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MODIFICATION AND INACTIVATION OF RHODANESE BY 2,4,6-TRINITROBENZENESULPHONIC ACID

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Bovine liver rhodanese (thiosulphate sulphurtransferase, EC 2.8.1.1) is modified by 2,4,6-trinitrobenzenesulphonic acid, by the use of modifying agent concentrations in large excess over enzyme protein concentration. The end-point of the reaction, viz., the number, *n*, per enzyme protein molecule, of modifiable amino groups was determined graphically by the Kézdy–Swinbourne procedure. It was found that the value for *n* depends on the pH of the reaction medium, and ranges from 2, at pH 7.00, to 10.66, at pH 9.00. Again, the value for *n* increases with an increase in the concentration of 2,4,6-trinitrobenzenesulphonic acid used, with values ranging from 3.52, at 0.10 mM modifying agent, to 8.96, at 2 mM modifying agent. Rhodanese primary amino groups modification by 2,4,6-trinitrobenzenesulphonic acid is described by a summation of exponential functions of reaction time at pH values of 8.00 or higher, while at lower pH values it is described by a single exponential function of reaction time. However, the log of the first derivative, at initial reaction conditions, of the equation describing protein modification, is found to be linearly dependent on the pH of the reaction. An identical linear dependence is also found when the log of the first derivative, at the start of the reaction, of the equation describing modification-induced enzyme inactivation is plotted against the pH values of the medium used. In consequence, the fractional concentration of rhodanese modifiable amino groups essential for enzyme catalytic function is equal to unity at all reaction pH values tested. It is accordingly concluded that, when concentrations of 2,4,6-trinitrobenzenesulphonic acid in excess of protein concentration are used, all rhodanese modifiable amino groups are essential for enzyme activity. A number of approaches were used in order to establish a mechanism for the modification-induced enzyme inactivation observed. These approaches, all of which proved to be negative, include the possible modification of enzyme sulfydryl groups, disulphide bond formation, enzyme inactivation due to sulphite released during modification, modification-induced enzyme protein polymerization, syncatalytic enzyme modification and hydrogen peroxide-mediated enzyme inactivation.

KEY WORDS: Rhodanese, 2,4,6-Trinitrobenzenesulphonic acid, modification-induced enzyme inactivation.

INTRODUCTION

Rhodanese (thiosulphate sulphurtransferase, EC 2.8.1.1) modification, and inactivation, studies by 2,4,6-trinitrobenzenesulphonic acid, by the use of protein concentrations well in excess of modifying agent concentration, have shown that enzyme inactivation is brought about by the modification of primary amino groups, and further, that the fractional concentration of rhodanese group reactivities essential for catalytic function depends on the hydrogen ion concentration of the reaction medium.[1] However, the determination of the number of modifiable amino groups, per enzyme protein molecule, as well as the kinetic separation of these groups into classes of different reactivity towards the modifying agent, may only be accomplished by the

use of modifying agent concentrations well in excess of protein concentration. In this communication, results of rhodanese modification, and inactivation, by 2,4,6-trinitrobenzenesulphonic acid, at high modifying agent/enzyme protein ratios, are reported and it is shown that, under these conditions, all enzyme protein modifiable amino groups are essential for catalytic function, at all hydrogen ion concentrations of the reaction medium tested. These results indicate the existence of strong inactivation cooperativity, i.e., enzyme inactivation by modification at regions of the rhodanese molecule far from the enzyme active-site.

MATERIALS AND METHODS

Rhodanese, purified from bovine liver, alcohol dehydrogenase from baker's yeast, ribonuclease A from bovine pancreas, poly-L-lysine, glycine, 5,5′-dithiobis(2-nitrobenzoic acid), and 2,4,6-trinitrobenzenesulphonic acid were purchased from Sigma Chemical Co., St. Louis, MO., U.S.A. Catalase was obtained from Boehringer.

Rhodanese activity determination was carried out as described previously.[1] Studies of the modification, and/or inactivation of rhodanese by 2,4,6-trinitrobenzenesulphonic acid were carried out by preparing an enzyme/modifying agent mixture, the final composition of which was: 0.1 M sodium phosphate buffer, of the required pH, 0.01 M thiosulphate, 9.12 μM rhodanese, and 2,4,6-trinitrobenzenesulphonic acid in the range of 0.10 to 2 mM. Inclusion of one of the substrates, namely thiosulphate, in the reaction medium was considered necessary for the enzyme to be maintained in the sulphur-rhodanese form; in the absence of thiosulphate the persulphide group of rhodanese is easily oxidized, and the enzyme is thereby rendered inactive.[2] After incubation of the enzyme/modifying agent mixture in a water bath at 25°C, for the time periods required, aliquots were withdrawn and used for the determination of rhodanese activity. The extent of the reaction of 2,4,6-trinitrobenzenesulphonic acid with rhodanese was monitored by taking absorbance readings at 345 nm.[3] It should be noted that sodium phosphate buffer was used for the pH 9.00 modification, and inactivation, experiments without fortification by added borate buffer, as was done previously.[1] This was so because it was felt that, although phosphate is a poor buffer at pH 9.00, the shift in pH likely to be brought about by product formation during rhodanese modification, must needs be of lesser importance than the anion and/or ionic strength-mediated effects on the reaction system necessitated by added buffer.

Results of rhodanese inactivation experiments were studied by plotting the log fractional enzyme activity value *vs.* reaction time. Since, in all cases studied, modifying agent concentration was in large excess over the enzyme concentration used, reaction kinetics are expected to be first-order with regard to enzyme concentration.[4]

Results of rhodanese modification experiments were studied by plotting the fractional concentration of unreacted primary amino groups *vs.* reaction time. The end-point of the reaction of 2,4,6-trinitrobenzenesulphonic acid with rhodanese, at each particular pH value of the reaction medium tested, was determined graphically by the Kézdy–Swinbourne procedure.[5,6] This method was chosen, in preference to the widely used Guggenheim procedure,[7] because in cases where the EQ describing protein groups modification consists of a summation of exponential functions of reaction time, rather than of a single exponential function of reaction time, data analysis according to Guggenheim[7] yields a value equal to the reaction end-point multiplied by the coefficient of the slowest exponential, while the Kézdy–Swinbourne procedure yields the true value for the reaction end-point.[8] The fractional concentra-

tion of unreacted amino groups was determined from the end-point of the reaction and from the value of the reaction product at each particular time. The number, n, of modifiable amino groups per protein molecule was determined from the absorbance value at the end-point of the reaction by the use of the extinction coefficient for trinitrophenylated amino groups of rhodanese i.e. 11, 500 $M^{-1}cm^{-1}$ at 345 nm, and of the molecular weight of the protein under study.[1] Molecular weight values of the proteins studied are: rhodanese, 32,900,[9] alcohol dehydrogenase, 150,000, ribonuclease A, 13,680, and poly-L-lysine, 139,000. Graphical analysis of protein modification data was carried out by fitting such data to the EQ:[10]

$$((n[E]_{n(0)} - [E]_{mod})/n[E]_{n(0)}) = \sum_i c_i e^{-k_i t} \quad (1)$$

where $[E]_{mod}$ is the concentration of modified protein groups, t is reaction time, $[E]_{n(0)}$ is unmodified protein concentration at $t = 0$, k_i are the constants, and c_i the coefficients of the protein modification equation.

RESULTS AND DISCUSSION

Enzyme inactivation studies

Plots of log fractional rhodanese activity *vs.* reaction time, at reaction pH values ranging from 7.00 to 9.00, are shown in Figure 1. It will be seen that all of these plots are rectilinear, and also that they pass through the origin of the graph, indicating that rhodanese inactivation is a single exponential function of reaction time. When the log of the reciprocal of the first-order enzyme inactivation rate constant is plotted *vs.* the pH of the reaction medium, a straight line is obtained with a slope of unity (Figure 2). The dependence of the experimentally determined first-order rate constant of the enzyme inactivation reaction, k_{inact}, on the hydrogen ion concentration used, is, when enzyme inactivation is due to the modification of one kind of protein reactive groups only, given by the relationship:

$$k_{inact} = jk/(1 + [H^+]/K_a) \quad (2)$$

where k is the first-order rate constant for the modification of the unprotonated, or dissociated, form of these groups, K_a is the dissociation constant of the protonated, or undissociated, form of these groups, and j is a proportionality constant.[4,11] A slope of unity in the log $(1/k_{inact})$ *vs.* pH plot indicates that the pK_a value of the electrophilic form of the protein reactive groups is well above the reaction pH values tested, viz., the inequality $([H^+]/K_a) \gg 1$ applies throughout the hydrogen ion concentration range used. A similar dependence of the apparent first-order enzyme inactivation reaction rate constant on the pH of the reaction medium used, has been found for the inactivation of thymidylate synthetase by phenylglyoxal.[12]

When rhodanese inactivation was tested at different 2,4,6-trinitrobenzenesulphonic acid concentrations, fractional enzyme activity was again a single exponential function of reaction time at all modifying agent concentrations tested. The dependence of the enzyme inactivation first-order reaction rate constant on the 2,4,6-trinitrobenzenesulphonic acid concentration used is shown in Figure 3. It will be seen that a linear relationship exists between the rate of enzyme inactivation and the concentration of the modifying agent, and accordingly that rhodanese inactivation, under the reaction conditions tested, follows pseudofirst-order kinetics.

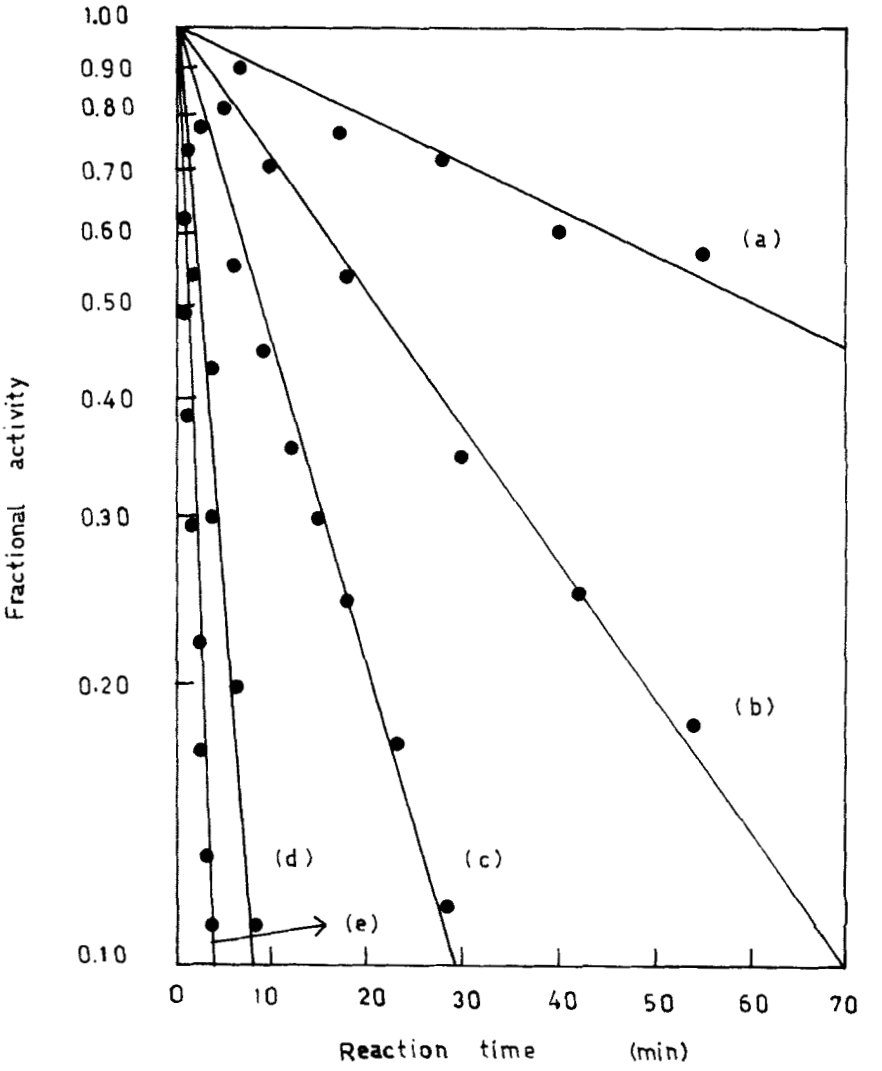

FIGURE 1 Inactivation of rhodanese by 2,4,6-trinitrobenzenesulphonic acid. Incubation mixtures contained sodium phosphate buffer 0.1 M, of the required pH, rhodanese 9.12 μM, 2,4,6-trinitrobenzenesulphonic acid 0.25 mM, and sodium thiosulphate 0.010 M. After incubation in a water bath at 25°C for the time periods shown, aliquots were taken and assayed for rhodanese activity. Reaction pH was 7.00 (a), 7.50 (b), 8.00 (c), 8.50 (d) and 9.00 (e).

Protein modification studies

The dependence of the extent of rhodanese amino groups modification on reaction time, at two pH values of the reaction medium tested, is shown in Figure 4, while a representative determination of the end-point of the reaction is shown in Figure 5. The dependence of the number of modifiable amino groups, per enzyme protein

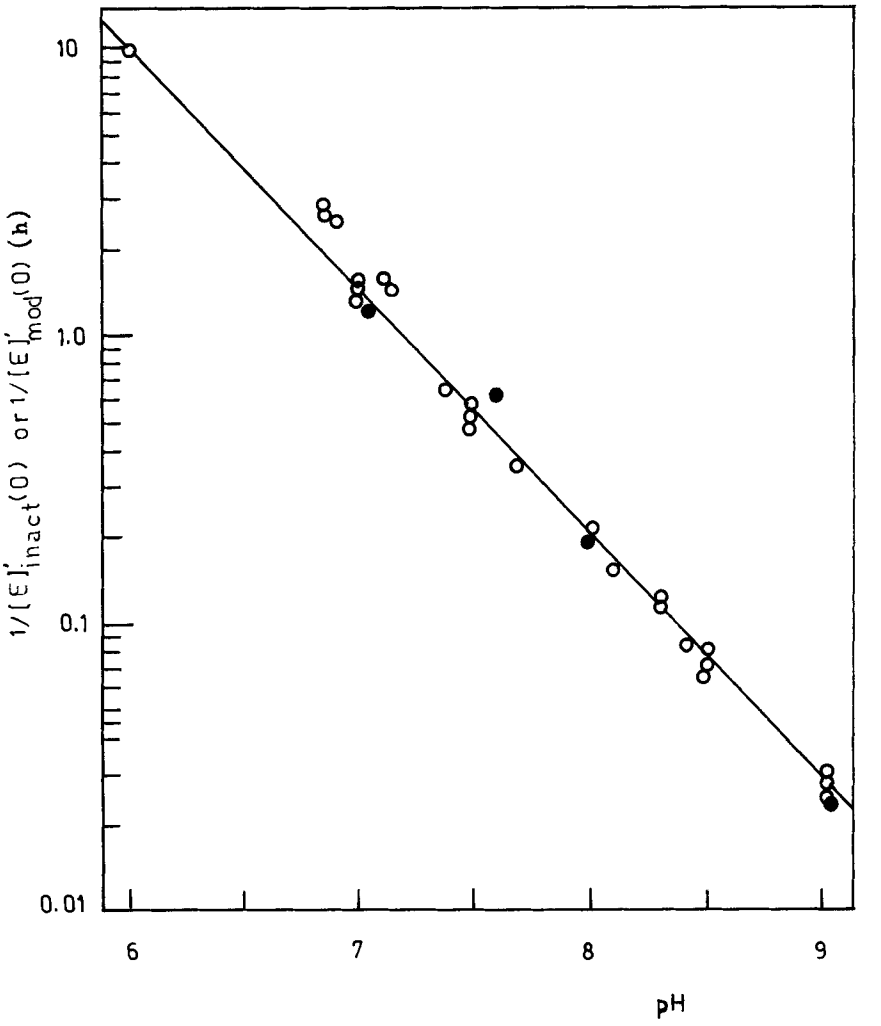

FIGURE 2 Dependence of rhodanese inactivation, and modification, parameters on reaction pH. Reaction conditions were as described in Figure 1. The reciprocal of the first derivative, at the start of the reaction, of the equation describing enzyme inactivation (○) or enzyme primary amino groups modification (●), was plotted against the pH of the reaction medium.

molecule, on pH is shown in Figure 6. It will be seen from Figure 6 that the number of groups modified, per enzyme protein molecule, is linearly dependent on the pH of the reaction medium. Apparently, dissociation of rhodanese electrophilic groups brings about a conformational change of the protein, in such a manner that primary amino groups become increasingly exposed to the modifying agent used. An increase in the number of exposed functional groups, consequent upon an increase in the pH of the reaction medium, has been observed for the nitration of the tyrosine residues of carbonic anhydrase by tetranitromethane.[13]

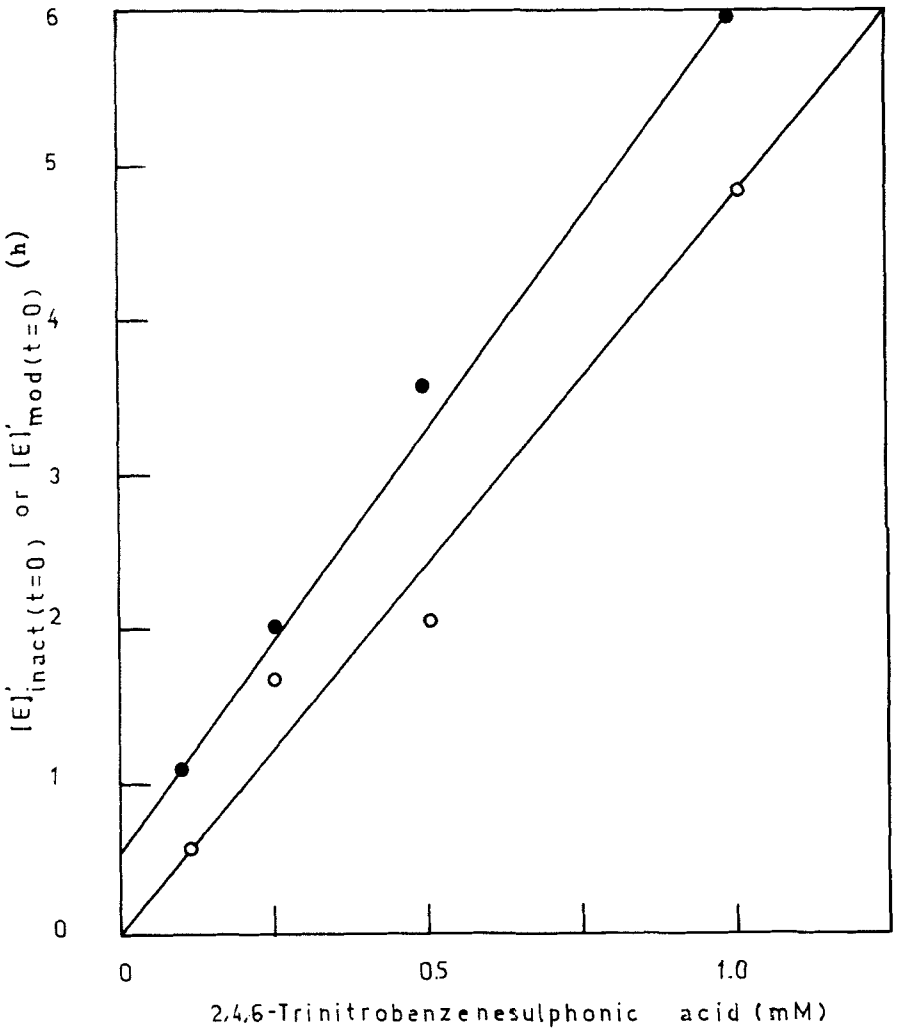

FIGURE 3 Dependence of rhodanese inactivation, and modification, parameters on 2,4,6-trinitrobenzenesulphonic acid concentration. Reaction conditions were as described in Figure 1. The first derivative, at the start of the reaction, of the equation describing enzyme inactivation (○) or protein modification (●), was plotted against the concentration of 2,4,6-trinitrobenzenesulphonic acid used.

The kinetic description of rhodanese modification by 2,4,6-trinitrobenzenesulphonic acid, at different pH values of the reaction medium, is given in Table I. It will be noted that, for the range of pH values of the reaction medium from 8.00 to 9.00, the kinetic description of rhodanese primary amino groups modification consists of a summation of exponential functions of reaction time rather than of a single exponential (Figure 7). The question arises whether the effect of low hydrogen ion concentration on rhodanese amino groups reactivity is due to the structural separation of rhodanese groups into independent sets of groups of different reactivity towards the

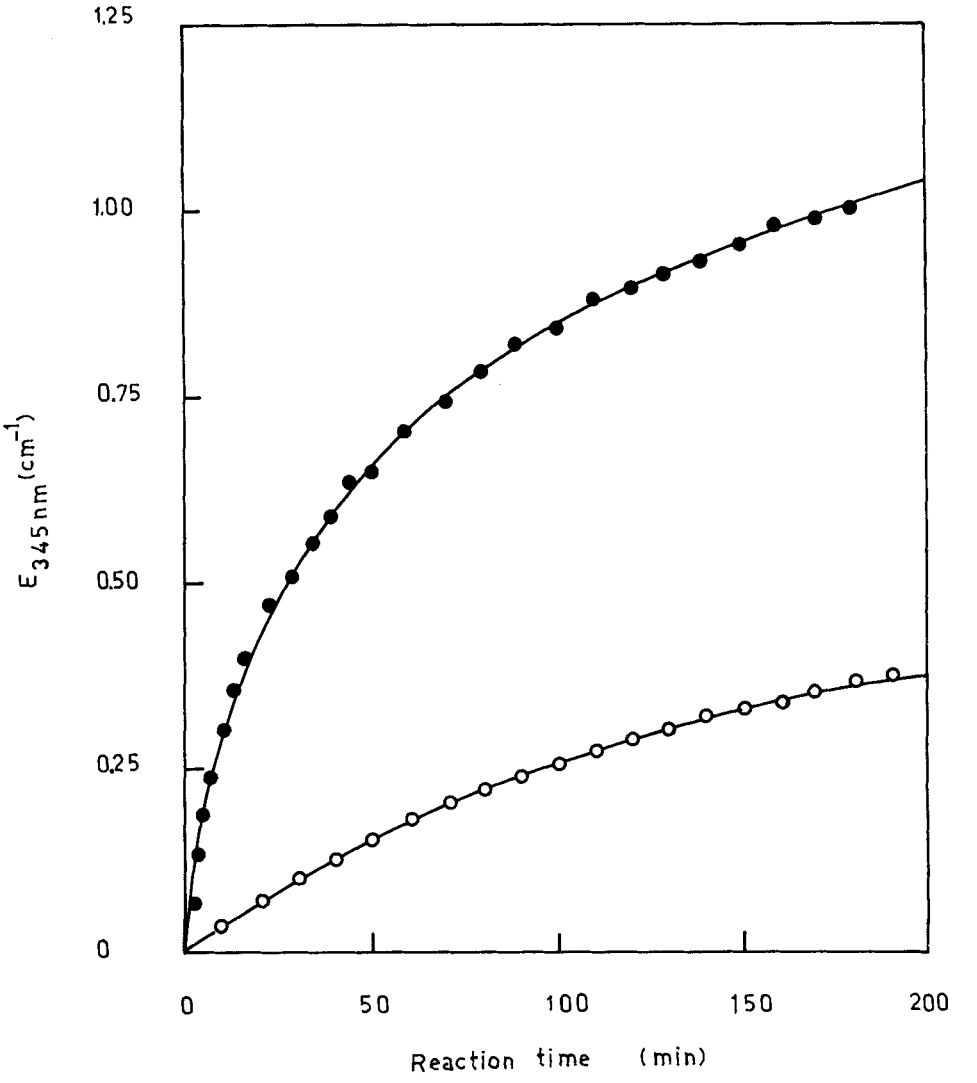

FIGURE 4 Modification of rhodanese by 2,4,6-trinitrobenzenesulphonic acid: dependence of the absorbance at 345 nm of the rhodanese-modifying agent reaction mixture on reaction time. Reaction conditions were as described in Figure 1. The pH of the reaction medium was 7.50 (○), and 9.00 (●).

modifying agent used, or to one of the situations presenting with sets of interdependent protein reactive groups.[14] Of these situations, stoichiometric protein modification cooperativity may be ruled out or, alternatively, may be shown to be compatible with the kinetic description of protein modification.[2,15] However, protein stoichiometric modification cooperativity models have been elaborated to apply to proteins of which the total number of modifiable groups remains constant during the course of the modification reaction.[15]

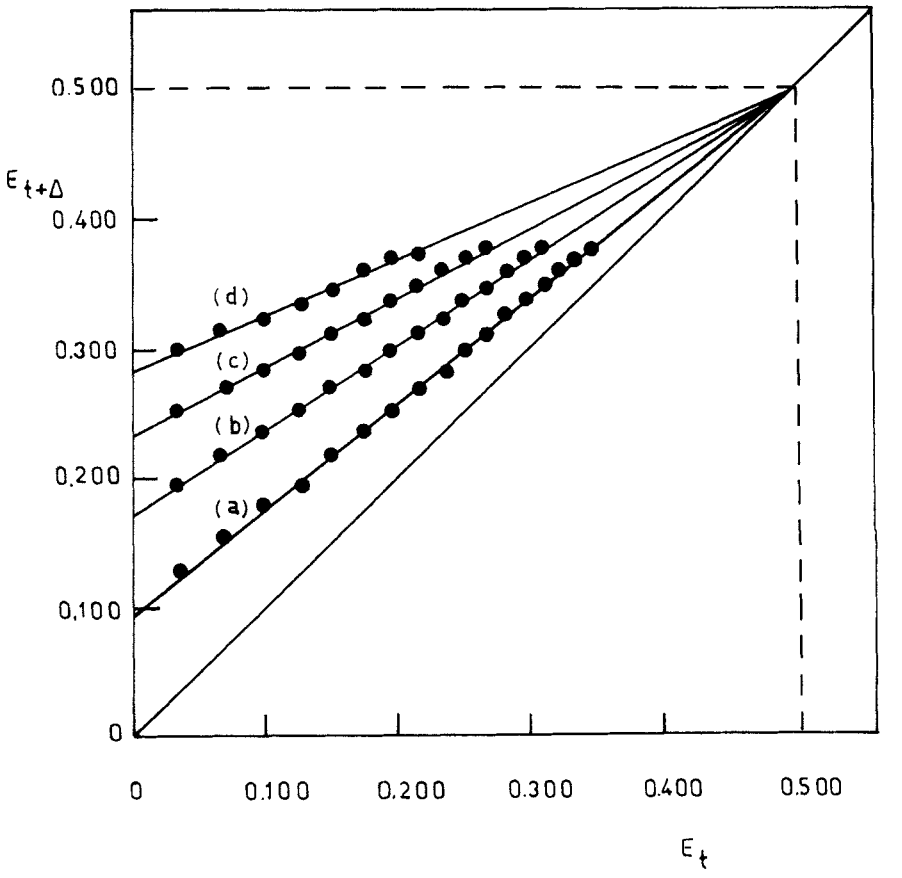

FIGURE 5 Modification of rhodanese by 2,4,6-trinitrobenzenesulphonic acid: determination of the end-point of the reaction by the Kézdy-Swinbourne procedure. Modification data are those of Figure 4 (pH 7.50 sample). The constant time increment, Δ, used in the construction of the plot is 30 (a) 60 (b), 90 (c), and 120 min (d).

In order to evaluate the effect of hydrogen ion concentration on rhodanese modification, use was made of the first derivative value, at the start of the reaction, of the protein modification process, $[E]'_{mod(0)}$. It has been shown that when a protein modification reaction is described by a summation of exponential functions of reaction time, the $[E]'_{mod(0)}$ value is the preferred value to use since it relates to the protein species present at the start of the reaction.[16] It will be noted from Figure 2 that, when $[E]'_{mod(0)}$ is plotted *vs.* the pH of the reaction medium, the values obtained are identical with the corresponding enzyme inactivation values. This means that the ratio $[E]'_{inact(0)}/[E]'_{mod(0)}$ is equal to unity and, accordingly, that all rhodanese modifiable amino groups are essential for enzyme catalytic activity at all pH values tested. The same conclusion may be arrived at by plotting the k_{inact}/k_{mod} value (where $k_{mod} = \Sigma c_i k_{mod(i)}$) *vs.* the number of protein modifiable groups in each instance (Figure 8).

Since at 2,4,6-trinitrobenzenesulphonic acid concentrations equal to or lower than rhodanese concentration, the fractional concentration of rhodanese group reactivities

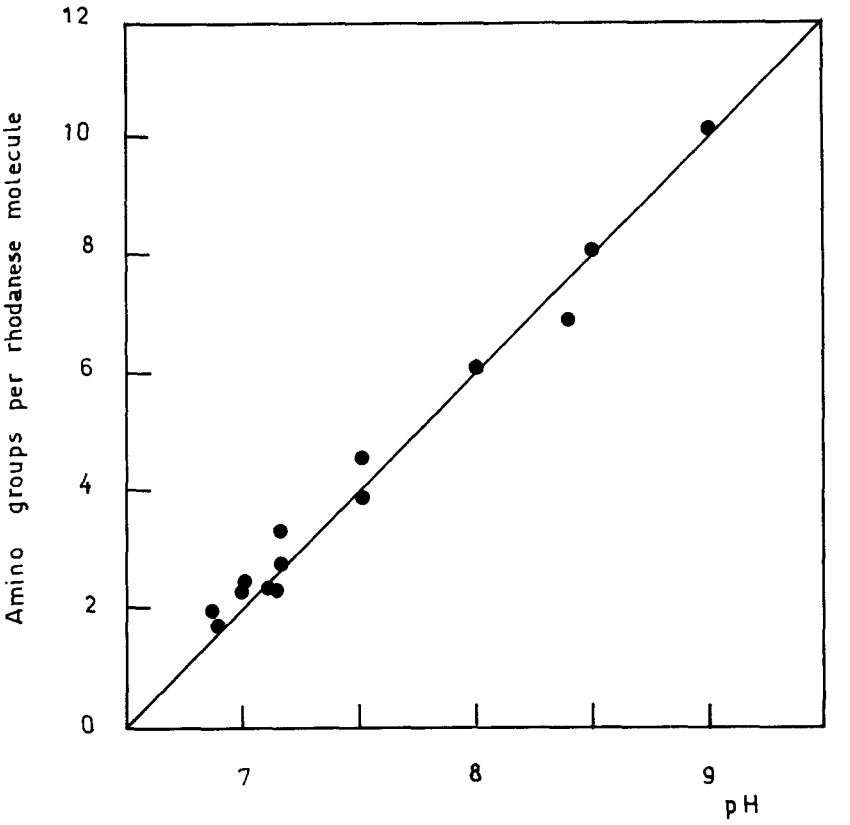

FIGURE 6 Modification of rhodanese by 2,4,6-trinitrobenzenesulphonic acid: dependence of the total number, per enzyme protein molecule, of modifiable amino groups on the pH of the reaction mixture. Reaction conditions were as described in Figure 1. The number of modifiable amino groups, per enzyme protein molecule, at each pH value tested, was determined by the graphical procedure shown in Figure 5.

essential for enzyme catalytic function has been found to decrease with decreasing pH values of the reaction medium used,[1] an examination of the number of enzyme protein modifiable amino groups per enzyme protein molecule , at different concentrations of modifying agent, was undertaken. It will be noted from Table II that the number of modifiable amino groups, per enzyme protein molecule, increases with an increase in the concentration of modifying agent used. It will be seen from Figure 8 that the fractional concentration of rhodanese group reactivities essential for enzyme catalytic function is close to unity for all concentrations of 2,4,6-trinitrobenzenesulphonic acid tested (see also Figure 8, for the ratio k_{inact}/k_{mod} at different modifying agent concentrations). Apparently, at high 2,4,6-trinitrobenzenesulphonic acid concentrations, reversible association of enzyme protein with the modifying agent brings about a change in the extent of rhodanese inactivation cooperativity (inactivation by modification far from the enzyme active-site[17]).

To establish whether the effect of different 2,4,6-trinitrobenzenesulphonic acid concentrations on the number of modifiable amino groups, per enzyme protein molecule, is restricted to rhodanese or is a generalized phenomenon, the effect of this

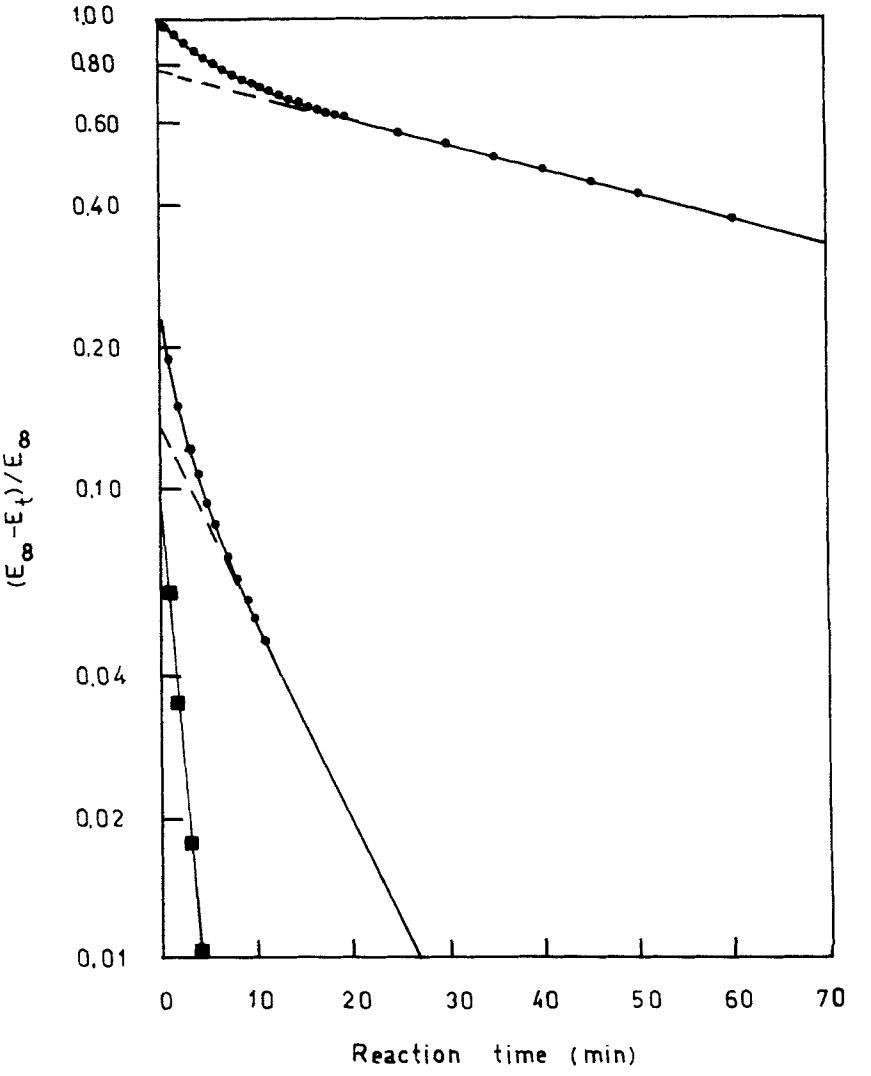

FIGURE 7 Modification of rhodanese by 2,4,6-trinitrobenzenesulphonic acid: graphical analysis of protein modification data as a summation of exponential functions of reaction time. Modification data are those of Figure 4 (pH 9.00 sample). After determination of the end-point of the reaction by the Kézdy-Swinbourne procedure, modification data were fitted to eqn. (1) as described in Frost and Pearson.[10]

modifying agent on ribonuclease A, alcohol dehydrogenase, poly-L-lysine and glycine was investigated. It will be noted from Table III that in all of these cases the number of modifiable amino groups, per enzyme protein molecule increases with an increase in the 2,4,6-trinitrobenzenesulphonic acid concentration used. It will also be noted from Table III that this is not the case for the reaction of 2,4,6-trinitrobenzenesulphonic acid with glycine, thus precluding the possibility of an increase in the number of modifiable amino groups being an artefact of the kinetic analysis applied. A

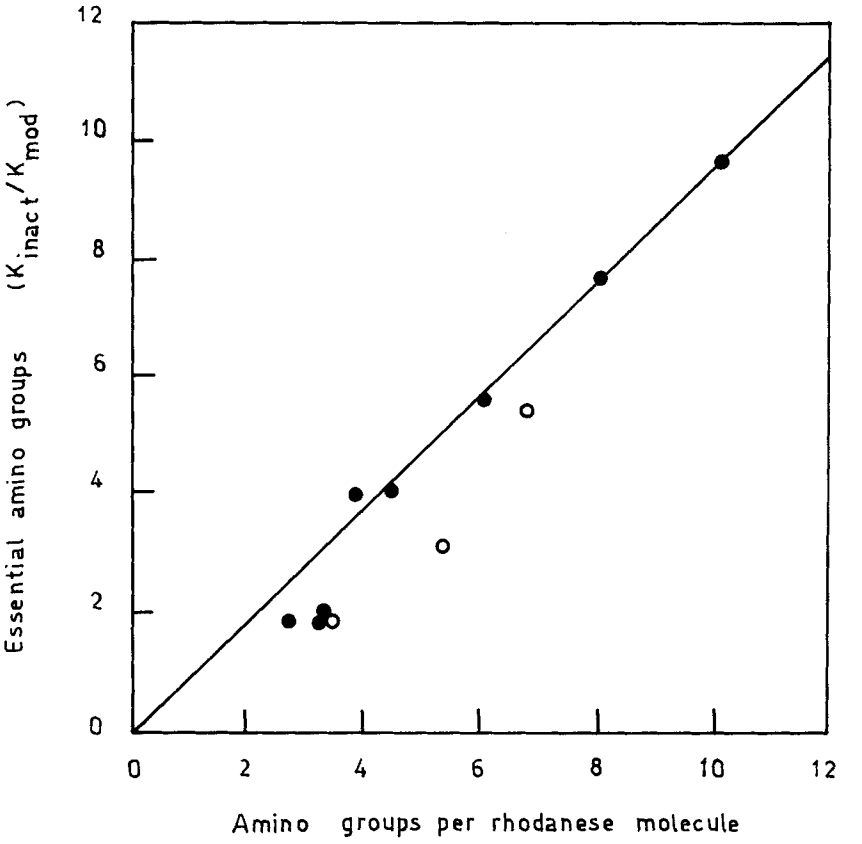

FIGURE 8 Determination of the fractional concentration of rhodanese primary amino groups essential for enzyme catalytic function. The ratio k_{inact}/k_{mod} is equal to the number of groups per enzyme protein molecule essential for catalytic function (k_{mod} is defined as $\Sigma_i c_i k_i$). The total number of modifiable amino groups, per rhodanese molecule, is obtained from studies at different pH values (●) (data from Table I), and from studies at different 2,4,6-trinitrobenzenesulphonic acid concentrations (○) (data from Table II).

theoretical analysis of a two-sited modification-induced protein unfolding model has been presented.[18]

A comparison of the $[E]'_{mod(0)}/[E]_0$ values, at different modifying agent concentrations (Tables II and III), indicates that a "rate saturation effect" is to be observed in all cases studied, except that of glycine modification. It is accordingly likely that 2,4,6-trinitrobenzenesulphonic acid, in a large number of cases, forms a reversible adsorptive complex with the protein prior to protein reactive amino groups modification (an alternative interpretation of the "rate saturation effect" has been put forward).[19] It will also be noted from Table III that 2,4,6-trinitrobenzenesulphonic acid exhibits different reactivities with the compounds tested. The reactivity of 2,4,6-trinitrobenzenesulphonic acid with several amino acid, peptides, and proteins has been studied.[20] The "intrinsic reactivity" of the compounds tested, i.e., the value for k in eqn. (2), has been calculated by the use of the pK_α values for the amino groups modified.[20] The "intrinsic reactivity" of the $\Sigma_i c_i k_i/[MA]$ value, where [MA] is 2,4,6-trinitrobenzenesulphonic acid concentration, of the compounds tested in the present

TABLE I

Dependence of rhodanese modification on pH. Reaction conditions are as described in the text. Reaction constants, k_i, and coefficients, c_i, are as described in Eq. (1). Reaction constants are in h^{-1}. After determination of the end-point of the reaction by the Kézdy–Swinbourne procedure,[5,6] modification data were fitted to Eq. (1) by graphical analysis[10] aided by the determination of the values of k_i and c_i by the method of least squares (Eq. (7.33) of P.G. Francis).[36] The error in k_i and c_i values was determined by the use of Eqs. (7.44), and (7.47), respectively, of P.G. Francis.[36] The k_i values are given immediately below the c_i values. The $[E]'_{mod(0)}/[E]_0$ value is defined as $n\,\Sigma_i c_i k_i$[16] and is in h^{-1}. The standard error of the $[E]'_{mod(0)}/[E]_0$ value (the standard error of the sum, as well as of the product, of two or more variables) was calculated as described by M.L. Boas.[37] The 2,4,6-trinitrobenzenesulphonic acid concentration used was 0.25 mM.

pH	n	c_1 & k_1	c_2 & k_2	c_3 & k_3	$[E]'_{mod(0)}/[E]_0$
7.10	2.48	–	–	0.967 ± 0.003	
		–	–	0.556 ± 0.002	1.33 ± 0.006
7.50	4.76	–	–	1.07 ± 0.120	
		–	–	0.508 ± 0.067	2.59 ± 0.447
8.00	6.35	–	0.078 ± 0.004	0.913 ± 0.004	
		–	5.00 ± 0.270	0.491 ± 0.003	5.32 ± 0.597
8.50	8.48	–	0.101 ± 0.002	0.932 ± 0.018	
		–	4.43 ± 0.120	0.709 ± 0.011	9.39 ± 0.648
9.00	10.56	0.092 ± 0.005	0.161 ± 0.003	0.724 ±0.008	
		29.6 ± 0.970	6.96 ± 0.118	0.659 ± 0.007	45.6 ± 1.85

TABLE II

Dependence of rhodanese modification on 2,4,6-trinitrobenzenesulphonic acid concentration. Reaction conditions are as described in the text. Reaction constants, k_i, and coefficients, c_i, are as defined in Eq. (1). Reaction constants are in h^{-1}. MA, modifying agent, is 2,4,6-trinitrobenzenesulphonic acid. $[E]'_{mod(0)}/[E]_0$ is as defined in Table I and is in h^{-1}. The pH of the reaction medium is 7.5. The values, as well as the error, of k_i, and of c_i, were determined as described in Table I.

MA (mM)	n	c_1 & k_1	c_2 & k_2	$[E]'_{mod(0)}/[E]_0$
0.10	3.52	–	0.970 ± 0.001	
		–	0.277 ± 0.006	0.947 ± 0.021
0.50	5.72	0.052 ± 0.00003	0.950 ± 0.008	
		6.30 ± 0.293	0.379 ± 0.005	3.94 ± 0.092
1.00	7.63	0.100 ± 0.0001	0.892 ± 0.006	
		4.19 ± 0.051	0.391 ± 0.004	5.86 ± 0.046
2.00	8.96	0.099 ± 0.001	0.895 ± 0.015	
		5.48 ± 0.150	0.600 ± 0.003	9.68 ± 0.161

investigation, as well as for some compounds studied by other authors,[20] is given in Table IV.

Studies on the mechanism of modification-induced enzyme inactivation

Rhodanese modification experiments, at modifying agent concentrations equal to or lower than enzyme protein concentration, have established that trinitrophenylation of primary amino groups is causally related to enzyme inactivation. The question arises,

TABLE III

Dependence of alcohol dehydrogenase, ribonuclease, poly-L-lysine, and glycine modification on the concentration of 2,4,6-trinitrobenzenesulphonic acid used. Reaction conditions are as described in the text. Reaction constants are in h^{-1}. $[E]'_{mod(0)}/[E]_0$ is as defined in Table I, and is in h^{-1}. MA is 2,4,6-trinitrobenzenesulphonic acid. The values, as well as the error, of k_i, and of c_i, were determined as described in Table I.

MA (mM)	n	c_1 & k_1	c_2 & k_2	$[E]'_{mod(0)}/[E]_0$
Alcohol dehydrogenase				
0.275	50.4	–	1.013 ± 0.003	
		–	0.439 ± 0.002	22.4 ± 0.101
1.00	74.5	0.083 ± 0.002	0.925 ± 0.006	
		4.35 ± 0.203	0.516 ± 0.004	62.5 ± 1.49
Ribonuclease A				
0.25	4.30	0.206 ± 0.005	0.783 ± 0.006	
		5.79 ± 0.191	0.470 ± 0.005	6.71 ± 0.211
0.50	5.41	0.283 ± 0.031	0.769 ± 0.006	
		6.93 ± 0.583	0.511 ± 0.004	12.7 ± 1.46
1.00	6.50	0.246 ± 0.006	0.772 ± 0.003	
		7.27 ± 0.187	0.527 ± 0.003	14.3 ± 0.410
Poly-l-lysine				
0.50	425[b]	0.389 ± 0.009	0.671 ± 0.027	
		6.64 ± 0.009	0.980 ± 0.023	1,376 ± 18.7
1.00	449[b]	0.356 ± 0.018	0.606 ± 0.030	
		7.00 ± 0.263	0.984 ± 0.028	1,387 ± 72.3
2.00[a]	472[b]	0.125 ± 0.004	0.288 ± 0.004	
		25.5 ± 0.739	10.8 ± 0.123	3,233 ± 72.6
Glycine				
0.50	1.05	–	1.006 ± 0.012	
		–	0.705 ± 0.006	0.745 ± 0.011
1.00	1.02	–	0.995 ± 0.009	
		–	1.125 ± 0.006	1.14 ± 0.012

[a] Modification described by a summation of three exponentials equation. Additional exponential is: $c_3 = 0.605 \pm 0.038$, $k_3 = 0.909 \pm 0.035\,h^{-1}$.

[b] Calculated number of lysine residues per poly-L-lysine molecule is 1,086.

however of the nature of the mechanism of this modification-induced enzyme inactivation effect. Since unequivocal evidence concerning amino groups involvement in the enzyme catalytic mechanism itself has only been adduced for Lys-247 of rhodanese,[21] the conclusion is inescapable that modification of the remaining amino groups brings about the loss of enzyme catalytic function by action far from the enzyme active-site (inactivation cooperativity).[17] To answer the question of the nature of this "action at a distance", several possibilities were investigated; all of these possibilities proved to be negative.

(a) Modification of rhodanese sulfhydryl groups. Although modification of groups other than primary amino groups has been ruled out by studies of the stoichiometry of modification-induced enzyme inactivation, the possibility of sulfhydryl groups reacting at the same rate as amino groups with the modifying agent used has been left open.[1] To determine the extent of possible sulfhydryl groups modification, a solution

TABLE IV

Determination of the intrinsic reactivity of amino acids and proteins with 2,4,6-trinitrobenzenesulphonic acid. Instrinsic reactivity is the reactivity of the unprotonated or dissociated, form of modifiable nucleophilic groups[20] (see Eq. (2)). Data used for the determination of intrinsic group reactivity are from Tables II, and III. Data given with an asterisk (*) are from Freedman and Radda.[20] MA is 2,4,6-trinitrobenzenesulphonic acid. Intrinsic modifiable group reactivity is given as $\Sigma_i c_i k_i[H^+]/K_a[MA]$, and is in $M^{-1}s^{-1}$.

Compound	Amino group pK_a	pH	MA (mM)	$\Sigma_i c_i k_i[H^+]/K_a[MA]$
Glycine	9.60*	8.20	0.50	9.89
Glycine*	9.60*	7.40*	–	11.3
Lysine-α*	8.95*	7.40*	–	1.50
Lysine-ε*	10.53*	7.40*	–	51.7
Rhodanese	10.53*	7.50	0.10	800
Alcohol dehydrogenase	10.53*	8.06	0.275	132
Ribonuclease A	10.53*	8.00	1.00	588
Ribonuclease A*	10.53*	7.60*	–	71.6
Poly-L-lysine	10.53*	8.06	0.50	531
Insulin*	10.53*	7.40*	–	230
α-Chymotrypsinogen*	10.53*	7.40*	–	81.1

was prepared containing (in a 2 ml final volume): rhodanese 5 mg/ml, sodium phosphate buffer 0.01 M, pH 7.6, and 2,4,6-trinitrobenzenesulphonic acid 0.25 mM. The preparation was incubated in a water bath at 25°C for 30 min, by which time all enzyme activity was lost. The preparation was then placed on a Sephadex G-75 column (1 × 30 cm), and was eluted with sodium phosphate buffer 0.01 M, pH 7.6. Sulfhydryl groups concentration was determined on the eluent fractions taken, by adding to each fraction 5,5′-dithiobis (2-nitrobenzoic acid) to a final concentration of 1 mM, and taking absorbance readings at 412 nm. An extinction coefficient of 14,000 $M^{-1}cm^{-1}$ was used for 3-carboxylato-4-nitrothiophenolate production.[22] A control sample was prepared as above, with the omission of 2,4,6-trinitrobenzenesulphonic acid. Both the control and the trinitrophenylated sample yielded a value of 0.92 sulfydryl groups per rhodanese molecule. This value agrees with the findings of Pensa *et al.*,[23] who obtained a value of 0.6 sulfhydryl groups per rhodanese molecule; the full sulfhydryl content of rhodanese (four groups per enzyme protein molecule) could only be determined after protein denaturation in 8 M urea.[23]

(b) Disulphide bond formation in the rhodanese molecule. Disulphide bond formation has been found to take place when rhodanese is modified with iodoacetic acid or with phenylglyoxal.[21,24] Disulphide bonds so formed may be reduced with an excess of thiosulphate, with complete restitution of enzyme activity.[2,24] To test for possible disulphide bond formation, rhodanese (9.12 μM) was modified by the use of 2,4,6-trinitrobenzenesulphonic acid (0.25 mM), in 0.1 M sodium phosphate buffer, pH 9.00, containing sodium thiosulphate 0.01 M. After incubation of the preparation at 25°C for 40 min it was dialysed overnight against water at 4°C. After dialysis the preparation was made 1 M with respect to sodium thiosulphate and left to stand at room temperature for 24 h. No reactivation of rhodanese catalytic activity was observed.

(c) Rhodanese inactivation due to sulphite released during modification. To rule out this possibility, use was made of the ability of trinitrophenylated glycine to tightly bind sulphite.[25] Trinitrophenylated glycine was prepared according to Dower *et al.*[26] Trinitrophenylated glycine, at a concentration equal to the concentration of 2,4,6-tri-

nitrobenzenesulphonic acid used, was included in the reaction mixture in a typical rhodanese modification experiment. It was found that the rate of rhodanese activity loss was not affected by the presence of trinitrophenylated glycine in the reaction mixture. Trinitrophenylated glycine, however, was found to completely prevent enzyme inactivation when equal concentrations of sulphite and trinitrophenylated glycine were included in the reaction mixture.

(d) Modification-induced rhodanese polymerization. Rhodanese inactivation might be due to enzyme polymerization, with loss of catalytic activity resulting from the inclusion of modified rhodanese molecules into the polymers formed. Enzyme portein polymerization has been observed with glyceraldehyde-3-phosphate dehydrogenase,[27] lactate dehydrogenase,[28] and insulin.[29] To test for this possibility, rhodanese was modified at pH 9.00 with 2,4,6-trinitrobenzenesulphonic acid, for a time period long enough to bring about complete enzyme inactivation, and was then dialysed overnight against 5 l of water containing sodium thiosulphate 0.01 M. After dialysis, the preparation was mixed with an equimolar amount of unmodified rhodanese and enzyme catalytic activity was determined. All of the enzyme activity assayed in the preparation could be accounted for by the amount of unmodified rhodanese used.

(e) Syncatalytic modification. When one or more of the protein modification steps is identical with the substrate-to-product transformation steps, in an enzyme utilizing two or more substrates, then enzyme catalytic function promotes enzyme protein modification and inactivation.[30,31] To test for this possibility, two time-dependence-of-rhodanese-inactivation experiments were run in parallel. These experiments differed only in the length of time used for rhodanese activity determination of the aliquots taken out of the enzyme-modifying agent mixture (7 and 10 min of incubation of the enzyme substrate mixture, in the enzyme activity determination procedure). The per cent activity remaining *vs.* incubation time plot was identical in the two parallel experiments, thus precluding syncatalytic enzyme modification.

(f) Rhodanese inactivation due to hydrogen peroxide formation during enzyme protein modification. The autoxidation, as well as the enzyme-mediated oxidation, of sulphite to sulphate results in the production of hydrogen peroxide.[32,33] Rhodanese inactivation due to hydrogen peroxide, as well as the superoxide radical, generated during enzyme protein modification by phenylglyoxal and of cyanide ions has been reported.[34,35] To test for the possibility of hydrogen peroxide-mediated rhodanese inactivation during modification by 2,4,6-trinitrobenzenesulphonic acid, catalase (0.1 mg/ml) was included in the enzyme-modifying agent reaction mixture. No effect of catalase was observed during the inactivation of rhodanese by 2,4,6-trinitrobenzenesulphonic acid. However, the possible involvement of free radicals in modification-induced rhodanese inactivation is a serious possibility and merits further, independent investigation.

CONCLUSION

The salient features of the study of rhodanese modification, and inactivation, by 2,4,6-trinitrobenzenesulphonic acid, presented in this communication, are: (1) The dependence of the extent of primary amino groups modification on pH, as well as on

the modifying agent concentration of the reaction medium. (2) The linear dependence of the first derivative, at the start of the reaction, of the equation describing protein modification on pH, as well as on modifying agent concentration. This indicates that the primary modification event is an effect of hydrogen ion concentration, as well as of modifying agent concentration, on the initial state of the rhodanese molecule. This initial state should, in all probability, involve only a small number of modifiable amino groups per enzyme protein molecule. Clearly, every subsequent transformation of the enzyme protein molecule is a consequence of the effect of hydrogen ion concentration, as well as of modifying agent concentration, on the enzyme initial conditions. (3) In distinction to results obtained at modifying agent concentrations comparable to enzyme protein concentration,[1] when modifying agent concentrations in large excess over enzyme concentration are used, all enzyme modifiable amino groups are shown to be essential for enzyme catalytic function. It is concluded that rhodanese modification, and inactivation, by 2,4,6-trinitrobenzenesulphonic acid is a process highly sensitive to the enzyme microenvironment and it may reasonably be assumed that the aforementioned effects are part of a larger regulatory mechanism of probable physiological significance.

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