Impairment of the Catalytic Activity of *Escherichia coli* Ribonucleic Acid Polymerase by a Unique Reaction of 4-Chloro-7-nitrobenzofurazan

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Escherichia coli RNA polymerase loses 55–65% of its catalytic activity on reaction with Nbf-Cl (4-chloro-7-nitrobenzofurazan). This partial inactivation was shown to be the result of specific impairment of RNA-chain elongation, since initiation of RNA chains was not altered after treatment with Nbf-Cl. The site of reaction was shown to be a unique thiol on the β -subunit. This thiol is not accessible to reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). No protection of the enzyme against reaction with Nbf-Cl could be obtained with the inhibitor rifamycin nor with calf thymus DNA, GTP or 1,10-phenanthroline, indicating that the unique thiol is probably not within the active site. The specific impairment of RNA-chain elongation thus appears to be the result of a local conformational change which leaves chain initiation unimpaired. Changes observed in the tryptophan fluorescence spectrum of the enzyme or reaction with Nbf-Cl are consistent with this proposal. The kinetics of reaction of the enzyme with Nbf-Cl are consistent with formation of a Meisenheimer complex of the reagent with a nucleophilic group on the enzyme near the reactive thiol. It is proposed that formation of such a complex and a subsequent conformational change renders this thiol unusually susceptible to reaction with Nbf-Cl.

The effect of chemical modification of thiol groups of *Escherichia coli* RNA polymerase on its catalytic activity has been studied by a number of investigators (Ishihama & Hurwitz, 1969; Nicholson & King, 1973; Harding, 1974; Yarbrough & Wu, 1974). The general conclusion to be drawn from these observations is that there are no such groups essential for the catalytic activity of this enzyme. This conclusion is exemplified by the observation that six to ten thiol groups will react with tetrathionate or with iodoacetic acid (Yarbrough & Wu, 1974) or with Nbs₂ (Nicholson & King, 1973) with little or no loss in enzyme activity. Loss of activity that is observed on reaction with mercurials such as *p*-chloromercuribenzoate (Nicholson & King, 1973) appears to result from dissociation of the enzyme into subunits (Harding, 1974) or disruption of its characteristic three-dimensional structure. The gradual loss of activity associated with reaction of 10–20 thiol groups of the enzyme with tetrathionate and iodoacetate (Yar-

brough & Wu, 1974) and with Nbs₂ (Nicholson & King, 1973) appears likewise to be due to gross structural changes in the enzyme rather than reaction at one or more essential thiol groups.

In attempting to prepare covalently linked fluorescent derivatives of RNA polymerase by reaction of Nbf-Cl with the thiol groups of the enzyme, we observed that preferential reaction took place at a single thiol group with concomitant loss of 55–65% of its catalytic activity. A preliminary account of these observations has already been given (Bratcher & Kronman, 1977), but they are presented in greater detail in the present paper, and we will provide an explanation for the unusual reactivity of this thiol group with Nbf-Cl in terms of formation of a Meisenheimer (1902) complex of the reagent and a nucleophilic group in the enzyme. This mechanism is based in part on our previous observations (Nitta *et al.*, 1979) that such complexes form between Nbf-Cl and thiolate ion and the observations of Baines *et al.* (1977) of formation of similar complexes with OH⁻.

Although the fluorescence of the Nbf-S derivative has been shown to be considerably weaker than that formed with the amino group (Birkett *et al.*, 1970), it is sufficiently intense to be useful in characterizing molecular environmental properties of labelled thiol groups in proteins. Although Nbf-S fluorescence has been shown to be 'environmentally sensitive', no

Abbreviations used: Nbf-Cl, 4-chloro-7-nitrobenzofurazan (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole); Nbf-S, the 4-thio derivative of Nbf-Cl; Nbf-OHETs, 4-(2-thiohydroxyethyl)-7-nitrobenzofurazan; Nbf-EtS, 4-(thioethyl)-7-nitrobenzofurazan; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); SDS, sodium dodecyl sulphate.

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systematic quantitative study has been made of the relationship of such fluorescence to solution parameters that would reflect the molecular environments found in proteins. In our attempts to derive molecular information from the fluorescence properties of the Nbf-S derivative of RNA polymerase we have carried out such studies using both steady-state and nanosecond-fluorescence-decay techniques.

Materials and Methods

RNA polymerase preparation

RNA polymerase was prepared from *E. coli* B cells (three-quarters exponential phase) obtained from Grain Productions Corp., Muscatine, IA, U.S.A. by a modification of the procedure of Burgess & Jendrisak (1975). We have found that the homogeneity of the enzyme obtained routinely is improved by the use of heparin-Sepharose affinity chromatography after DNA-cellulose affinity chromatography (Burgess & Jendrisak, 1975). The buffers referred to below are those described in the original procedure of Burgess & Jendrisak (1975). The RNA polymerase fraction from the DNA-cellulose chromatography step was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (35 g/100 ml), dissolved in a minimum volume of buffer A [0.01 M-Tris/acetic acid (pH 7.5)/0.01 M-magnesium acetate/1 mM-dithiothreitol/10% (v/v) glycerol] and dialysed overnight at -20°C against buffer A + 50% (v/v) glycerol + 0.3 M- NH_4Cl . The dialysed solution was diluted with 4 vol. of buffer A, and its conductivity adjusted to that found for buffer A + 0.3 M- NH_4Cl . The solution was applied to an affinity column (2.5 cm $\times$ 20 cm) of heparin-Sepharose prepared as described by Sternbach *et al.* (1975), and washed with buffer A + 0.3 M- NH_4Cl . The enzyme was eluted with buffer A + 0.7 M- NH_4Cl , precipitated with $(\text{NH}_4)_2\text{SO}_4$ (35 g/100 ml), dissolved in a minimum of TGED buffer + 10% (v/v) glycerol and finally dialysed at -20°C against storage buffer. The latter buffer contained 1 mM-dithiothreitol and 50% (v/v) glycerol. SDS/polyacrylamide gel electrophoresis showed that enzyme preparations were homogeneous and contained σ -subunit comparable with that found by Burgess & Jendrisak (1975). Concentrations of enzyme were determined from the A_{280} corrected for light scattering, by using a molar absorption coefficient of $3.05 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (mol.wt. 500 000) (Burgess, 1976). Enzyme activity was assayed by a procedure previously described (Bratcher & Kronman, 1977).

Other materials

Unlabelled Nbf-Cl was obtained from Pierce Chemical, Rockford IL, U.S.A. Its melting point was found to be 97°C , in exact correspondence with the reported value (Ghosh & Whitehouse, 1968). $[\text{U}-^{14}\text{C}]$ Nbf-Cl was obtained from Service de Mole-

cules Marquis, CEN Saclay, Gif-sur-Yvette, France. Triethanolamine, obtained from Calbiochem Corp., East Rutherford, NJ, U.S.A., was recrystallized twice from hot water before use. Nbf-OHEtS and Nbf-EtS were prepared by a method similar to that of Allen & Lowe (1973); 4-(*p*-methoxybenzylamino)-Nbf was synthesized by the procedure of Kenner & Aborderin (1971), or was purchased from ICN Pharmaceutical Co., Cleveland OH, U.S.A. These Nbf derivatives were shown to be homogeneous by t.l.c. on silica-gel plates in several solvent systems. 'Affi-Gel Blue' and the Bio-Gel filtration media were products of Bio-Rad Corp., Rockville Center, NY, U.S.A. Organic solvents were of the highest grade available, generally spectroscopic grade; they were dried over Linde 4B molecular sieve before use. All other chemicals were of reagent grade. All aqueous solutions were prepared with deionized charcoal-filtered water. Dithiothreitol and mercaptoethanol were obtained respectively from Calbiochem and Eastman Chemicals, Rochester, NY, U.S.A. Rose Bengal was obtained from the latter source. Unlabelled ATP, UTP, CTP and GTP were purchased from P.-L. Biochemical Corp., Milwaukee, WI, U.S.A. or from Miles Laboratories, Elkhart, IN, U.S.A. $[\text{U}-^{14}\text{C}]$ ATP was obtained from New England Nuclear Corp., Boston, MA, U.S.A., as was $[\gamma\text{-}^{32}\text{P}]$ -ATP. Calf thymus DNA was supplied by Worthington Biochemical Corp., Freehold, NJ, U.S.A., or by Miles Laboratories. T2 and T4 DNA were obtained from the latter source.

Reaction of thiol-group reagents with RNA polymerase

Before reaction of Nbf-Cl or Nbs₂ with RNA polymerase the dithiothreitol of the enzyme-storage buffer was removed by gel filtration on a column (1.5 cm $\times$ 6 cm) of Bio-Gel P-100 which had been equilibrated with reaction buffer [pH 7.50; 0.05 M-triethanolamine hydrochloride/NaOH, 0.2 M-NaCl, 1 mM-EDTA, 10% (v/v) glycerol]. The kinetics of reaction of Nbf-Cl with the thiol groups of the protein were determined spectrophotometrically in a Cary 118C spectrophotometer in 1 cm-pathlength semi-micro cells thermostatically controlled at 15°C . A portion (1 ml) of the dithiothreitol-free enzyme solution was equilibrated in the spectrophotometer and its u.v. spectrum scanned from 350 to 250 nm for use in determining its concentration (see above) before addition of 0.02 ml of an Nbf-Cl solution in 95% ethanol. We had shown previously that a concentration of ethanol of 2% had no observable effect on catalytic properties of the enzyme. The amount of Nbf incorporated was determined, as a function of time, from the A_{420} , a molar absorption coefficient of $13000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ being used (Dwek *et al.*, 1972). Kinetic analysis was restricted to data obtained at times at which no more than 6% of the Nbf-Cl had been consumed.

In some instances incorporation of Nbf was determined with ^{14}C -labelled reagent. In such cases 0.02 ml of the reaction mixture was pipetted at approximate times into 3.00 ml of cold 3.5% (w/v) HClO_4 . The precipitate was collected on Whatman GF/C filters and washed with 3.5% HClO_4 . The filters were dried under a heat lamp and the amounts of Nbf incorporated determined by scintillation counting of the filters. The spectrophotometric and radioactive-isotope-incorporation methods gave identical results.

The reaction of Nbs_2 was followed spectrophotometrically at 412 nm in a Cary 118C spectrophotometer, a molar absorption coefficient of $1.36 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ being used (Ellman, 1959). In experiments where reaction of RNA polymerase was carried out in the presence of both Nbf-Cl and Nbs_2 , incorporation of Nbf label was done by the radioactive-isotope method. The Nbs_2 reaction was not followed in this case, since the absorption bands for the Nbf-Cl and Nbs_2 reaction products lie too close together.

Spectroscopic measurements

Solutions of the Nbf-Cl-modified enzyme were freed of excess Nbf-Cl before spectroscopic measurements by gel filtration of the reaction mixture on a column ($1.5 \text{ cm} \times 6 \text{ cm}$) of Bio-Gel P-100.

Absorption spectra were measured with a Cary 118C spectrometer. Emission spectra were measured at 25.0°C in an instrument described previously (Kronman *et al.*, 1972). Since that report the fluorimeter has been fitted with digital scanning controls and computer interfaced for data-logging. Emission-spectral data were collected at 2 nm intervals with both analogue filtering (lock-in amplification) and digital averaging at each wavelength increment. Correction of spectra and calculation of quantum yields were done as described previously (Kronman *et al.*, 1972), except where solvents other than aqueous ones were used; corrections for refractive index (Brand & Witholt, 1967) were also made. The working standard for protein quantum yields (u.v.) was tryptophan ($Q = 0.13$), and for Nbf-S fluorescence, it was 4-(*p*-methoxybenzylamino)-Nbf in either ethanol or methanol ($Q = 0.358$ and 0.215 respectively). The latter values were obtained by Kenner & Aborderin (1971) using quinine as a standard.

Excited-state-lifetime measurements were performed by the single-photon method (Yguarabide, 1972) by using an Ortec 9200 nanosecond fluorescence spectrometer equipped with a sample compartment designed by Dr. P. Rosen and fabricated in the Rockefeller University Instrument Shops. This sample chamber is provided with lenses to ensure high efficiency of excitation and high flux of fluorescent light, as well as with extensive baffling to

reduce the level of stray light reaching the detector. This has been particularly important with Nbf-S fluorescence, which is weak and short-lived.

Excitation was with a free-running air-gap lamp with a Corning 7-60 excitation filter; emission was viewed through a Schott GG 495 filter. The time scale was calibrated with a delay line and verified by measurement of the lifetime of quinine sulphate.

Lifetimes were calculated by the method of moments (Yguarabide, 1972; Isenberg & Dyson, 1969) implemented in a program written in BASIC for the Hewlett-Packard 9830A computer. The results were output from the nanosecond spectrometer on to paper punch tape, which was read by the high-speed tape reader of the computer. The data were routinely analysed as one- and two-component decays.

The low intensity of fluorescent light obtained on excitation of the Nbf-S fluorophore necessitated the use of a broad-pass excitation filter rather than a narrow-band interference filter or a monochromator for measurements of lifetimes of such groups in a protein. Thus a very low degree of labelling of amino groups might give rise to significant contribution to the decay curve. Since the quantum yield for the amino derivative is much larger than that of the Nbf-S derivative, serious errors could occur. For this reason we used a solution of the dithiothreitol-treated Nbf derivative of the enzyme as background. This procedure is justified by the observation that the fluorescence-decay curve of a solution of Nbf-OHETS on treatment with dithiothreitol reduced to the level of the solvent background. Portions (1 ml) of the enzyme derivative were pipetted into $0.5 \text{ cm} \times 1 \text{ cm}$ fluorescence cells. We then added 0.02 ml of 1 M-dithiothreitol to one cell and 0.02 ml of the buffer to the other. After thiolysis was complete (a period of about 15 min) sample and background were counted alternately for relatively short intervals to ensure that fluctuations in the lamp would be averaged out over both solutions.

Results

Inactivation of RNA polymerase by Nbf-Cl

We have reported previously that a single thiol group of *E. coli* RNA polymerase reacts rapidly with Nbf-Cl with partial loss of activity of the enzyme (Bratcher & Kronman, 1977). We have also shown by spectrophotometric techniques that reaction does not occur with the amino groups of the enzyme. The details of the kinetics of this reaction are considered below. However, anticipating these results, we can state that the reaction of thiol group is relatively rapid and occurs on a 'background' of reaction of other thiol groups at a much lower rate.

The relationship of inactivation and reaction with thiol groups is illustrated in Table 1. In these experiments the kinetics of reaction of Nbf-Cl with the

Table 1. *Relationship between inactivation of RNA polymers and reaction of thiol groups*

The reaction was carried out at 15°C in reaction buffer (see the Materials and Methods section). The times for maximum inactivation, t_1 , were obtained from plots of percentage of original activity versus time. The total number of thiol groups (column 6) reacting at $t = t_1$ were determined from plots of A_{420} versus time. The number of 'fast' thiol groups at $t = t_1$ (column 7) were calculated from the rate constants obtained in the complete kinetic analysis (see the text) by using only the exponential term of eqn. (1) for such calculations.

RNA polymerase concn. (μM)	Nbf-Cl concn. (mm)	Maximum inactivation (%)	t_1 (s)	$10^3 \times B_1$ (s^{-1})	Total thiol groups reacting at $t = t_1$	'Fast' thiol groups reacting at $t = t_1$
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1.66	0.110	56.4 ± 7	1600	1.53	2.0	0.91
1.77	0.118	53.7 ± 0.7	1500	1.71	2.2	0.95
1.88	0.176	54.1 ± 7	1200	2.10	2.1	0.90
2.35	0.252	61.4 ± 1.4	900	2.04	2.7	0.82

thiol groups of RNA polymerase and of inactivation by the reagent were simultaneously carried out with identical enzyme and Nbf-Cl solutions. Since the dithiothreitol used to stabilize the enzyme had to be removed before reaction with Nbf-Cl, there was a risk of spontaneous inactivation. We showed in independent experiments that no significant loss of activity occurred in the 30–60 min required to complete the kinetic and inactivation measurements. In order to minimize further the possibility of spontaneous inactivation, a fresh enzyme solution was prepared, for each Nbf-Cl concentration used, by gel filtration before use. The reactions thus were not carried out at constant enzyme concentration (Table 1).

In addition to the unique thiol group, about one to two groups (difference between data of columns 6 and 7 in Table 1) react by $t = t_1$. As shown below, these are averages for partial reaction of a larger number of thiol groups.

The data of Table 1 and the results of numerous experiments not shown here indicate that the activity on reaction with Nbf-Cl always falls to a minimum non-zero value, i.e. about 35–45% of the original [see Bratcher & Kronman (1977) for detailed plots of activity versus time]. We have reported (Bratcher & Kronman, 1977) that whereas the standard assay procedure demonstrated 55–65% loss of catalytic activity, an initiation assay with [γ - ^{32}P]ATP as substrate showed no loss of activity on modifications with Nbf-Cl. Since product release is not favoured by the conditions of our standard assay, it appears that the decrease in activity that we observe reflects specific impairment of the RNA-elongation step.

The data of Table 1 indicate, as might be expected, that the time for maximum inactivation decreases with increasing concentrations of Nbf-Cl. However, the kinetic analysis shows that inactivation attains a maximum value when reaction with the single 'fast' thiol group is complete within experimental error.

We have reported earlier (Bratcher & Kronman, 1977) that neither GTP, ATP, rifamycin, calf thymus

DNA nor Rose Bengal protected RNA polymerase against inactivation by Nbf-Cl. No protection was observed by 1 mM-1,10-phenanthroline in the present study.

E. coli RNA polymerase has been shown to contain 2g-atoms of Zn^{2+} /mol of enzyme (Scrutton *et al.*, 1971; Wu *et al.*, 1977); 1,10-phenanthroline produces 50% inhibition of the enzyme at concentrations of about 1 mM (Scrutton *et al.*, 1971). The two zinc atoms are located on β - and/or β' -subunits (Wu *et al.*, 1977). The role of these metal ions is not clear, nor do we know their relative proximity within the intact enzyme. Our observations suggest, however, that the rapidly reacting thiol group is not near the Zn^{2+} site(s).

The failure of Rose Bengal to protect the enzyme against inactivation is of particular interest. Rose Bengal has been shown to be inhibitor of RNA polymerase activity (Wu & Wu, 1973a,b). It inhibits non-competitively with respect to nucleoside triphosphates, does not alter template binding and preferentially inhibits elongation. The latter observation in particular suggests that the site for Rose Bengal binding might be near the rapidly reacting thiol group. The fact that Rose Bengal does not protect RNA polymerase against reaction with Nbf-Cl at concentrations at which the dye binds to the enzyme (Wu & Wu, 1973b) might seem to militate against the near correspondence of the two sites. However, these observations themselves do not rule out the possibility that Rose Bengal is displaced from the enzyme by the Nbf-Cl.

In order to determine if such displacement does occur, a binding experiment was carried out that was similar to that employed by Wu & Wu (1973a). Our use of their gel-filtration technique demonstrated binding of 1.0 mol of the dye/mol of enzyme in a solution containing 1.08 μM -Rose Bengal and 2.3 μM -RNA polymerase. A column (1.5 cm $\times$ 9 cm) of Bio-Gel P-100 was employed and concentrations of dye and enzyme were determined by measurements of A_{545} and A_{280} respectively. A portion of the same

Rose Bengal/RNA polymerase solution was made 400 μ M in Nbf-Cl and promptly gel-filtered. The latter procedure was followed to minimize the reaction of Nbf-Cl with the enzyme, so as to determine if displacement might have occurred before reaction with the unique thiol group. The spectra of fractions containing the enzyme showed that no more than 0.2 mol of thiol had reacted per mol of enzyme. Binding of Rose Bengal to RNA polymerase was identical in the presence or absence of Nbf-Cl within experimental error. It would thus appear that the binding site for Rose Bengal and a putative binding site for Nbf-Cl (see the Discussion section) are not the same.

Reactivation of Nbf-Cl-inactivated RNA polymerase

The activity lost on reaction of Nbf-Cl with RNA polymerase can be restored by treatment with dithiothreitol or mercaptoethanol (Bratcher & Kronman, 1977). However, failure to remove excess Nbf-Cl by gel filtration before addition of the thiol results in irreversible inactivation. In a typical experiment carried out at 15°C, reaction of 1.81 μ M-RNA polymerase with 200 μ M-Nbf-Cl resulted in loss of 50% of the original activity in 15 min. After addition of dithiothreitol to a concentration of 2 mM without removal of excess Nbf-Cl, 66% of the original activity was first rapidly restored, but then decreased such that by 70 min only 16% of the original activity remained. A control solution of unused enzyme containing no dithiothreitol lost only 10% of its activity during a comparable period. Dialysis of the reaction mixture plus dithiothreitol against buffer in the cold for 24 h failed to restore activity.

The irreversible inactivation is probably due to reaction of the enzyme with an ill-defined reaction product formed between Nbf-Cl and an excess of thiol. This reaction product, referred to earlier as P2 (Nitta *et al.*, 1979), has been shown to undergo a non-specific reaction with proteins such as bovine serum albumin as well.

Identification of subunits with Nbf-labelled thiol groups

Separation of Nbf-Cl-labelled RNA polymerase into individual subunits was unsuccessful by a single method or by a sequence of steps. We therefore used two methods which showed that (a) the unique thiol group was located in the $\beta+\beta'$ -subunit fraction and (b) that it was most probably located on the β -subunit. In Expt. I (Table 2) the reaction between RNA polymerase and Nbf-Cl was followed spectrally until about 2.5 mol of Nbf had been incorporated/mol of enzyme. At that point excess reagent was removed by gel filtration (see the Materials and Methods section) and the enzyme dissociated in 6 M-urea before application to a Bio-Gel P-60 gel-filtration column. The earliest protein fraction containing $\beta+\beta'+\sigma$ -subunit had 88% of the Nbf label, with about 12% being found in the later fraction containing the α -subunit. The total incorporation of Nbf label was about 3.1 mol/mol of enzyme. This value is somewhat larger than the value of 2.5, the degree of reaction before removal of reagent. This difference is reasonable, since reaction is not actually terminated until the Nbf-Cl is separated from the enzyme within the gel-filtration column. Fraction A (Table 2) was rechromatographed on Bio-Gel P-100. The early protein fraction containing $\beta+\beta'$ -subunit had 96% of the Nbf label. Thus we can conclude that of the 3.1 Nbf groups introduced into the enzyme, 2.6 are found in β - and/or β' -subunit. This, of course, must include the unique thiol group.

In an independent experiment (Expt. II, Table 2) Nbf-Cl and dithiothreitol-free RNA polymerase were allowed to react until 1.5 Nbf groups had been introduced. After removal of excess reagent, the enzyme was dissociated in 6 M-urea before fractionation on an 'Affi-Gel-Blue' affinity column. The flow-through, containing $\beta+\alpha+\sigma$ -subunit, had 73% (1.3 groups) of the Nbf label, and β' -subunit, obtained by elution with 1 M-NH₄Cl, contained 27% (0.49 group).

Since we showed in Expt. I (Table 2) that the unique thiol group was in neither the σ - nor the α -subunit,

Table 2. *Identification of the site of reaction of Nbf-Cl with RNA polymerase*

RNA polymerase was labelled with Nbf-Cl as described in the text. Incorporation of the Nbf label was determined spectrophotometrically. Blue Dextran affinity chromatography was carried out as described by Wu *et al.* (1977), with the omission of dithiothreitol from the buffers.

Expt.	Separation method	Fraction	Subunits in fraction	Nbf incorporated (mol/mol of enzyme)	Percentage of label incorporated
I	P-60 gel filtration of urea-denatured enzyme	A	$\beta+\beta'+\sigma$	2.73	88
		B	α	0.36	12
		A+B	$\alpha+\beta+\beta'+\sigma$	3.09	(100)
	P-100 gel filtration of fraction IA	C	$\beta+\beta'$	2.62	96
		D	σ	0.11	4
		C+D = A	$\beta+\beta'+\sigma$	2.73	(100)
II	Sephacrose-Blue Dextran affinity chromatography	Flowthrough	$\alpha+\beta+\sigma$	1.34	73
		1 M-NH ₄ Cl eluate	β'	0.49	27
		Sum	$\alpha+\beta+\beta'+\sigma$	1.83	(100)

the observation that 73% of the label is found in the $(\alpha + \beta + \sigma)$ fraction indicates that it must be located in the β -subunit.

A total of approx. 1.8 mol of Nbf/mol of enzyme had been incorporated into the enzyme in Expt. II. The kinetics of reaction (see below) indicate that when 1.8 groups have reacted, 0.8–0.9 mol/mol of label must be on the rapidly reacting thiol group, and the remainder, 0.9–1.0 mol, is an average for partial reaction of a larger number of thiol groups. Thus the non-specific reaction of thiol groups in Expt. II must correspond to an average of 0.49 group for the β' -subunit and 0.44–0.54 group for $\alpha + \sigma$ -subunit.

The time course for the reaction of Nbf-Cl with RNA polymerase has been published previously (Bratcher & Kronman, 1977). The kinetic data were analysed by a Gauss-Newton non-linear regression technique (Fraser & Suzuki, 1973) for the function:

$$A_{420} = A_0 - C_1 e^{-k_1 t} + Bt \quad (1)$$

where t is time, C_1 is the absorbance change for a single group, k_1 the apparent pseudo-first-order rate constant for this group, and B and A_0 are parameters obtained from the fit. We have shown earlier (Bratcher & Kronman, 1977) that the reactive groups in RNA polymerase are thiols. We have therefore set C_1 equal to the absorbance change for a single thiol group for the enzyme concentration employed (see the Materials and Methods section). One test of the quality of the fit of the equation to the experimental data is the numerical correspondence of A_0 and C_1 . In our analyses A_0 and C_1 differed typically by about $\pm 4\%$. Convergence of fits was obtained with the above function in seven to ten iterations with standard deviations of experimental and calculated absorbances of the order of 5×10^{-4} .

Eqn. (1) was derived as follows. In general:

$$A_{420} = \sum_{i=1}^n A_i (1 - e^{-k_i t}) \quad (2)$$

where A_i is the change in absorbance for reaction with the i th thiol group with rate constant k_i . The sum is taken over all n reactive groups. If we expand all exponentials save one as Taylor Series we obtain:

$$A_{420} = A_1(1 + e^{-k_1 t}) + \sum_{i=2}^n [A_i k_i t + A_i (k_i t)^2 + \text{higher terms}] \quad (3)$$

For low degrees of reaction we can neglect terms beyond kt and obtain:

$$A_{420} = A_1(1 - e^{-k_1 t}) + t \sum_{i=2}^n A_i k_i \quad (4)$$

if we assume that the absorbance per group, A_i , is a constant:

$$A_{420} = A_0(1 - e^{-k_1 t}) + A_0 t \sum_{i=2}^n k_i \quad (5)$$

Comparison of eqns. (1) and (5), which have the same form, indicates that:

$$B = A_0 \sum_{i=2}^n k_i \quad (6)$$

Attempts to analyse the data by the Gauss-Newton non-linear least-squares procedure by a two-exponential function in place of eqn. (1) were unsuccessful, i.e. no convergence of fit could be obtained for experimental data obtained at any pH or Nbf-Cl concentration. The fact that the data could be described by the function given in eqn. (1) for the time range examined over the range of experimental conditions employed indicates that under these conditions almost complete reaction occurs at a single thiol group with concomitant slow partial reaction with a large number of such groups.

Examination of the kinetics of reaction as a function of reagent concentration and of pH revealed anomalies which, as we shall see, shed light on the origin of the unusual selectivity of the reagent for a single thiol group of RNA polymerase. Shown in Fig. 1 are plots of k_1 and B versus Nbf-Cl concentration obtained at pH 7.5. Note that neither the concentra-

Fig. 1. Dependence of kinetic parameters on Nbf-Cl concentration for reaction of Nbf-Cl with RNA polymerase at 15°C

(a) Dependence of the parameter B (eqn. 1). (b) Dependence of the apparent first-order rate constant for the rapidly reacting thiol group. The buffer employed was pH 7.50 (0.05 M-triethanolamine hydrochloride/NaOH/10% (v/v) glycerol/0.2 M-NaCl/1 mM-EDTA. The enzyme concentration was 1.77–2.35 μ M.

Fig. 2. Dependence of the apparent first-order rate constant on the parameter $[Nbf-Cl]/[H^+]$, for reaction of Nbf-Cl with RNA polymerase

The solid line represents the smoothed experimental data. The broken lines give the dependence of $\log k_1$ on the parameter $\log ([Nbf-Cl]/[H^+])$ calculated on the assumption that the reactive species is S^- and that the Nbf-S derivative is the only reaction product (see the text). k_0 is the pH-independent second-order rate constant ($\text{litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$). The pH range spanned in the plot is 7–8.5. The unfilled and filled circles represent data obtained at Nbf-Cl concentrations of 0.103 and 0.189 mM respectively. The other conditions were as specified in Fig. 1.

tion-dependence observed for k_1 nor for B are what might be expected for a pseudo-first-order reaction, i.e. linearity, with the curve passing through the origin.

The pH-dependence of the reaction of Nbf-Cl and RNA polymerase likewise exhibits an unexpected character. The pH range studied, 7–8.5, was unfortunately somewhat restricted because of the instability of the enzyme (Novak & Doty, 1970). The kinetic data (Fig. 2) were plotted in the form $\log k_1$ versus $\log ([Nbf-Cl]/[H^+])$ for reasons that will be explained below. The broken lines give the expected dependence of the reaction of a thiol group with pH-independent second-order rate constant, k_0 , as indicated. These curves are what would be expected if the S^- ion is the reactive species and the sole reaction product is the Nbf-S derivative (Nitta *et al.*, 1979). The value of k_0 observed for the reaction of Nbf-Cl and mercaptoethanol, in the absence of Meisenheimer complex-formation (see below), was found to be $2.8 \times 10^{-7} \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ (Nitta *et al.*, 1979). The data of Fig. 2 cannot be described by a linear relationship comparable with those shown as broken lines, the actual curve exhibiting a decided flatness in the pH region accessible to measurement.

A similar-shaped curve was observed for the pH-dependence for the rate constant for reaction of Nbf-Cl and mercaptoethanol (Nitta *et al.*, 1979). The

Scheme 1. Reaction of Nbf-Cl with a nucleophile RX^- . The scheme is taken from Baines *et al.* (1977).

characteristic form of the curve was the consequence of a reaction competitive with formation of the Nbf-Cl derivative. This competitive reaction appeared to be formation of a complex of the Meisenheimer (1902) type with S^- (structure I, Scheme 1) similar to that observed by Baines *et al.* (1977) with OH^- (structure II, Scheme 1). This process, which gave rise to the same kind of anomalous pH-dependence and dependence of k_1 on Nbf-Cl concentration observed with RNA polymerase, is due to formation of a Meisenheimer-type complex with thiol groups. We believe that such complex-formation can account for the unexpected action of Nbf-Cl with a specific thiol group (see the Discussion section).

Competitive reaction of Nbs_2 and Nbf-Cl with the thiol groups of RNA polymerase

The unique character of the rapidly reacting thiol groups of RNA polymerase can also be demonstrated by examining the competitive reaction of the thiol groups of the enzyme with Nbs_2 and Nbf-Cl. In the absence of Nbf-Cl, reaction of the thiol groups of the enzyme with 8.33 mM- Nbs_2 is rapid; approx. 16 of 28–34 thiol groups (Nicholson & King, 1973; Yarbrough & Wu, 1974) of the enzyme react in 20 min (curve A, Fig. 3). During the same time interval 0.223 mM-Nbf-Cl alone reacts with slightly

Fig. 3. Competitive reaction of Nbf-Cl and Nbs_2 with the thiol groups of RNA polymerase

Curve A (right-hand scale) represents the reaction of 8.33 mM- Nbs_2 with enzyme in the absence of Nbf-Cl as determined spectrophotometrically (see the Materials and Methods section). Curve B (left-hand scale) represents the reaction of 0.223 mM-Nbf-Cl in the absence of Nbs_2 . Curve C (left-hand scale) represents the reaction of 0.223 mM-Nbf-Cl in the presence of 8.33 mM- Nbs_2 . The extent of reaction of Nbf-Cl with enzyme was determined by the ^{14}C -incorporation technique (see the Materials and Methods section). Other experimental conditions have been described in Fig. 1.

more than two groups (curve B, Fig. 3). Nonetheless, when both reagents are present at the same concentrations as cited above, a single thiol group still reacts with Nbf-Cl during the 20 min interval (curve C, Fig. 3). The rate constant estimated from the data of curve C (Fig. 3) is of the order of 2×10^{-3} – $3 \times 10^{-3} s^{-1}$, which is comparable with that obtained for the rapidly reacting thiol group in the absence of Nbs_2 . These observations suggest that the unique thiol group of RNA polymerase is not intrinsically more reactive with thiol-group reagents, but rather that Nbf-Cl possesses unusual selectivity for this side-chain moiety. We believe that the thiol group in question may indeed be sterically inaccessible to reagents such as Nbs_2 (see the Discussion section).

Spectroscopic properties of Nbf-Cl-treated RNA polymerase

The u.v.-fluorescence spectrum of Nbf-Cl-treated polymerase is compared in Fig. 4 with that measured for native enzyme. The fluorescence parameters derived from such spectra excited at 280 nm are given in Table 3 for two independent experiments. The spectral properties of these derivatives were obtained immediately after gel filtration of the Nbf-Cl/RNA polymerase reaction mixture (see the Materials and Methods section).

RNA polymerase has 90 tyrosine and 20 tryptophan residues per molecule of mol.wt. 500000 (Fujika & Zurek, 1975), all of which should be excited at 280 nm. As is usually the case, even where the tyrosine content is very high relative to that of tryptophan, fluorescence

Fig. 4. U.v. emission spectra of native (curve I) and Nbf-Cl-modified (curve II) RNA polymerase

The spectra were measured at 25°C at an excitation wavelength of 280 nm. The Nbf-Cl-modified enzyme free of excess reagent was prepared as described in the Materials and Methods section and contained 2.0–2.7 mol of Nbf/mol of enzyme. The curves correspond to Expt. II (Table 3).

Table 3. *U.v. fluorescence properties of native and Nbf-Cl-modified RNA polymerase at 25°C*

RNA polymerase was labelled with Nbf-Cl by reaction at 15°C to the degree indicated in column (1). The extent of reaction was followed spectrophotometrically. The numbers of Nbf groups given in parentheses (column 1) were obtained from the change in absorbance when the reaction was terminated before gel filtration (see the Materials and Methods section), and the other values were calculated from the A_{420} of the gel-filtered derivative before measurement of fluorescence. Fluorescence parameters were obtained from corrected emission spectra excited at 280nm. The buffer employed was that given in Fig. 1.

Expt.	Nbf (mol/mol of enzyme) (1)	Emission maximum (nm)		Quantum yield	
		Native (2)	Nbf-Cl-modified (3)	Native (4)	Nbf-Cl-modified (5)
I	2.4 (2.4)	336 ± 1	336 ± 1	0.25	0.072
II	2.7 (2.0)	335 ± 1	335 ± 1	0.31	0.085

from tryptophan residues dominates the spectrum, i.e. tyrosine fluorescence is largely quenched. The quantum yield of the native enzyme (0.28–0.31; Table 3) is rather high compared with that observed for most proteins, e.g. of the 17 proteins examined by Kronman & Holmes (1971), only pepsinogen had a yield comparable with that obtained with native RNA polymerase. Quantum yields found for proteins were more commonly 0.05–0.15.

Formation of the Nbf-S derivative of the enzyme results in a 3–4-fold decrease in yield with no change

in the position of the emission maximum (Table 3). Indeed, it is possible to normalize the spectra to unit intensity at a given wavelength and achieve almost exact correspondence of emission spectra for native and Nbf-Cl-modified enzyme. The significance of these observations will be considered in the Discussion section. The possibility that the decrease in yield is the result of the energy transfer from tryptophan to the Nbf-S moiety of the enzyme is ruled out by the observation that no Nbf-S fluorescence (see below) could be detected with excitation at 280nm.

Fig. 5. Absorption and emission spectra of the Nbf group in Nbf-Cl modified RNA polymerase and in Nbf-OHETs. Absorption (—) and emission (---) spectra for Nbf-Cl-modified RNA polymerase (curves B) and absorption (----) and emission (----) for the Nbf-OHETs derivative (curves A) are shown. The emission spectra (right-hand curves) were excited at 420nm. The Nbf-enzyme derivative was the same used to obtain the spectrum of Fig. 4. Other conditions were as in Fig. 4.

A likely explanation for the large change in u.v.-fluorescence properties observed on reaction of Nbf-Cl with the enzyme is a localized conformational change. A more extensive conformational change involving extended regions of the RNA polymerase molecule or dissociation into subunits is ruled out by the observation that initiation of the transcription process (see above) is not at all impaired on reaction with Nbf-Cl.

Nbf-S fluorescence

In attempting to interpret the visible-fluorescence observations of Nbf-Cl-modified RNA polymerase, we were hampered by the paucity of detailed quantitative data on the spectral properties of the Nbf-S chromophore itself. For this reason we carried out extensive studies of the spectral properties of the Nbf-S chromophore (M. J. Kronman & J. Imperato, unpublished experiments), which are summarized below. We have also prepared Nbf-Cl derivatives of ovalbumin and bovine serum albumin to provide 'baseline' values of the spectral parameters for the chromophore in proteins. The latter protein is of particular interest in that it has a single thiol group (Peters, 1975).

The absorption and emission spectra of Nbf-Cl-modified RNA polymerase are compared in Fig. 5 with those observed with Nbf-OHEtS. The spectral parameters obtained with Nbf-OHEtS-enzyme derivative and those for ovalbumin and bovine serum

albumin are summarized in Table 4. The absorption maximum observed with the Nbf-S derivative of RNA polymerase lies about 13 nm lower than that observed with Nbf-OHEtS in water, whereas those for serum albumin and ovalbumin lie at 18–20 nm lower. The absorption maxima observed with serum albumin and ovalbumin correspond to that observed with papain (Allen & Lowe, 1973).

The emission maximum for Nbf-Cl-treated RNA polymerase lies at about 10 nm lower than that found for Nbf-OHEtS in water, and its quantum yield is about 13 times greater. The yield observed for the Nbf-S derivative of bovine serum albumin is about 6 times greater than that found for Nbf-OHEtS in water, and that obtained for ovalbumin is only about 50% greater. The emission maxima obtained for these two protein derivatives would appear to correspond to that observed for the low-molecular-weight thiol derivative.

Since all three parameters, absorption maxima, emission maxima and quantum yields, appear to be 'environmentally sensitive', these should be potentially useful in providing information about the molecular environment of the Nbf-S chromophore. The relationship between the spectral parameters for the Nbf-S parameter and solvent environment, however, is extraordinarily complex. Such properties were determined for Nbf-OHEtS and the Nbf-Cl derivative of ethylmercaptan in a wide range of solvents (M. J. Kronman & J. Imperato, unpublished experiments). These observations are summarized as follows.

Table 4. Visible-fluorescence parameters for Nbf-Cl-modified RNA polymerase, ovalbumin, bovine serum albumin and Nbf-OHEtS

Emission maxima and quantum yields were obtained from corrected spectra obtained with excitation at 420 nm. The buffer employed was the same as given in Table 3. The difference between Nbf-RNA polymerase-decay and Nbf-RNA polymerase+dithiothreitol background decay curve was too small to calculate lifetimes, but significant differences in counts were observed in early channels, suggesting the presence of a short-lived component (see the text).

$$f_1 = \frac{a_1}{a_1 + a_2}$$

where a_1 and a_2 are the pre-exponential amplitudes obtained in fit of decay data.

Protein	Nbf (mol/mol of protein)	Absorption maximum (nm)	Emission maximum (nm)	Quantum yield	Lifetime parameters		
					f_1	τ_1 (ns)	τ_2 (ns)
RNA polymerase	1.9	—	—	—	0.93	0.3	4
	3.8†	—	—	—	—	—	—
	2.4	413	521 ± 1	0.021	—	—	—
	2.7	413	521 ± 1	0.021	—	—	—
Bovine serum albumin	0.61	408	—	0.01	1.0	4	—
	0.49	408	530 ± 1	—	1.0	6	—
Ovalbumin	0.60	407	—	—	1.0	0.8	—
	1.0	406	530 ± 1	0.0025	0.75	0.8	2
Nbf-OHEtS	—	426	531 ± 1	0.0016	0.99	0.3	5

(a) Both absorption and emission maxima exhibit an increasing short-wavelength shift with decrease in solvent polarity. The emission maxima show dependence on the Kosower Z parameter (Kosower, 1968) comparable with that observed for other dye systems (Turner & Brand, 1968; Seliskar & Brand, 1971; Wu & Wu, 1973b). It would thus appear that the position of the emission maximum for the Nbf-S chromophore may be a good measure of the polarity of the protein molecule in the region of the fluorophore. Thus it would appear that the Nbf-S groups in RNA polymerase are in regions of relatively low polarity, whereas those in ovalbumin and bovine serum albumin are in contact with water (compare emission maxima with that observed for Nbf-OHETs, Table 4).

(b) The quantum yield of the fluorescence of Nbf-OHETs, by contrast, shows no systematic dependence on solvent polarity. The highest yield (0.015) was observed in 80–100% glycerol and the lowest was found in cyclohexane (0.001), with those for other solvents being intermediate between the values observed with glycerol and water.

(c) Lifetime measurements of the Nbf-S derivatives show two emitting components, with lifetimes of 0.3–2 and 6–12 ns. Magnitudes of τ and the relative contributions of the two components depended on the solvent, with nearly all of the emission in water coming from the short-lived component and nearly all of the emission in cyclohexane coming from the long-lived species.

(d) Comparison of the quantum yields and lifetimes obtained for the range of solvents studied leads to the conclusion that considerable quenching of fluorescence must occur in the ground state for both Nbf-OHETs and the Nbf-Cl derivative of mercaptoethanol. It seems likely that this is due to self-association of these compounds, since the magnitude of the molar absorption coefficient at the 260–290 nm band is quite dependent on solvent and on chromophore concentration, unlike that observed at the 420 nm band. Formation of self-association complexes in the excited state probably also occurs.

Environmental effects appear to influence strongly the yield and/or the lifetime of the Nbf-S chromophore in proteins as well, where presumably self-association is absent. The yields for the Nbf-Cl derivatives of RNA polymerase, bovine serum albumin and ovalbumin are all significantly higher than that observed with Nbf-OHETs (Table 4). Emission from the RNA polymerase derivative is predominantly from a short-lived component, whereas for bovine serum albumin it is from an emitter with a longer lifetime. The change in distribution of emitting components that occurs with ovalbumin with increase in mol of Nbf incorporated per mol of protein must reflect reaction at thiol groups with different molecular environments (Table 4). These observations indicate that, although it may not be possible to

derive molecular information from quantum yield and lifetime data for proteins, the sensitivity of these parameters to changes in environment may make Nbf-S derivatives of proteins serve a phenomenological function in signalling changes in molecular environment of these regions of the molecule.

Discussion

Before the observation reported earlier (Bratcher & Kronman, 1977) and in the present paper, there had been no indication that *E. coli* RNA polymerase had a 'unique' thiol group which on chemical reaction would give rise to altered catalytic properties. As we have indicated in the introduction, previous studies have shown that fairly substantial labelling of the most reactive thiol-groups can be carried out with a variety of thiol group reagents with little or no effect on the catalytic properties of the enzyme. Our observations with Nbf-Cl and RNA polymerase indicate, on the contrary, that the enzyme has a thiol group that on modification can give rise to a highly specific impairment of RNA-chain elongation.

However, subsequent to our original report (Bratcher & Kronman, 1977), a paper by Kamzolova *et al.* (1972) came to our attention. This publication appears to have escaped the notice of previous investigators concerned with the potential role of thiol groups in RNA polymerase catalysis. Kamzolova *et al.* (1972) found that on allowing *E. coli* RNA polymerase to react with the spin-label reagent 2,2,6,6-tetramethyl-4-(*p*-chloromercuricarboxy)benzoxy)piperidine-1-oxyl a single thiol group was labelled, with loss, at most of 50% of the catalytic activity and with no impairment of template binding or initiation of transcription. The correspondence of these features of the reaction with spin label to those observed with Nbf-Cl in the present study is striking. It seems likely that the same thiol group is involved in reaction with both reagents and that reaction at this thiol group gives rise to the same molecular events that alter the catalytic properties of the enzyme.

Although we have referred to the 'unique' thiol group of polymerase as a 'rapidly' reacting group, this is true only with respect to the rate of reaction relative to the other thiol groups of the enzyme. If we ignore for the moment the deviation of the kinetics of reaction of Nbf-Cl with RNA polymerase from true pseudo-first-order (Fig. 1) (see below for further consideration of this apparent kinetic anomaly), we can compare the rate constant for reaction of the 'unique' group with that observed previously (Nitta *et al.*, 1979) for the low-molecular-weight thiol, 2-mercaptoethanol, under comparable conditions of pH and Nbf-Cl concentration. This calculation shows that the rate constant for 2-mercaptoethanol is three times higher than observed with the 'rapidly' re-

acting thiol group of RNA polymerase. This is in contrast with the observations of Birkett *et al.* (1970), who found that the apparent rate constant for the 'fast' group of thiols in glyceraldehyde 3-phosphate dehydrogenase was about 10 times higher than that observed for low-molecular-weight thiol derivatives. Thus it would appear that Nbf-Cl possesses a unique 'selectivity' for reaction with a particular thiol group in RNA polymerase.

What then is the basis for the unique selectivity of Nbf-Cl for a particular thiol group in RNA polymerase? The kinetic anomalies observed on reaction of Nbf-Cl with RNA polymerase provide a clue in formulating an answer to this question. First of all, neither the parameter B , which eqn. (6) indicates is proportional to the rate constants for reaction of the 'slow' groups, nor the apparent rate constant for the rapidly reacting group show a dependence on Nbf-Cl concentration that might be expected for a pseudo-first-order reaction (Fig. 1). Secondly, a plot of $\log(k_{app.})$ versus $\log([Nbf-Cl]/[H^+])$ shows marked deviations from the linear dependence predicted on the assumption that the reactive species is S^- and that no products are formed other than the Nbf-S derivative of the enzyme (Fig. 2). Identical anomalous kinetic features were observed for the reaction of Nbf-Cl and 2-mercaptoethanol (Nitta *et al.*, 1979). These kinetic measurements, as well as the observation of characteristic absorption difference spectra in mixtures of reagent and thiol, demonstrated that a product other than the Nbf-S compound was also formed.

This product is most likely a Meisenheimer (1902) complex of Nbf-Cl and the thiolate ion, similar to the complex found with this reagent and OH^- ion by Baines *et al.* (1977). Scheme 1 (Baines *et al.*, 1977) illustrates the reaction paths for Nbf-Cl and a nucleophile RX^- to form the Nbf-X product (III) and the reversible Meisenheimer complex (IV). Analysis of the dependence of the apparent first-order rate constant for reaction of Nbf-Cl and 2-mercaptoethanol (Nitta *et al.*, 1979) on H^+ and Nbf-Cl in a form similar to that of Fig. 2 permitted calculation of the equilibrium constant for the equilibrium between compound I, RX^- and compound IV of Scheme 1, where RX^- was the thiolate form of 2-mercaptoethanol.

We propose that the anomalous kinetics of reaction observed with Nbf-Cl and RNA polymerase is the consequence of similar formation of reversible Meisenheimer complexes with the enzyme. Unfortunately, kinetic data could not be obtained over a sufficiently wide range of pH without denaturation to permit the kind of analysis that was done with the data for Nbf-Cl and 2-mercaptoethanol.

Nucleophilic groups in RNA polymerase that potentially might form Meisenheimer complexes with Nbf-Cl include, in addition to thiol groups,

tyrosine, histidine and tryptophan residues. We observed earlier (Nitta *et al.*, 1979) that thiol compounds and indole gave absorption difference spectra with Nbf-Cl, which we have taken to be indicative of Meisenheimer-complex-formation. No difference spectra were observed with Nbf-Cl and imidazole or anisole. The possibility of complex-formation with tryptophan groups is an attractive one in view of the dramatic change in the fluorescence of these groups of the enzyme (Fig. 4, Table 4) on reaction with Nbf-Cl (see below for further discussion).

There are several ways by which formation of a Meisenheimer complex with Nbf-Cl might lead to selective reaction with the unique thiol group. The Nbs₂- and Nbf-Cl-competition experiments (Fig. 3) suggest that this thiol group may not be available to most thiol reagents. Nbs₂ alone reacts with about 15 thiol groups in 20 min, whereas Nbf-Cl reacts with only 2.5 groups in the same time interval (compare curves A and B, Fig. 3). Nonetheless, Nbf-Cl can compete very effectively with Nbs₂ for reaction at the unique group (curve C, Fig. 3).

These observations suggest that the unique thiol group is normally masked in the enzyme and not available for reaction. Formation of a Meisenheimer complex of Nbf-Cl with a nucleophilic group in the enzyme with a concomitant conformational change might then make this thiol group available for reaction. Such a reaction could occur in one of several ways: (a) dissociation of the complex to yield a free Nbf-Cl molecule, which would then attack the newly exposed thiol group; attack of a second molecule of Nbf-Cl on the exposed thiol group while the first Nbf-Cl molecule in Meisenheimer complex maintains the enzyme in an 'open' conformation.

The first explanation seems less likely, since the rate of reaction of the Nbf-Cl molecule dissociated in the immediate vicinity of the unique thiol group might be expected to be high, higher indeed than reaction with a free Nbf-Cl molecule in solution. This is to be contrasted with the observation (see above) that the rate of reaction of Nbf-Cl with the unique thiol group is much smaller than that observed with the reagent and low-molecular-weight thiol. Parenthetically, it should be noted that this same consideration would seem to militate against the Meisenheimer-complex-forming nucleophiles being thiol groups, since such thiol groups would likewise be expected to react rapidly with the reagent. The above conclusions must be regarded, however, as very tentative, since at present we have no information as to the relative rates of complex-formation, conformational change of the enzyme and attack of the thiolate ion.

The absence of any protective effect of GTP, rifamycin and DNA template on reaction of RNA polymerase with respect to reaction with Nbf-Cl indicates that the unique thiol group is not located within the active-site region. The absence of total

abolition of catalytic activity suggests that the catalytic site was not directly affected and thus must be the consequence of an indirect effect, as might result from a conformational change having its origin at a locus some distance from the active site. The 3–4-fold decrease in the tryptophan quantum yield (Table 3) observed on reaction of the enzyme with Nbf-Cl supports this hypothesis. We cannot, however, distinguish between the putative conformational change occurring on formation of the Nbf-Cl–RNA polymerase Meisenheimer complex (see above) and the conformational change that might occur on forming the Nbf-S derivative.

The 3–4-fold decrease in tryptophan quantum yield took place without a detectable change in the wavelength maximum of the emission spectrum or in the band shape (Fig. 4, Table 4). These observations suggest first of all that, although the enzyme contains 20 tryptophan residues (Fujika & Zurek, 1975), the fluorescence from relatively few of these must dominate the emission spectrum. Secondly, the molecular environment of these few residues must be rather profoundly altered on Nbf-Cl modification of the enzyme. Our conclusion that fluorescence from relatively few tryptophan residues must dominate the emission spectrum is not without precedent. Tryptophan emission from hens'-egg-white lysozyme which contains six such residues, appears to occur, for example, primarily from residues 62 and 108 (Imoto *et al.*, 1972).

Although there are some significant differences in the feature of inhibition of RNA polymerase by Rose Bengal (Wu & Wu, 1973*a,b*) and Nbf-Cl, there are some similarities that make comparison worthwhile. Both Rose Bengal (Wu & Wu, 1973*a*) and Nbf-Cl preferentially inhibit RNA-chain elongation. The characteristics of the inhibition led Wu & Wu (1973*a,b*) to conclude that the Rose Bengal-binding site is not within the active-site region and that the inhibition of elongation must be the consequence of local conformational change. Such a conformational change might be expected to give rise to changes in the molecular environment of vicinal tryptophan residues. Binding of Rose Bengal does indeed lead to a nearly 2-fold decrease in the quantum yield for tryptophan fluorescence with no apparent change in the wavelength maximum of the emission spectrum or in emission band shape (Wu & Wu, 1973*b*). These observations likewise suggest that the tryptophan fluorescence spectrum of *E. coli* RNA polymerase is dominated by emission from relatively few residues. Wu & Wu (1973*b*) concluded from these observations that changes in tryptophan fluorescence resulted entirely from tryptophan–Rose Bengal energy transfer. Their published observations, although demonstrating that such energy transfer occurs, did not show that the decrease in yield is quantitatively the result of such a transfer. It seems likely that the

change in fluorescence properties must result directly, at least in part, from their proposed conformational change.

The features of the inhibition of elongation by Rose Bengal and by Nbf-Cl suggest that they may share a common basis for their action. If indeed the tryptophan fluorescence is dominated by relatively few residues of high quantum yield, the large changes with both reagents suggest that the conformational changes may involve the same region of the enzyme molecule. We have shown that reaction of Nbf-Cl with the unique thiol is unaffected by Rose Bengal binding and that formation of the putative Nbf-Cl–enzyme Meisenheimer complex does not displace Rose Bengal from the enzyme. Thus it is very unlikely that the unique thiol group is located within the Rose Bengal-binding site. Independent evidence for the latter conclusion is also found in the absence of Nbf–tryptophan energy transfer in the Nbf-S derivative of the enzyme. If we use the average distance obtained by Wu & Wu (1973*b*) for Rose Bengal–tryptophan transfer and the value of the overlap integral J (Forster, 1947) as calculated from the tryptophan emission spectrum and the Nbf-S absorption spectrum, we find that the transfer efficiency should be about 10%, contrary to what was observed. The above observations suggest that a similar conformational change leading to impairment of RNA-chain elongation can be initiated by a molecular event(s) from different loci on the enzyme molecule.

It is not clear, however, where in the enzyme molecule the conformational change takes place. We have shown that the unique thiol group is located on the β -subunit (Table 2). Studies with RNA polymerase prepared from mutant strains of *E. coli* have shown that a particular amino acid sequence of the β -subunit is requisite for the binding of the inhibitor rifamycin (Rabussay & Zillig, 1969). However, it has not been possible to identify unambiguously the catalytic site with any single subunit; isolated subunits and subcomplexes are devoid of activity. The smallest species that will catalyse RNA-chain elongation is core enzyme, $\alpha_2 + \beta + \beta'$. Even in this case a particular arrangement of subunits is required for activity, i.e. studies with reassembly of enzyme from isolated subunits have shown that an inactive 'premature' enzyme molecule can form in which the arrangement of subunits is probably different from that in native enzyme (Fukuda & Ishihama, 1974; Palm *et al.*, 1975; Harding & Beychok, 1974). Cross-linking studies by Hillel & Wu (1977) demonstrated that β – β' -, α – β - and α – β' -subunit contacts are found in the holoenzyme. Reaction of the unique thiol group on the β -subunit might thus give rise to structural changes at subunit interfaces that could then be propagated within other subunits whose elements make up the RNA-chain-elongation subsite.

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