Anomalous Reaction of 4-Chloro-7-nitrobenzofurazan with Thiol Compounds

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(Received 25 April 1978)

The kinetics of reaction of 4-chloro-7-nitrobenzofurazan with thiol groups at pH values above 5 cannot be accounted for solely on the basis of formation of a single product, the 4-thio derivative. Spectroscopic observations indicate that, in addition to the 4-thio derivative, at least two other products are formed. One of these, referred to as P_1 , is most likely a reversible complex of thiol compound and 4-chloro-7-nitrobenzofurazan of the Meisenheimer type. The other product, P_2 , which forms primarily when thiol compound is in a large excess, does not appear to result from direct reaction of thiol group and 4-chloro-7-nitrobenzofurazan, but may be a reaction of product P_1 and thiol compound. The coloured product, P_2 , will react further with proteins, such as bovine serum albumin and *Escherichia coli* RNA polymerase. This reaction irreversibly destroys the catalytic activity of RNA polymerase. The implications of these observations for utilization of 4-chloro-7-nitrobenzofurazan as a protein-modifying agent are discussed.

Nbf-Cl has proved to be a very useful reagent for introducing a fluorescent label into proteins. Reaction appears to occur primarily at thiol groups (Birkett *et al.*, 1970a), although reports have appeared indicating reaction at amino (Birkett *et al.*, 1970a) and tyrosyl (Ferguson *et al.*, 1974) groups. One of the attractive features of this reagent is that the site of labelling can be readily determined from the spectroscopic properties of the product (Birkett *et al.*, 1970a). Reactivity of thiol groups has been studied for papain, bromelain and ficin (Shipton *et al.*, 1976), phosphorylases *a* and *b* (Dwek *et al.*, 1972; Birkett *et al.*, 1971), glyceraldehyde phosphate dehydrogenase (Birkett *et al.*, 1970b), adenylate kinase (Price, 1972), and of tyrosyl groups for bovine heart mitochondrial adenosine triphosphatase (Ferguson *et al.*, 1974), chloroplast coupling factor 1 (Cantley & Hammes, 1976) and *Escherichia coli* (Mg^{2+}/Ca^{2+})-stimulated adenosine triphosphatase (Nelson *et al.*, 1974). Since Nbf-Cl is stable and will react over a wide range of pH values, the choice of pH for reaction seems to have been based in general on the intrinsic properties of the protein, such as its stability. Other than a preferential pH-dependence for the Nbf-S to Nbf-N transfer (Birkett *et al.*, 1970a), there seems to

be no hard and fast rule that relates pH and specificity of reaction. With the exception of the plant-derived proteolytic enzymes (Shipton *et al.*, 1976), all of the proteins cited above were allowed to react with Nbf-Cl in the pH range 7–8.

Considerations of stability led us to restrict studies of thiol reactivity in *Escherichia coli* RNA polymerase to pH values above 6. We have shown that a single thiol group reacts very rapidly at pH 7.5 with impairment of the elongation step of the transcription process catalysed by this enzyme (Bratcher & Kronman, 1977). A parallel reaction of a relatively large but indeterminate number of thiol groups occurs at a much lower rate. To determine the basis for the unique reactivity of the single 'active-site-related' thiol group, we studied the kinetics of reaction in some detail, including an examination of the pH-dependence of the reaction rate (M. J. Kronman, K. Nitta & S. C. Bratcher, unpublished work). Kinetic anomalies observed in these studies with RNA polymerase led us to examine comparable kinetic properties for the reaction of Nbf-Cl with a simple thiol derivative, 2-mercaptoethanol. The anomalous kinetic behaviour observed with this simple system, together with spectroscopic observations that explain these results, are the subject of this report.

Subsequent to completion of our studies the report by Baines *et al.* (1977) came to our attention. Their investigations showed that a Meisenheimer-type complex (Meisenheimer, 1902) could be formed reversibly with OH^- at alkaline pH at position C-6 of Nbf-Cl. The anomalous kinetics that we report in

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Abbreviations used: Nbf-Cl, 4-chloro-7-nitrobenzofurazan (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole); Nbf-S, 4-nitro-7-thiobenzofurazan; Nbf-OHEtS, 4-(2'-hydroxyethylthio)-7-nitrobenzofurazan; Nbf-N, 4-amino-7-nitrobenzofurazan.

the present paper support the view that a similar complex can be formed with thiol compounds. However, we also show that an additional product or products form that can react in an irreversible manner with proteins.

Experimental

Nbf-Cl and 2-mercaptoethanol were obtained from Pierce, Rockford, IL, U.S.A., and Matheson-Coleman-Bell, Norwood, OH, U.S.A., respectively. The m.p. of the Nbf-Cl was found to be 97°C, corresponding to that reported previously (Ghosh & Whitehouse, 1968). Dithiothreitol (grade A) and triethanolamine were products of Calbiochem, La Jolla, CA, U.S.A. All other chemicals were of reagent grade. With the exception of triethanolamine, which was recrystallized twice from hot water, all chemicals were used as received. Deionized charcoal-filtered water was used in preparing buffers. Nbf-OHEtS was prepared by a method similar to that of Allen & Lowe (1973).

Absorption spectral measurements were made with a Cary 118C spectrophotometer with the most sensitive scales and zero suppression appropriate to the spectral changes encountered. Kinetic measurements were made at 15.0°C in thermostatically controlled cell holders. To initiate reaction of Nbf-Cl with 2-mercaptoethanol or with RNA polymerase, a 1 ml sample of the thiol species was equilibrated in the thermostatically controlled semi-micro cell, after which 0.02 ml of concentrated Nbf-Cl solution (15–95 mM) in 95% ethanol was added and the cell contents were mixed by inversion against a plastic-film cell cover. Concentrations of Nbf-OHEtS formed in the reaction were calculated from the A_{420} by using a molar absorption coefficient of 13000 (Dwek *et al.*, 1972).

Results

Kinetics of reaction of Nbf-Cl with 2-mercaptoethanol

The anomalous kinetic behaviour of RNA polymerase manifested itself in two kinds of observations: (a) plots of the apparent first-order rate constant against Nbf-Cl concentration did not go through zero for either class of reactive thiol; (b) the apparent second-order rate constants showed an unexpectedly small dependence on pH in the 7–8.5 range. Measurements could not be extended to much lower pH because of instability of the enzyme.

Similar kinds of measurements were therefore made for the reaction of Nbf-Cl with 2-mercaptoethanol over the pH range 4–7.5. With the exception of a group of experiments carried out with an excess of 2-mercaptoethanol (see Fig. 2), all of the kinetic observations were made with a minimum molar ratio of Nbf-Cl to 2-mercaptoethanol of 2:1. Observations were restricted to times corresponding to less than 4%

consumption of Nbf-Cl. The kinetics were analysed as pseudo-first-order, but since such kinetics were anomalous (see below), we will refer to the rate constant, $k_{app.}$, as an apparent rate constant.

Plots of the apparent rate constant against Nbf-Cl concentration at a constant 2-mercaptoethanol concentration are shown in Fig. 1. The concentration-dependencies at pH 7.00 and 7.50 are highly anomalous; at pH 7.50 the rate constant is nearly independent of Nbf-Cl concentration over a 7-fold range. At pH 7.00 a very weak dependence on concentration is observed, independent of the buffer employed. However, the best line through the experimental points by no means goes through zero. The rate constant at pH 6.00 shows a much greater dependence on Nbf-Cl concentration, but the curve likewise does not go through zero. The concentration-dependence of $k_{app.}$ at pH 5.00 appears to be normal for a pseudo-first-order reaction. Similar observations were made below pH 5 (results not shown here). A more detailed kinetic analysis of the data is reserved for the Discussion section of this paper.

The kinetics of reaction where 2-mercaptoethanol was in excess were even more surprising (Fig. 2). Under conditions where we would expect a nearly 20-fold increase in rate constant for the range of 2-mercaptoethanol concentrations employed, a 6-fold decrease in rate constant is observed!

The above kinetic observations lead to the conclusion that the consumption of Nbf-Cl and of 2-

Fig. 1. Dependence of the apparent first-order rate constant on Nbf-Cl concentration for reaction of 2-mercaptoethanol with excess reagent at 15°C

The numerical values of the pH of measurement are given in the Figure. The 2-mercaptoethanol concentrations were 0.034 mM at pH 7.00 and 7.50, 0.068 mM at pH 6.00 and 0.137 mM at pH 5.00. The buffer compositions were: 0.05 M-sodium citrate/citric acid at pH 7.50 (Δ) and at 7.00, 6.00 and 5.00 (●), 0.05 M-triethanolamine HCl/NaOH at pH 7.50 (Δ) and 0.05 M-KH₂PO₄/Na₂HPO₄ at pH 7.00 (○).

Fig. 2. Dependence of the apparent first-order rate constant on 2-mercaptoethanol concentration for reaction of Nbf-Cl with excess 2-mercaptoethanol at 15°C

The buffer was 0.05 M-triethanolamine HCl/NaOH (pH 7.50). The Nbf-Cl concentrations were 0.076 mM (Δ), 0.057 mM ($\bullet$), 0.039 mM ($\circ$) and 0.019 mM ($\bullet$).

Fig. 3. Absorption spectra of Nbf-Cl (curve 1) and Nbf-OHEtS (curve 2) in water adjusted to pH 7.1

mercaptoethanol must give rise to products other than the Nbf-S derivative. For this reason we have examined the spectra of mixtures of Nbf-Cl and 2-mercaptoethanol during the course of the reaction, to detect products other than the Nbf-S derivative.

Spectra of Nbf-Cl/2-mercaptoethanol and Nbf-Cl/dithiothreitol reaction products

To magnify spectral changes of reactant and products a difference spectral technique was employed by using tandem cylindrical cells (2.00 cm path length per compartment) of the kind usually used for solvent perturbation measurements (Herskovits & Laskowski, 1962). The usual arrangement of solutions was: reference beam (Nbf-Cl, buffer); (2-mercaptoethanol, buffer); sample beam: (Nbf-Cl+2-mercaptoethanol,

Fig. 4. Difference spectra of mixtures of Nbf-Cl and 2-mercaptoethanol at ambient temperature

The spectra were scanned at a rate of 30 nm/min from high to low wavelength. The times given below are for the initial wavelength in the scan. The solution containing the Nbf-Cl+2-mercaptoethanol was in the sample beam (see the text for details). The spectra in set I were obtained in 0.05 M-triethanolamine HCl/NaOH (pH 7.5) at an Nbf-Cl concentration of 0.102 mM and a 2-mercaptoethanol concentration of 2.28 mM. The times for the individual curves in set I were 2 min (curve 1), 12 min (curve 2), 22 min (curve 3), 42 min (curve 4) and 72 min (curve 5). The spectra in set II were obtained in 0.05 M-triethanolamine HCl/NaOH buffer (pH 7.5) at an Nbf-Cl concentration of 0.104 mM and a 2-mercaptoethanol concentration of 2.28 mM. The times for the individual curves in set II were 3 min (curve 1), 15 min (curve 2), 35 min (curve 3) and 60 min (curve 4). The spectra in set III were obtained in 0.05 M-sodium citrate/citric acid buffer (pH 5.0) at an Nbf-Cl concentration of 0.112 mM and a 2-mercaptoethanol concentration of 2.28 mM. The times for the individual curves of set III were 3 min (curve 1), 27 min (curve 2), 57 min (curve 3), 87 min (curve 4), 117 min (curve 5), 147 min (curve 6), 177 min (curve 7), 207 min (curve 8) and 237 min (curve 9).

buffer); (buffer). The expected difference spectrum where Nbf-S is the only product should exhibit a

positive band at approx. 425nm, corresponding to product formation, and a negative band at approx. 343nm, corresponding to disappearance of Nbf-Cl (see Fig. 3 for absolute spectra of Nbf-Cl and of the Nbf-S derivative of 2-mercaptoethanol). Furthermore, the ratio A_{425}/A_{343} should be independent of time. The difference spectra obtained at pH 5.00 (curves III, 1-9, Fig. 4) appear to correspond solely to formation of the Nbf-S derivative of 2-mercaptoethanol. When the observed absorbances were plotted as a function of time and values of A interpolated for corresponding times, the ratio A_{425}/A_{343} was equal to 1.693 ± 0.015 for the entire time range examined, approx. 250min. The difference spectra at pH 7.50 (curves I, 1-5, and II, 1-4, Fig. 4) showed by contrast bands that could not be accounted for on the basis of formation of Nbf-S. With a 20-fold excess of 2-mercaptoethanol (curves I, Fig. 4), a positive band is observed at about 420nm that shifts with increasing time toward longer wavelengths. It seems likely that this band corresponds, at least in part, to formation of the Nbf-S derivative of 2-mercaptoethanol. The negative band at 358nm, which shifts toward 350nm with increasing time, reflects, in part, disappearance of Nbf-Cl.

The most striking feature of these spectra is the positive band observed at 305-307nm. The amplitude of this band changes very little with increasing time, attaining its near-maximal value at the earliest time observed. This band must correspond to a product other than the Nbf-S adduct. The fact that this difference band attains its near-maximal amplitude at the earliest time observed suggests that this product is formed more rapidly than Nbf-S.

The product corresponding to the 307nm band is formed with the release of protons. Addition of Nbf-Cl to thiol compound (molar ratio, thiol compound/Nbf-Cl, 50:1) in an unbuffered solution maintained at pH 7.00 in a pH-stat gave rise to a very rapid titration with base to compensate for proton release. Subsequent addition of base was much slower as protons were released during formation of Nbf-S.

The exact shape of the 307nm band cannot be determined directly, since it is obscured by the spectral changes corresponding to disappearance of Nbf-Cl and appearance of Nbf-S. The shifts of the 358nm and the 425 to 430nm bands relative to the peak positions for Nbf-Cl and the Nbf-S adduct of 2-mercaptoethanol (see Fig. 3), however, suggest that the 307nm band in the difference spectrum may correspond in a component-resolved absolute spectrum to a band extending to much longer wavelengths or to formation of a second product (see below).

When the molar ratio of 2-mercaptoethanol to Nbf-Cl was increased to 220:1 (curves II, 1-4, Fig. 4), the band near 420nm was almost completely suppressed, indicating that virtually no Nbf-S adduct is formed. This is in accord with the kinetic observation

Fig. 5. Difference spectra of mixtures of Nbf-Cl and dithiothreitol at ambient temperature

Spectra were scanned at a rate of 30nm/min from high to low wavelength. The times given below were for the initial wavelength in the scan. The solution of Nbf-Cl+thiol compound were in the sample beam (see the text for details). The buffer used was 0.05M-triethanolamine HCl/NaOH (pH 7.5). The spectra of set I were obtained at an Nbf-Cl concentration of 0.148mM and a dithiothreitol concentration of 9.97×10^{-3} mM at 3min (curve 1), 13min (curve 2), 23min (curve 3), 38min (curve 4), 58min (curve 5) and 88min (curve 6). The spectra in set II were obtained at an Nbf-Cl concentration of 0.0742mM and a thiol concentration of 9.38mM at 2min (curve 1), 12min (curve 2), 22min (curve 3), 42min (curve 4), and 72min (curve 5).

(Fig. 2) that the rate constant for formation of Nbf-S decreased markedly with increase in 2-mercaptoethanol concentration when 2-mercaptoethanol was in excess. The negative difference spectral band near 360nm and the large positive band observed at about 307nm are comparable with those observed at the lower thiol compound concentration (cf. curves I and

II, Fig. 4). The broadness of the positive band above 400nm may be due to a 'tail' from the 307nm band as suggested above. More likely, however, as will be seen with dithiothreitol+Nbf-Cl difference spectra (see below), the broad band is due to product(s) other than that corresponding to the 307nm band or to the Nbf-S adduct. It is especially interesting that the 307nm peak begins to decrease in amplitude at the longest time observed, seemingly paralleling an increase in amplitude of the broad band above 400nm (curves II, Fig. 4). The spectral changes that we observe, at least below 425nm, appear to correspond to those alluded to by other investigators, as summarized by Baines *et al.* (1977).

In looking at spectra of mixtures of dithiothreitol and Nbf-Cl in buffer, it is apparent that the pattern of reaction of these substances resembles that observed with 2-mercaptoethanol (Fig. 4). In the presence of a large excess of Nbf-Cl compared with dithiothreitol (curves I, 1-6, Fig. 5), a positive difference peak at about 310nm is seen (compare with spectra for 2-mercaptoethanol, Fig. 4). The amount of this component disappears with time as an increasing amount of what appears to be the Nbf-S derivative is formed (425nm band). A broad band extending out to 700nm underlies the Nbf-S peak. When dithiothreitol is in excess with respect to Nbf-Cl, the Nbf-S peak, if present at all, appears to be overwhelmed by the broad band extending from 400 to 700nm (curves II, 1-5, Fig. 5), the band at approx. 307nm being resolved only at the earliest time observed. (Such solutions have a purple-brown colour.) The amplitude of the very broad band is more than 10 times greater than that observed when Nbf-Cl is in excess (cf. curves I and II, Fig. 5; note difference in scales for the two sets of curves). Thus the extent of formation of the coloured product is higher when dithiothreitol is in excess. Comparison of curves I and II of Fig. 5 likewise would suggest that as the thiol concentration is increased, coloured product is formed at the expense of Nbf-S adduct.

It is noteworthy that indole and Nbf-Cl at pH 5.00 and 7.50, at concentrations of 458 and 134 μM respectively, give rise to difference spectra (results not shown) with positive bands at about 300nm and negative ones at approx. 360nm. These spectra developed more slowly than those seen with Nbf-Cl and thiol compound and were clearly time-dependent. No such spectra were observed in solutions of Nbf-Cl and anisole or imidazole. These observations with indole are of interest in terms of our conclusion that Nbf-Cl and 2-mercaptoethanol form a reversible Meisenheimer-type complex (see the Discussion section).

Effect of Nbf-Cl/thiol compound reaction products on proteins

Although virtually complete reversal of inhibition

of RNA polymerase activity by Nbf-Cl can be achieved on addition of 2-mercaptoethanol or dithiothreitol when excess Nbf-Cl is removed before addition of thiol compound (Bratcher & Kronman, 1977), if thiol compound is added without such removal a purple-brown colour develops and irreversible inactivation occurs. The visible spectrum of the product shows a broad band extending from 700nm down to the u.v. region. The spectral features closely resemble those observed with excess of thiol compound and Nbf-Cl (Figs. 4 and 5). Similar observations were made with bovine serum albumin that had been treated with Nbf-Cl and then with dithiothreitol in a manner similar to that used with RNA polymerase. The characteristic visible spectral features were retained for both serum albumin and RNA polymerase even after the proteins were denatured and dialysed against 6M-guanidine hydrochloride. These observations suggest that the coloured product is either very strongly bound to the protein or is covalently attached. The significance of these observations will be considered in the Discussion section.

Discussion

Our kinetic and spectroscopic observations indicate that Nbf-Cl and low-molecular-weight thiol compounds form products above pH 5.00 other than the Nbf-S compound. The product or products, which we designate P_2 , appear to form in significant amounts when thiol compound is present in a large excess. We shall consider the properties of P_2 subsequently.

The rate of formation of the second product, designated P_1 , corresponding to the 307nm band in the difference spectra (Figs. 4 and 5) appears to be faster than that of the Nbf-S product. The rapid release of protons in the pH-stat experiments in mixtures of Nbf-Cl and thiol compound and the small amplitude of the 307nm band at pH 5.00 (Fig. 4) suggest that the rate of formation of P_1 is pH-dependent. The detailed study of the reaction of OH^- with Nbf-Cl by Baines *et al.* (1977) provides a useful framework in considering the formation of Nbf-OHETs and P_1 . Their observations strongly support the view that in addition to the C-4 alkylation product (III, Scheme 1) a reversible Meisenheimer complex (Meisenheimer, 1902) forms at C-6 (IV). If we identify P_1 with this Meisenheimer complex with RX^- equal to RS^- , we can describe the reaction of 2-mercaptoethanol and Nbf-Cl by Scheme 2.

The following expression for the time-dependence of the concentration of Nbf-SR can be derived, assuming Nbf-Cl to be in excess.

$$\begin{aligned} [\text{Nbf-SR}] = \\ [\text{R-SH}]_0 \left[1 - \exp \left(\frac{k_0 K_1 [\text{Nbf-Cl}]_0 / [\text{H}^+]}{1 + K_0 K_1 [\text{Nbf-Cl}]_0 / [\text{H}^+]} \right) t \right] \end{aligned} \quad (1)$$

Scheme 1. Reaction of NbF-Cl with a nucleophile RX^- (taken from Baines *et al.*, 1977)

Scheme 2. Reaction of NbF-Cl with a thiol compound R-S- to form the covalent product NbF-SR and the Meisenheimer complex P_1

The equilibrium constant, K_0 , for formation of the P_1 complex is defined as an association constant.

Fig. 6. Dependence of the apparent first-order rate constant on the parameter $\log ([\text{NbF-Cl}]/[\text{H}^+])$ for reaction of NbF-Cl with 2-mercaptoethanol at pH 7.50 ($\circ$), 7.00 (Δ), 6.00 ($\square$) and 5.00 (∇)

The data are identical with those of Fig. 1.

where $[\text{R-SH}]_0$ and $[\text{NbF-Cl}]_0$ are the initial concentrations of the reactants and K_1 is the ionization constant for 2-mercaptoethanol. The apparent first-order rate constant, k_{app} , for formation of NbF-SR will thus be:

$$k_{\text{app}} = \frac{k_0 K_1 [\text{NbF-Cl}]_0 / [\text{H}^+]}{1 + K_0 K_1 [\text{NbF-Cl}]_0 / [\text{H}^+]} \quad (2)$$

Fig. 6 shows a plot of the kinetic data obtained with 2-mercaptoethanol for the pH range 5–7.5, NbF-Cl concentration, 0.2–2 mm. The line was fitted to the data by the method of Kita *et al.* (1976). $\log (k_{\text{app}})$ approached a linear dependence on $\log ([\text{NbF-Cl}]_0 / [\text{H}^+])$ at low values of this parameter, in accord with

the predictions of eqn. (2). The linear portion of the curve had a slope of 0.91 in reasonable agreement with the stoichiometry of Scheme 2. Eqn. (2) likewise predicts that with an increase in $[\text{NbF-Cl}]_0 / [\text{H}^+]$, k_{app} should approach a limiting value, k_0 / K_0 . $\log (k_{\text{app}})$ does approach such a limiting value (Fig. 6). By using a value of 3.16×10^{-10} for the ionization constant, K_1 , of 2-mercaptoethanol (Tanford, 1962) and the data of Fig. 6, we obtain values of 2.8×10^{-7} litre $\cdot$ mol $^{-1} \cdot$ s $^{-1}$ and 1.8×10^6 litre $\cdot$ mol $^{-1}$ for k_0 and K_0 respectively. The value of 1.8×10^6 obtained for K_0 , the association constant for formation of the

putative C-6 Meisenheimer complex of Nbf-Cl and 2-mercaptoethanol, is about 250-fold greater than the value obtained by Baines *et al.* (1977) for the OH⁻ complex.

The kinetics observed for formation of Nbf-OHEtS are inconsistent with the direct formation of P₂ from Nbf-Cl and thiol compound (Scheme 3).

In this pathway, the apparent rate of formation of Nbf-SR should have a comparable dependence on Nbf-Cl and on 2-mercaptoethanol concentration, irrespective of which is in excess. What we observe, however, is that the apparent first-order rate constant shows an increase with increase in Nbf-Cl concentration when this reagent is in excess (Fig. 1) and a decrease with increase in 2-mercaptoethanol concentration when the thiol compound is in excess (Fig. 2).

Our observations do not allow us to specify a unique pathway for formation of P₂. The broad absorption spectrum extending to rather long wavelengths (Figs. 4 and 5) suggests that the product is of much greater complexity than P₁ or Nbf-SR.

Some remarks are in order on interpretation of kinetic observations with RNA polymerase in terms of the present study. Reaction of Nbf-Cl with this enzyme has been shown to be radically different (Bratcher & Kronman, 1977) from that observed with other thiol-group reagents. The reaction of RNA polymerase with Nbf-Cl gives unique reaction at a single thiol group to specifically impair the elongation phase of RNA synthesis. No such unique thiol group has been observed with other thiol reagents. Baines *et al.* (1977) have suggested that Nbf-Cl might form a Meisenheimer complex with a highly nucleophilic centre in a protein, bringing about a conformational change that would then give rise to direct alkylation at the usual C-4 position. The unusual reactivity of RNA polymerase with Nbf-Cl appears to be an example of this type of process [detailed experimental spectroscopic and kinetic evidence has been obtained (M. J. Kronman, K. Nitta & S. C. Bratcher, unpublished work)].

The complex reaction pattern of Nbf-Cl with low-molecular-weight thiol compounds would appear to complicate the use of this reagent in characterizing thiol-group reactivity in proteins. Although the protein concentrations employed are frequently much lower than those of the low-molecular-weight thiol

compounds used in the present study, it is apparent from our studies with *E. coli* RNA polymerase that reaction(s) similar to those observed in this study can influence the kinetics of formation of the Nbf-S product.

Great care must be employed in the use of this reagent in kinetic studies above pH 5 to ensure that complex-formation does not obscure the rate of formation of the Nbf-S derivative. Determination of the dependence of rate constants on Nbf-Cl concentration should provide an indication if such complex-formation does occur.

Finally, we address ourselves to the question of the apparent reversibility of formation of the Nbf-S derivative of proteins. We have shown that when high concentrations of thiol compound, such as 2-mercaptoethanol or dithiothreitol, are added to proteins in the presence of relatively high concentrations of Nbf-Cl, irreversible reactions occur with such proteins, giving rise to coloured products having broad spectral bands between 400 and 700 nm. The consequence of this reaction for RNA polymerase was irreversible inactivation (Bratcher & Kronman, 1977). This inactivation could be avoided by gel filtration of the Nbf-Cl/enzyme reaction mixture to remove free reagent before addition of low-molecular-weight thiol compound.

Since the nitrobenzofurazan derivative of an amino group cannot be thiolysed, it is particularly important to follow the above procedure when restoration of enzymic activity after addition of dithiothreitol or 2-mercaptoethanol is used as a means of distinguishing catalytically significant reaction at thiol and at amino groups. Application of this gel-filtration procedure, together with use of spectroscopic criteria (Birkett *et al.*, 1970a) for the reaction at these two kinds of groups should provide unambiguous evidence for the identification of thiol as a group that can influence catalytic activity. This procedure was followed with RNA polymerase (Bratcher & Kronman, 1977).

This research was supported by a grant from the National Institutes of Health (no. GM21187 and Institutional grant no. 580PR0540216). K. N. thanks the Yoshida Kagaku-Gijutsu Zaidan (Yoshida Science and Engineering Foundation) for a transportation fund. We thank Dr. Robert Stach for many helpful discussions of these observations.

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Scheme 3. Reaction of Nbf-Cl with a thiol compound R-S⁻ to form the coloured product P₂ as well as the Meisenheimer complex P₁ and covalent product Nbf-SR

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