerol).

Polyacrylamide gel electrophoresis was carried out as described elsewhere (1).

RESULTS AND DISCUSSION

Number of SH Groups Per Mole of Enzyme

The reaction of DTNB with SH groups can be followed spectrophotometrically at 412 nm (6). In order to determine the number of cysteine residues in RNA polymerase, the enzyme, after denaturation in 8M urea, was incubated with a 1000 fold excess of DTNB and the increase in absorbance was measured as a function of time. The number of SH groups was determined using an extinction coefficient of $13,600 \text{ M}^{-1}\text{cm}^{-1}$ (6). Figure 1 shows that the reaction between SH groups of the enzyme and DTNB is a time dependent process and reaches a plateau after 25 min, when 45 cysteine residues per mole of enzyme have reacted. Taking into account the larger molecular weight of RNA polymerase I, this number is in the range of that found for bacterial RNA polymerases. The enzymes from *E. coli* and from *A. vinelandii* each have 32 residues (11,12).

Inactivation of RNA Polymerase I by Incubation with DTNB

The enzyme was incubated with $5 \times 10^{-6} \text{ M}$ DTNB for the time periods indicated in Figure 2 and aliquots were assayed for enzymatic activity. It can

FIGURE 1. Time course of the reaction between DTNB and RNA polymerase I in 8M urea. Enzyme (0.04 mg/ml) was incubated in buffer A and 8M urea at 30°C with 5×10^{-5} M DTNB in 1 ml. The increase of absorbance at 412 nm was determined as a function of time. The reference cuvette contained 4×10^{-5} M DTNB in the same buffer.

be seen that RNA polymerase rapidly loses its activity following pseudo first order kinetics. The inactivation is completed within 15 min. Addition of a reducing agent such as 1mM DTT results in a reversal of the inhibition suggesting that the loss of activity is caused by DTNB blocking of cysteine residues important for enzymatic activity. Control enzyme incubated in the absence of DTNB gradually loses its activity during the incubation period and this effect is not reversed by the addition of DTT, indicating that the enzyme is unstable under these conditions. Figure 3 shows the effect of incubating RNA polymerase I with increasing amounts of DTNB (between 1×10^{-7} and 5×10^{-6} M). The results indicate that DTNB is a very effective reagent. Con-

FIGURE 2. Inactivation kinetics of RNA polymerase I by DTNB and reversal by DTT. Enzyme (0.06 mg/ml) was incubated in buffer B at 30°C with (●---●) or without (o---o) 5×10^{-6} M DTNB in a final volume of 0.5 ml. At the indicated times aliquots of 40 μ l were withdrawn for determination of enzyme activity. At 25 min, 1mM DTT was added to the reaction mixture.

FIGURE 3. Reaction of RNA polymerase I with DTNB. Inactivation (○---○). Enzyme (0.06 mg/ml) was incubated in buffer B with the indicated concentrations of DTNB for 15 min at 30°C in a volume of 40 μ l. The enzyme activity was measured in the same test tube in a final volume of 60 μ l. Spectrophotometric titration (●---●). Enzyme (0.08 mg/ml) was treated in buffer A with 5 μ l aliquots of 6×10^{-3} M DTNB in a final volume of 1 ml. The absorbance was measured after 15 min of each addition.

centrations as low as $1-5 \times 10^{-6}$ M DTNB produce 60-100% loss of activity.

Spectrophotometric Titration of Reactive SH Groups with DTNB

The enzyme was incubated for 15 min with increasing concentrations of DTNB (between 2 and 25×10^{-5} M) under non-denaturing conditions, and the reactive SH groups were determined spectrophotometrically in each case. It can be seen in Figure 3 that at increasing concentrations of DTNB, more cysteine residues react and follow a saturation-type curve. Of the 45 SH groups, only 20 react under these conditions, indicating that they are accessible to the reagent and that the others are buried when the enzyme molecule is in its native form.

Reaction with pCMB

Another specific reagent, pCMB, was also used to study the SH groups of yeast RNA polymerase I. The results of Figure 4 show that under non-denaturing conditions increasing amounts of pCMB react with a maximum of 20 SH groups per mole of enzyme. This number is identical to that found with DTNB although the DTNB concentration used to obtain this value is almost 40 times higher than the concentration of pCMB. The reaction of RNA polymerase I with

FIGURE 4. Binding of ^{14}C -pCMB to RNA polymerase I. Enzyme (0.06 mg/ml) was incubated in buffer B with the indicated concentrations of ^{14}C -pCMB for 15 min at 30°C in a final volume of 0.1 ml. Then 2 ml of cold buffer C were added to the reaction mixture and filtered through wet Millipore filters. The filters were washed with 2x5 ml of buffer C, dried and counted. The insert shows a magnification of the first part of the curve. Twenty moles of ^{14}C -pCMB/mole enzyme correspond to 7000 cpm.

pCMB is sigmoidal, suggesting that the binding of the first pCMB molecule facilitates the binding of the following ones.

The inhibition of RNA polymerases by pCMB was also studied. The enzyme was incubated for 15 min at 30°C with different concentrations of pCMB (between 1×10^{-7} and $1 \times 10^{-6}\text{M}$) and assayed for residual activity. The results are shown in Figure 5. The loss of activity is dependent on the concentration of the modifying agent, and at $1 \times 10^{-6}\text{M}$ pCMB the enzyme is completely inactivated. This concentration is 5 times less than that of DTNB needed to produce the same effect, indicating again that pCMB is more effective than DTNB. This is probably due to steric hindrance of the latter reagent to reach the reactive cysteine residues. DTNB has two aromatic rings joined together by disulfide bond and each ring has bulky nitro and carboxylic groups and a reactive Hg head.

As in the case of DTNB, inhibition by pCMB can be reversed by reducing agents. Table I lists the activity of the enzyme after incubation with various concentrations of the reagent for 15 min, as well as the activity reco-

FIGURE 5. Inactivation of RNA polymerase I by pCMB. Enzyme (0.06 mg/ml) was incubated in buffer B with the indicated concentration of pCMB for 15 min at 30°C in a final volume of 40 μ l. Then enzymatic activity was measured in the same test tube in a final volume of 60 μ l.

vered after the addition of 10mM 2-mercaptoethanol to the modified enzyme. This again confirms that both reagents act in the same way, that is, by modifying SH groups.

Determination of the Number of pCMB Molecules Necessary to Completely Inactivate Yeast RNA Polymerase I

From the data of Figures 4 and 5 one can correlate the number of pCMB

TABLE I: Effect of 2-Mercaptoethanol on the Inactivation of RNA Polymerase I by pCMB

pCMB	% Control Activity	
	with 2-Mercaptoethanol	without 2-Mercaptoethanol
$1 \times 10^{-7} \text{ M}$	90	--
$4 \times 10^{-7} \text{ M}$	25	--
$8 \times 10^{-7} \text{ M}$	0	--
$1 \times 10^{-6} \text{ M}$	0	95
$5 \times 10^{-6} \text{ M}$	0	70
$2 \times 10^{-5} \text{ M}$	0	55

Enzyme (0.06 mg/ml) was incubated in buffer B with the indicated concentrations of pCMB for 15 min at 30°C. Then 10mM 2-mercaptoethanol was added to the reaction mixture and enzymatic activity was measured after another 5 min incubation. A reference experiment was done adding buffer instead of 2-mercaptoethanol. The control was without pCMB.

FIGURE 6. Determination of the number of pCMB molecules bound to the enzyme necessary to completely inactivate RNA polymerase I. The data of this figure were calculated from the moles of pCMB bound per mole of enzyme (Fig. 4) and the corresponding enzyme activity (Fig. 5) at a given pCMB concentration.

molecules bound per mole of polymerase and the activity of the enzyme. The results indicate that the modification of only a few SH groups results in a significant loss of activity (Figure 6). Extrapolation to zero activity shows that the reaction of two SH groups produces the complete inactivation

TABLE II: Protection by Substrates and DNA on the Inactivation of Yeast RNA Polymerase I by pCMB and DTNB

Addition	% Control Activity	% Protection
pCMB $3.3 \times 10^{-7} \text{M}$	47	
+ ATP, GTP and CTP 1mM each	60	24
+ calf thymus DNA 0.25 mg/ml	66	36
+ ATP, GTP and CTP 1mM each and calf thymus DNA 0.25 mg/ml	85	72
DTNB $1 \times 10^{-6} \text{M}$	50	
+ ATP, GTP and CTP 1mM each	64	28
+ calf thymus DNA 0.25 mg/ml	77	54
+ ATP, GTP and CTP 1mM each and calf thymus DNA 0.25 mg/ml	80	60

Enzyme (0.06 mg/ml) was incubated in buffer B with the indicated concentrations of modifying agent in the presence and absence of substrates and calf thymus DNA in a final volume of 40 μl for 15 min at 30°C. Enzymatic activity was measured in a final volume of 60 μl in the same test tube.

of the enzyme. This suggests that they are located at the active site. Additional evidence comes from experiments showing that nucleotide substrates and DNA in concentrations normally used in the assay protect the enzyme against inactivation. The results are listed in Table II. They indicate that nucleotides as well as DNA prevent to some extent the inactivation of the enzyme by both SH reagents and that a greater protection is obtained when both compounds are added simultaneously. These results are consistent with the notion that the cysteine residues important for enzyme activity may be near or at the substrate and DNA binding site.

The results described above for yeast RNA polymerase I are markedly different to the results reported by others for *E. coli* RNA polymerase where the titration of the 4 most reactive SH residues has no effect on enzyme activity (13-15).

Effect on the Quaternary Structure

In order to determine if the chemical modification affects the quaternary structure, the modified enzyme was analyzed by sucrose gradient sedi-

FIGURE 7. Reaction of RNA polymerase I with NEM.

Inactivation (o---o): Enzyme (0.06 mg/ml) was incubated in buffer B with the indicated concentrations of NEM for 10 min at 30°C in a volume of 40 μ l. The enzyme activity was measured in the same test tube in a final volume of 60 μ l. Binding of ¹⁴C-NEM to RNA polymerase I (Δ---Δ): Enzyme (0.15 mg/ml) was incubated in buffer B with the indicated concentrations of ¹⁴C-NEM for 10 min at 30°C in a volume of 0.1 ml. Then, 10mM 2-mercaptoethanol was added to the reaction mixture followed by 2 ml of cold buffer C and filtered through wet Millipore filters. The filters were washed with 2x5 ml of buffer C, dried and counted. The insert was calculated from the above data.

FIGURE 8. Labeling pattern of polypeptide components of yeast RNA polymerase I after reaction with ^{14}C -NEM. Enzyme (15 μg) was incubated in 0.08 ml for 10 min at 30°C with a ^{14}C -NEM concentration of $1.2 \times 10^{-6}\text{M}$, and the reaction was stopped by adding 0.02 ml of a solution containing 10% 2-mercaptoethanol and 9% sodium dodecyl sulfate. A: Coomassie blue staining of the gel. B: Fluorography of enzyme subunits after treatment with $1.2 \times 10^{-6}\text{M}$ ^{14}C -NEM. The apparent discrepancy in the length of the gel is due to shrinkage of the gel after fluorography and drying.

mentation. The results (not shown) indicate that there is no alteration of the sedimentation coefficient of the enzyme upon modification. These results were confirmed by SDS gel electrophoresis analysis of all fractions of the gradients, which showed that the modified enzymes contain the same subunit composition as the nontreated enzyme and that free subunits are not found elsewhere in the gradient (results not shown).

Location of the Reactive SH Among the RNA Polymerase I Subunits

Inhibition and binding experiments were also performed with ^{14}C -NEM, an irreversible SH reagent suitable to identify the most reactive subunits. This

reagent completely inactivates the enzyme at a concentration of $1.5 \times 10^{-5} \text{M}$ after 10 min incubation at 30°C , and at a concentration of $5 \times 10^{-6} \text{M}$, the remaining activity is 20% (Fig. 7). Similar to the results obtained with pCMB, 2 moles of reagent bound to the enzyme are needed to completely inactivate the RNA polymerase (Fig. 7). In order to determine which subunit(s) bind(s) NEM, enzyme was incubated with a ^{14}C -NEM concentration of $1.2 \times 10^{-6} \text{M}$ and the reaction was stopped by adding 2-mercaptoethanol and sodium dodecyl sulfate. SDS electrophoresis was run and fluorography was performed as described by Bonner and Laskey (16). The results are on Figure 8. Radioactivity was found associated only with subunits of molecular weight 185,000, 137,000 and 41,000. Interestingly, the lysyl residues believed to be involved in catalysis in the active site of yeast RNA polymerase I (2,3) are also located in the two largest subunits of the enzyme.

In conclusion, the present studies indicate that yeast RNA polymerase I has three main classes of SH groups. Approximately 25 SH residues are not modified in the native enzyme and therefore are probably buried in the interior of the molecule. Twenty SH residues are accessible to the modifying reagents and probably located at or near the surface of protein. Finally, two of the accessible SH groups are absolutely required for polymerase activity. The modification of these residues is partially protected by substrates and DNA, suggesting that they are involved in catalysis or in the maintenance of the conformation of the active site. The most reactive SH groups are located in subunits of molecular weight of 185,000, 137,000 and 41,000.

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