# REACTION OF THE THIOL GROUPS OF E. COLI RNA POLYMERASE WITH 7 CHLORO-4-NITROBENZO-2 OXA-1,3 DIAZOLE

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Summary: A single thiol group of E. Coli RNA polymerase reacts rapidly with 7-chloro-4-nitrobenzo-2 oxa-1,3 diazole (NBD-Cl) at pH 7.5 with slower reaction of a much larger number of such groups. Reaction of the unique thiol is associated with maximal loss of about 60% of the catalytic activity. The extent of inactivation is independent of the template used in the assay. Initiation of RNA synthesis shows no inhibition, thereby implicating the elongation step as the impaired function. Neither substrate, template, rifampcin, nor the elongation inhibitor rose bengal protect the enzyme against reaction with NBD-Cl. These observations suggest that the partial inactivation of RNA polymerase on reaction with a thiol group is most likely due to a local conformational change outside of the active site which influences the active site in an indirect manner.

Covalent labeling of enzymes with chromophoric "reporter groups" has been a valuable way of studying the architecture of such molecules and has been useful in monitoring biochemical processes involving such enzymes. In our attempts to label E. Coli RNA polymerase (ribonucleoside triphosphate-RNA-nucleotidyl transferase, EC 2.7.7.6) with the fluorogenic reagent NBD-Cl, we have found that one of the 28-34 thiol groups (1,2) reacts at a markedly higher rate than any of the others with a parallel decrease in enzymatic activity. This pattern of reactivity and loss of enzymatic activity is in marked contrast with the observations of other investigators (1-4) who reacted RNA polymerase with a variety of sulfhydryl reagents.

## MATERIALS AND METHODS

RNA polymerase was prepared from E. Coli B cells obtained from Grain Products Corp. by a modification (S.C. Bratcher, to be published) of the procedure of Burgess and Jendrisak (5) and stored at -20° in a storage buffer containing 50% (v/v) glycerol and 0.1 mM DTT (5). SDS gel electrophoresis indicated that the enzyme was at least 95% pure. Prior to reaction of NBD-Cl with the enzyme, the DTT was removed by gel filtration on a 1.5 x 6 cm column of Biogel P-100 which

Abbreviations: NBD-Cl, 7 chloro-4-nitrobenzo-2 oxa-1,3 diazole; DTT, dithiothreitol; TEA, triethanolamine; ME, mercaptoethanol.





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had been equilibrated with pH 7.50, 0.05 M TEA, 1 mM EDTA, 10% (v/v) glycerol, 0.2 M NaCl. Concentrations of RNA polymerase were determined from absorbences at 280 nm which were corrected for light scattering (6).

The kinetics of reaction were determined spectrophotometrically in a Cary 118 C spectrophotometer at a temperature of 15°. Concentrations of NBD-Cl labeled thiol were determined as a function of time from the absorbence at 420 nm using an extinction coefficient of 13,000 (7). Kinetic analysis was restricted to times at which no more than 6% consumption of NBD-Cl had occurred thereby insuring psuedo first order kinetics. The time dependence of the absorption at 420 nm was analyzed using a Gauss-Newton non-linear least squares method (8) for the function:

$$A_{420} = A_1 + C_1 e^{-k_1 t} + bt \qquad 1.$$

where $-C_1$ is the absorbence change per reactive group; $k_1$, the psuedo first order rate constant; and $A_1$ and b are parameters obtained from the fit (see below). This function gave rapid convergence of fit with standard deviations of the order of $5 \times 10^{-4}$. Attempts to fit the data with a two exponential term expression were unsuccessful, i.e. convergence could not be obtained. The significance of the above type of fit will be considered below.

Enzymatic activity was generally assayed by the method of Chamberlin and Berg (9) using calf thymus DNA as template, but with the omission of ME or DTT in the assay mixture.

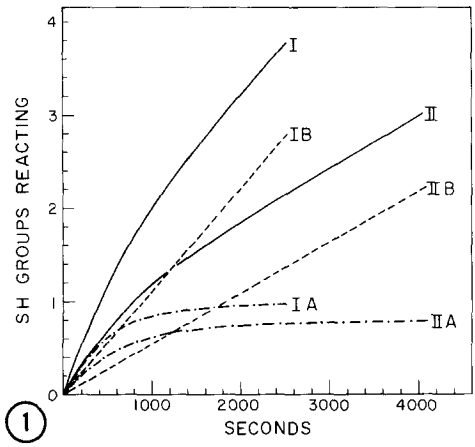
## RESULTS AND DISCUSSION

The time course of the reaction of NBD-Cl with RNA polymerase is illustrated in curves I and II of Figure 1. The number of thiols which had reacted with NBD-Cl were determined from the concentration of the NBD labeled thiols and the concentrations of the enzyme.

In general terms, the time dependence of the absorbence corresponding to reaction of the thiols (see below for justification for identifying the reactive groups as thiols) can be described by:

$$A_{420} = \sum_{i=1}^{n} A_i + C_i e^{-k_i t} \qquad 2.$$

where the sum is taken over all reactive groups. $A_i$, a parameter of the fit, should be equal to $-C_i$. Equation 1 is a special case of equation 2 for a system having a single rapidly reacting thiol and a large, but indeterminent, number of slowly reacting groups. Expansion of Equation 2 as a Taylor series for i=1 and discarding all terms larger than t leads to equation 1 where $b = \Sigma k_i$. The details of the kinetic study of the reaction will be published in due course (K. Nitta, S. C. Bratcher and M. J. Kronman, unpublished observations).



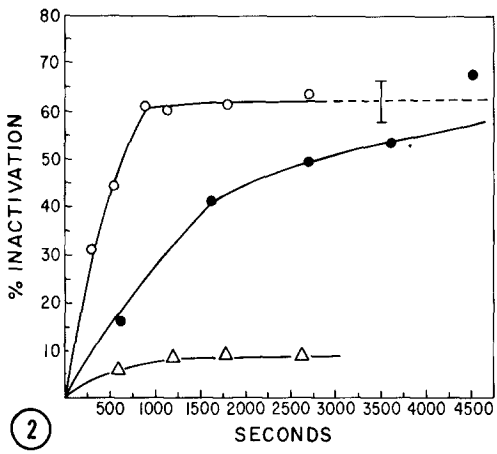




Figure 1: Time course of reaction of NBD-Cl with RNA polymerase. Reaction carried out at 15.0° in 0.05 M TEA buffer, pH 7.5, 10% glycerol (v/v), 0.2 M NaCl, 1 mM EDTA, Curve I, NBD-Cl conc., 252μM, RNA polymerase conc., 2.16 μM. Curve II, NBD-Cl conc., 110 μM, RNA polymerase conc., 1.16 μM. Curves IA and IIA were calculated from the first order rates observed for the "fast" group, while IB and IIB correspond to the reaction of the "slow" groups (see text).

Figure 2: Time course of inactivation of RNA polymerase by NBD-Cl. Experimental conditions as in Figure 1. Concentration of enzyme and reagent identical for Curves I (Figure 1) and O (Figure 2) and Curve II (Figure 1) and ● (Figure 2).

The time dependencies of the rapid and slowly reacting thiols are given as curves IA, IIA, and IB, IIB respectively of Figure 1. By 2500 seconds, about four thiols have reacted at an NBD-Cl concentration of 0.252 mM, of which one is the rapidly reacting group. Thus, there must be a minimum of three groups which react slowly. Since the time dependence of reaction for this class of thiols is linear over the time range studied (curve IB, Figure 1), there are probably considerably more than three slowly reacting thiols.

The time course for inactivation of the enzyme is illustrated in Figure 2. The inactivation and reactivity measurements were carried out in four paired experiments where the RNA polymerase and NBD-Cl solutions were identical for a given reagent concentration. At all four concentrations of NBD-Cl employed (range, 0.110 to 0.252 mM) the percent inactivation approached a limiting value of ca.

63%. The error bar shown in Figure 2 represents the average value for percent inactivation for the four concentrations. At an NBD-Cl concentration of $2.52 \times 10^{-4}$M maximum inactivation was attained by about 900 seconds. By 900 seconds the reaction of the single "fast" thiol is nearly complete (curve IA, Figure 1), while an average of Ca. 1 group has reacted from the "slow" class. Since the "slow" class contains a minimum of three thiols, only about 30% of each of these groups on the average has reacted by 900 seconds. These observations, together with similar ones made for the other concentrations of NBD-Cl employed, strongly suggest that the inactivation can be identified with reaction of the rapidly reacting thiol.

These observations are in marked contrast with those made by other investigators using different thiol reagents. Yarbrough and Wu (1), for example, have shown that four to five thiols of RNA polymerase can react with iodoacetamide with virtually no loss of activity. Reaction of eight thiols with tetrathionate (1) likewise leads to virtually no loss of activity while a loss of 30-50% is only observed after reaction with 12 groups. King and Nicholson (2) found that five thiols of RNA polymerase can be reacted with N-ethyl-malimide, or with 5.5'-dithiobis-(2-nitrobenzoic acid), with no loss of activity. Harding (3) has shown that three thiols of RNA polymerase can react with p-mercuribenzoate without loss of activity. The observations of Novak, et al. (4) are unusual in that reaction of 10 tryptophans and 6 thiols with 2-hydroxy-5-nitrobenzyl bromide leads to loss of a maximum of 50% activity. These authors likewise report a maximum inactivation of 50% with iodoacetic acid and acetyl imidazole although no indication is given of the extent of reaction in this case. Furthermore, acetyl imidazole reacts with tyrosyl and amino groups as well. Thus, the observations of Novak, et al. (4) do not provide convincing evidence for a correlation of loss of activity with a single thiol.

The spectral evidence per se strongly supports the view that the loss of activity is associated with reaction of NBD-Cl with thiols. The absorption maxima for the SH, amino (10) and tyrosyl (11) reaction products with NBD-Cl are 420-425, 475, and 385 nm respectively. The absorption spectrum of gel filtered NBD-Cl

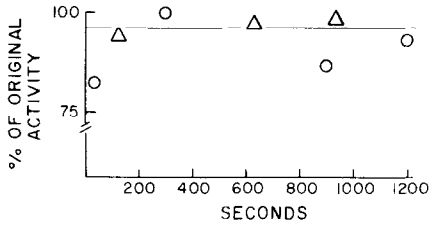



Figure 3: Time dependence of the reaction of NBD-Cl with RNA polymerase as measured by an initiation assay. Reaction conditions as in Figure 1. Assaying conditions the same as previously used except that ($\alpha$-$^{14}$C)ATP was replaced by ($\gamma$-$^{32}$P)ATP at a final concentration of 6.94 μM and a specific activity of 23.3 Ci/mmole.

reacted RNA polymerase shows no sign of maxima in either the 475 or 385 nm region. The observed absorption maximum is at about 415 nm, which is somewhat lower than the range cited above; however, environmental effects can produce this type of shift in the spectrum of the thiol derivative; the Thiol-NBD derivative of papain has an absorption maximum at 405 nm (10). The fluorescence emission spectrum observed with NBD-Cl treated RNA polymerase has a maximum at 521 nm corresponding to the value of 525 nm reported for the thiol derivative (10) and is markedly lower than the value of 545 nm found for the amino derivative (10). The spectral evidence thus provides support for the view that the reactive species in RNA polymerase is thiol.

Thiolysis of the NBD-Cl derivative of RNA polymerase with DTT or ME leads to nearly complete recovery of enzymatic activity; in a typical experiment modified RNA polymerase free of excess reagent was treated with DTT to a final concentration of 0.03 M. The enzyme regained at least 90% of the original activity within 15 minutes. The fact that thiolysis could restore virtually all of the activity rules out the possibility that reaction of NBD-Cl with amino groups is responsible for the loss of activity. Reaction of NBD-Cl with RNA polymerase, unlike the reaction of this enzyme with tetrathionate (1), does not lead to inactivation resulting from impairment of template binding. In a typical experiment, a 1.5 μM solution of the enzyme was reacted with a 200 μM solution of NBD-Cl and assayed as a func-

tion of time using calf thymus DNA (0.15 mg/ml), T2 DNA (0.10 mg/ml), and T4 DNA (0.10 mg/ml) as templates. In all three cases the course of the inactivation was the same, e.g. 67 ± 2% occuring by 1250 seconds. Since it is doubtful if the impairment of binding of all three templates would be exactly the same, it is reasonable to conclude that inactivation does not result from impairment of template binding.

In order to determine if initiation of RNA synthesis is impaired on reaction of the thiol group, the reaction of the enzyme with NBD-Cl was followed by the standard assay procedure and by an initiation assay where the ($\alpha$-$^{14}$C)ATP was replaced with ($\gamma$-$^{32}$P)ATP. The standard assay procedure showed ca. 60% inactivation by about 1200 seconds, but the initiation assay gave no indication of significant impairment (Figure 3). Since the conditions of the assay do not favor product release, it would appear that the reaction of NBD-Cl with a thiol of RNA polymerase leads to impairment of elongation.

The marked decrease in catalytic activity of RNA polymerase on reaction of a thiol with NBD-Cl seemed to suggest that this group could be at the active site of the enzyme. However, reaction of the enzyme preincubated with GTP (12 mM), ATP (12 mM), rifampcin (18 uM), calf thymus DNA (0.15 mg/ml) and rose bengal (20 to 50 μM) failed to protect the thiols of the enzyme against reaction, nor did it have a significant effect on the rates of reaction. The failure of rose bengal to protect against reaction is particularly interesting since it has been shown to be an inhibitor of elongation (12) which is the step in the catalytic process that seems to be impaired. Thus, the 60% loss of the overall catalytic activity of the enzyme would appear to be the result of a local conformational change which ultimately brings about a structural change in the active site region itself.

Preliminary studies have provided some evidence for this putative conformational change. Reaction of RNA polymerase with NBD-Cl brings about a three fold decrease in the quantum yield for tryptophan fluorescence, demonstrating a large change in the molecular environment of such groups. We have further demonstrated

that the subunit containing the rapidly reacting thiol is β or β′. The latter observation is not unexpected in view of the identification of these subunits as the locus of many of the active site functions (see ref. 13 for a recent summary of pertinent observations). These studies are currently being extended to establish in a definitive way the location of the unique thiol in the enzyme, as well as the nature of the structural change.

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