SULFHYDRYL REACTIVITY OF E. COLI DNA DEPENDENT RNA POLYMERASE*

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SUMMARY: Reaction of DNA dependent RNA polymerase with the sulfhydryl specific reagent p-mercuribenzoate is described. An exposed class consisting of approximately 19 out of a total of 32 sulfhydryl groups is demonstrated. There is no loss of enzymatic activity when 3-4 groups of this class are reacted; over 90% of activity is lost upon reaction of 4-5 additional groups. Inactivation is completely reversible after all of the 19 accessible groups are reacted. ATP, a substrate, protects a small number of groups from reaction with the mercurial.

Although data is accumulating about the mechanism of RNA synthesis catalyzed by DNA-dependent RNA polymerase (1, 2) little is known about the structural details of the enzyme and its constituent polypeptide chains. In connection with our investigations of subunit proteins which can be reversibly dissociated, we have investigated the sulfhydryl (SH) reactivity of RNA polymerase. P-mercuribenzoate (PMB), a reagent that reacts specifically with the SH groups of cysteine residues, can inactivate enzymes by binding to SH groups that are either necessary for maintenance of the native conformation or are active site residues. In the absence of published amino acid sequence and X-ray crystallographic data, studies utilizing such a reagent can be particularly useful in elucidating some features of enzyme structure and function .

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Ishihama and Hurwitz (3) examined the effect of a number of reagents on the E. coli W RNA polymerase holoenzyme (a complex of core enzyme and sigma factor, a dissociable subunit necessary for proper initiation of RNA synthesis on DNA templates). They found that PMB inactivated the enzyme when present in excess relative to the total half cystine content and that inactivation could be reversed completely by an excess of mercaptoethanol. Amino acid analysis indicates 32 half cystine residues per E. coli K12 core molecule (4). Krakow has shown that the A. vinelandii enzyme contains 30-32 cysteine residues (5).

In this communication, we report preliminary findings showing distinct classes of SH groups of differing reactivity to PMB. The role of these in enzyme activity is described and protection of a small number of reactive groups by ATP is demonstrated.

MATERIALS AND METHODS. RNA polymerase core enzyme was purified by the method of Burgess from E. coli K12 (6). All preparations were judged to be substantially pure when run on polyacrylamide gels containing sodium dodecyl sulfate (4). Prior to any given experiment, an aliquot of the enzyme in storage buffer was diluted with standard buffer containing 0.01 M Tris, 0.01 M $MgCl_2$, 0.2 M KCl, pH 7.9 and applied to a short column of Biogel P2, 200-400 mesh (Bio-Rad), equilibrated with the same buffer. The column was eluted at 4°C. After allowing the eluted enzyme to reach room temperature, the concentration was determined at 280 nm using an $E^{1\%} = 6.5$ (6) after correcting the extinction at 280 nm for the effects of light scattering when necessary (7). The molecular weight of RNA polymerase core was taken as 400,000 and the total half cystine content assumed to be 32 per molecule (4). RNA polymerase was assayed at room temperature by the method of Burgess using calf thymus DNA as template (6) except that dithiothreitol was not included in the assay mixture.

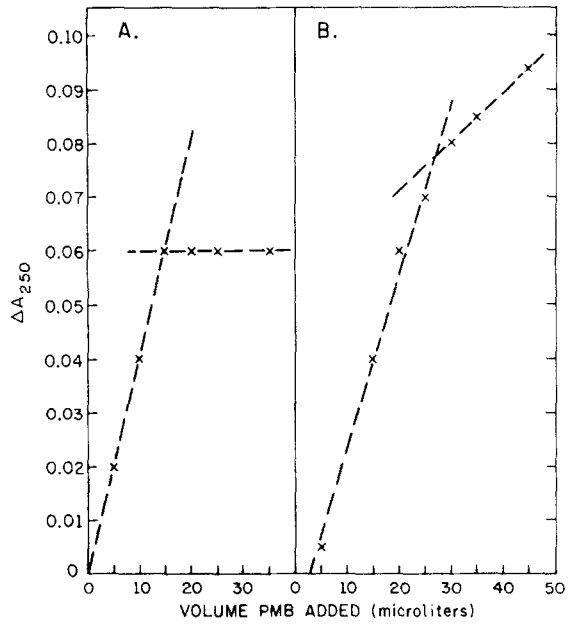


Figure 1. Titration of SH Groups.

A. In absence of ATP: 1.7 ml of 4.35 X 10^{-7} M RNA polymerase core in standard buffer was titrated with 9.82 X 10^{-4} M PMB at room temperature. Intersection of the two lines is at 8.59 X 10^{-6} M PMB; 19.7 SH per molecule were titrated.

B. In presence of ATP: 2.0 ml of 8.35 X 10^{-7} M RNA polymerase core in standard buffer containing 5 X 10^{-5} M ATP was titrated with 9.94 X 10^{-4} M PMB at room temperature. Intersection of the two lines is at 1.32 X 10^{-5} M PMB; 15.8 SH per molecule were titrated. A_{250} does not change if only ATP is present.

Two methods of sulfhydryl determination were used (8). In the first (Fig. 1) 5 microliter aliquots of a PMB solution are added sequentially to a solution of RNA polymerase and the increase in optical density at 250 nm measured five minutes after the addition of each aliquot. The change in extinction is plotted versus the amount of PMB added and the end point of the titration is determined at the intersection of two lines drawn through the points of the plot as indicated. An ε = 1.69 X 10^4 at 232 nm was used to determine the concentration of the PMB stock used in the titration. The second method of sulfhydryl determination (Figs. 2 and 3) utilizes the value of the mercaptide extinction directly. The $\Delta\varepsilon$ = 7.6 X 10^3 at 250 nm deter-

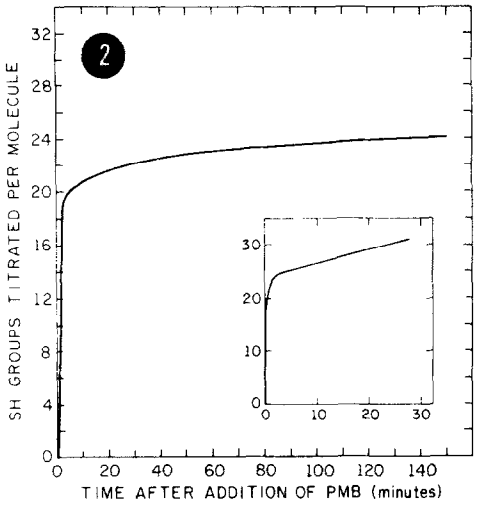
mined by Boyer for formation of the cysteine mercaptide (8) is used to calculate the number of sulfhydryl groups reacted. The average RNA polymerase mercaptide extinction determined by us is within 3% of this value.

RESULTS.

Sulfhydryl Reactivity and Effect of ATP. Figure 1 shows representative titrations of sulfhydryl groups in solutions of free enzyme and of enzyme in the presence of a 60 fold molar excess of ATP. With the free enzyme, the average of determinations on three different preparations gave a value of 18 ± 1.6 equivalents SH per mole enzyme. In the presence of ATP, an average of the same number of measurements using corresponding preparations gave a value of 14 ± 0.9 equivalents. In the experiments illustrated in Fig. 1 the breakpoints are at 19.7 and 15.8 SH titrated in the absence and presence of ATP, respectively. Thus, a relatively low level of ATP (one-third of that present during a typical assay) protects a small but measurable number of sulfhydryl groups. This effect is reproducible despite the experimental error which is caused primarily by the small amount of enzyme used in these experiments.

Classes of Sulfhydryl Groups. Figure 2 shows the reaction of RNA polymerase sulfhydryl groups with excess PMB as a function of time. At least two clases of sulfhydryls are evident. The most reactive class comprises about 19 groups that react within 3 minutes of the addition of PMB. The remaining groups continue to react over a much longer period until, after an overnight incubation, all of the half cystine groups of the molecule are complexed with PMB (inset, Fig. 2).

Relationship Between Groups Titrated and Enzymatic Activity. Figure 3 shows that approximately 3 groups can be reacted without loss of activity. As the 4th through 8th groups are titrated, the enzyme shows an almost



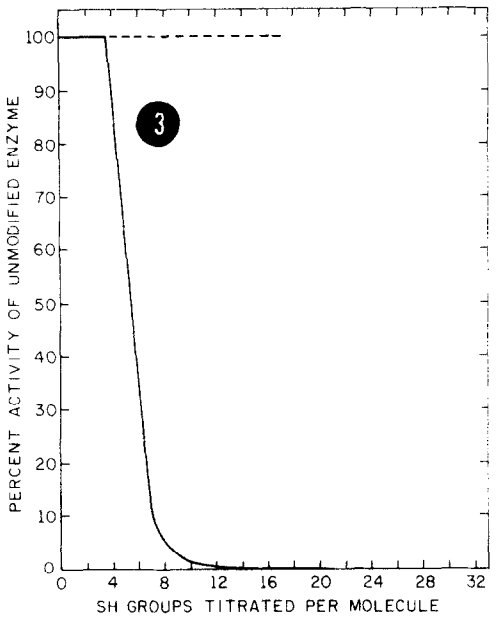


Figure 2. Reaction of SH Groups in Excess PMB.

Average curve obtained by adding an 80 fold molar excess of PMB (relative to protein concentration) to an RNA polymerase solution, approximately 4×10^{-7} M in standard buffer at room temperature. SH groups titrated per molecule were determined with $\Delta \epsilon_{250} = 7.6 \times 10^3$.

Inset: The reaction over a more extended time period. The abscissa is time after addition of PMB in hours; the ordinate is SH groups titrated per molecule.

Figure 3. Relationship Between Groups Titrated and Enzymatic Activity.

Solid line (———): An aliquot of PMB was added to an RNA polymerase solution (approximately 4×10^{-7} M) in standard buffer at room temperature. After 5 minutes part of the solution was assayed at room temperature and the ΔA_{250} was read from the remaining solution to determine the number of SH reacted. 100% activity is that of the unmodified enzyme before PMB addition. Unmodified enzyme does not lose activity over the total time of the experiment.

Dashed line (- - - - -): An aliquot of enzyme that had a given number of SH reacted was combined with an equal volume of 10^{-3} M mercaptoethanol in standard buffer and incubated for 5 minutes at 37°C. The reactivated enzyme was assayed at room temperature.

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linear decrease in activity. Although we show curvature in the graph at the lowest residual activities (8th to 13th groups titrated) it should be emphasized that this is the most uncertain region of the plot due to the reduced activity of the enzyme at room temperature relative to 37°C, the optimal assay temperature. Figures 2 and 3 represent two different types of experiments

since in the former PMB is present in excess relative to total protein sulfhydryl. However, there are two reasons to believe that the groups shown to be necessary for activity in Fig. 3 are among the 19 most reactive groups in Fig. 2. First, in data not shown, we have found that if the enzyme in Fig. 2 is assayed after 3 minutes of reaction with excess PMB all enzymatic activity is lost. Second, experiments of the kind shown in Figs. 1 and 3 are comparable since PMB is not in excess. In the absence of ATP, the same number of SH groups are titrated using the step method of Fig. 1 as there are fast reacting groups in Fig. 2. Although it is possible that excess PMB could cause the exposure of groups not normally measurable in a titration like that of Fig. 1, it is unlikely that it could at the same time make normally reactive groups unavailable for reaction.

Reactivation. Figure 3 also shows that enzymatic activity can be completely regenerated by treating the enzyme with excess mercaptoethanol at 37° C for 5 minutes before assay at room temperature. We have tested this up to 17 groups reacted. Similarly, enzyme that has been incubated at room temperature for three minutes in excess PMB (as in Fig. 2), can be reactivated. However, in some cases, particularly after prolonged exposure to PMB, reactivation could not be demonstrated under these conditions.

DISCUSSION. The results indicate that ATP, a substrate of the enzyme, protects four sulfhydryl groups from reaction with PMB. It is possible either that the enzyme has a slightly different overall conformation in the presence of ATP or that some sulfhydryls are actually in or close to the ATP binding sites. The former argument is favored by the fact that Ishihama and Hurwitz (3) have shown that holoenzyme completely inactivated by excess PMB can bind ATP. It should be noted that various studies have reported 1 (9), 2 (10) and 4-9 (3) purine binding sites per molecule in various RNA poly-

merase preparations. While these values are of the same order as the number of sulfhydryl groups protected by ATP, none of the binding studies has been done under the exact conditions of our experiments.

It is particularly interesting that the most reactive class of sulfhydryl groups shows a complex relationship to enzymatic activity. Our results are in good agreement with brief reports from other laboratories (11, 12, 5). The fact that reaction of the first 3-4 SH residues has no effect on enzyme activity whereas subsequent reaction leads to a total inactivation means that all of the approximately 19 accessible groups are not equivalent. Moreover, the steepness of the inactivation curve beginning with reaction of the 4th (on the average) group suggests additional division of the exposed groups, a small number being essential for activity. Whether this is because of a general conformational change or direct participation of such groups in activity can not presently be decided. Studies to resolve this question as well as to examine the effects of other substrates and inhibitors on sulfhydryl reactivity are currently in progress.

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