Chemical Modification of *Escherichia coli* RNA Polymerase by Diethyl Pyrocarbonate: Evidence of Histidine Requirement for Enzyme Activity and Intrinsic Zinc Binding†

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ABSTRACT: RNA polymerase (RPase) from *Escherichia coli* contains five subunits ($\alpha_2\beta\beta'\sigma$) and two intrinsic Zn ions located in the β and β' subunits. This enzyme was rapidly inactivated by diethyl pyrocarbonate (DEP) at pH 6.0 and 25 °C. The difference spectrum of the DEP-inactivated and native RPases showed a single peak at 240 nm indicating the formation of *N*-carbethoxyhistidines. No decrease in absorbance at 278 nm, due to *O*-carbethoxytyrosine, or modification of amino and sulfhydryl groups was observed. Inactivated RPase with six to nine histidines being modified could be fully reactivated by incubation with 0.5 M hydroxylamine at pH 6.0 and room temperature for 1 h. No structural difference was detected between the native and modified enzymes as evidenced by UV/visible and fluorescence spectra, sodium dodecyl sulfate–polyacrylamide gel electrophoretic pattern, or gel filtration properties. Substrate ATP at 0.11 and 1.14 mM concentrations provided, respectively, 25% and 90% protection against DEP inactivation, while template DNA did not. These results suggest that one or more histidine residues is/are in close proximity to the substrate binding site. The pH dependence of the DEP inactivation of RPase suggested the modification of histidine at the active site with a p*K* value of 6.9. The inactivation of RPase by DEP and the formation of *N*-carbethoxyhistidine displayed a similar second-order rate constant of $\sim$0.9 mM^{-1} min^{-1}. Treatment of RPase inactivation data using the statistical method of Tsou [Tsou, C. L. (1962) *Sci. Sin.* (*Engl. Ed.*) *11*, 1535] is consistent with one histidine residue being critical for the enzyme activity and reacting $\sim$7 times faster than the other nonessential histidines. Furthermore, the order of reaction with respect to inactivation by DEP was approximately 1, indicating the modification of a single histidine residue. Finally, modification of Zn-free $\alpha_2\beta$ and β' subunits of RPase with DEP and subsequent reconstitution with Zn indicated that histidine(s) is (are) at (or near) the Zn binding sites.

The DNA-dependent RNA polymerase (RPase)[1] from *Escherichia coli* catalyzes the sequential assembly of four ribonucleotides (NTPs) into RNA product in the presence of DNA template and an extrinsic divalent cation (Goldthwait et al., 1970; Kumar, 1981; Wu & Tweedy, 1982). *E. coli* RPase contains five subunits ($\alpha_2\beta\beta'\sigma$) and two Zn(II) ions (Scrutton et al., 1971) which are localized in the β and β' subunits (Wu et al., 1977; Miller et al., 1979). These intrinsic Zn ions are indispensable for the activity of RPase (Solaiman & Wu, 1984, 1985). By using in vitro Co- and Mn-substituted RPases, we have shown that the substituted metal in the β subunit is located at the initiation site and is in direct coordination with the base moiety of the substrate (Chatterji & Wu, 1982a,b; Chatterji et al., 1984).

In an in vivo metal substitution study, it was observed that the Co-substituted Co(II)–Co(II) RPase could be oxidized to Co(III)–Co(III) RPase by H_2O_2 (Wu et al., 1977). Since Co(III) in the Co(III) complexes is known to have high affinity for nitrogen donors (Cotton & Wilkinson, 1972), the imidazole nitrogens of histidine are likely candidates for these N donors. Furthermore, histidine residues have been found in the majority of Zn metalloenzymes, participating directly in enzyme catalysis or assisting in the maintenance of the proper conformation of enzyme by ligation to metal ion (Lindskog, 1983; Eklund & Branden, 1983). Recently, it has been found that the *Xenopus* transcription factor A (TFIIIA) contains intrinsic Zn ions (Hanas et al., 1983) and is a Zn metalloenzyme (Wu, 1986; Hazuda & Wu, 1986). On the basis of its amino acid sequence, Miller et al. (1985) speculated that the Zn ion in this transcription factor may be tetrahedrally coordinated to histidine and cysteine.

Diethyl pyrocarbonate (DEP) in aqueous solutions at neutral or slightly acidic pH values has been shown to modify histidinyl residues in proteins with considerable specificity (Melchior & Fahrney, 1970; Miles, 1977). The combination of chemical modification by DEP and NMR studies have been very elegantly used to identify the Zn binding imidazole ligands in aldolase (Smith & Mildvan, 1981). We have thus carried out the chemical modification of RPase by diethyl pyrocarbonate to explore the possibility that histidinyl residues might be located at the active site of *E. coli* RPase and/or involved in the coordination of intrinsic Zn ions.

An earlier attempt to modify the histidines of *E. coli* RPase by diazonium-1*H*-tetrazole (Ishihama & Hurwitz, 1969) concluded that the reagent inhibited the [^{14}C]ATP incorporation, but not the binding of [^{14}C]DNA or the pyrophosphate

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[1] Abbreviations: RPase, RNA polymerase; Co–Co RPase, RNA polymerase containing two intrinsic Co ions; NTP, ribonucleoside triphosphate; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; β-ME, β-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; DEP, diethyl pyrocarbonate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

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exchange reaction. Modification of RPase by DEP carried out later by Rozovskaya and Bibilashvili (1979) showed that RPase was inactivated by DEP and there was an exponential decrease in the enzymatic activity depending on DEP concentration. The modification of RPase at higher DEP concentrations (0.5–4 mM) led to an irreversible inactivation as a result of the modification of 16–18 histidines and the supplementary modification of sulfhydryl and amino groups. Such modified RPase lost the ability to bind single- and double-stranded DNA. With a change in quaternary structure, DEP-modified RPase was used to study the binding efficiency to DNA and its ability to destabilize the DNA helix at the binding site (Rozovskaya et al., 1981). In view of RPase having 78 histidine residues, no attempt was made in the above studies to distinguish the critical histidines required for the RPase activity from the nonessential ones. In addition, these studies were not designed to elucidate the role of histidine(s) at the metal binding site.

In the present studies, we have reexamined and further characterized the inactivation of *E. coli* RPase by DEP. We have for the first time correlated the loss of enzymatic activity to the degree of modification of RPase histidine(s) as judged by various criteria such as secondary, tertiary, and quaternary structure and the pK_a value of the group involved in the modification. In addition, the kinetics of DEP inactivation of RPase and formation of *N*-carbethoxyhistidine were also compared. We have subjected the kinetic data of inactivation to the statistical method of Tsou (1962) to differentiate the critical and nonessential histidines. Furthermore, we have investigated the possibility of histidine involvement in the coordination with Zn in RPase holoenzyme and its subunits.

Materials and Methods

Chemicals. Unlabeled nucleoside triphosphates (NTP) were obtained from P-L Biochemicals. ^{3}H-Labeled NTP was from either ICN Pharmaceuticals or New England Nuclear. Ultrapure Tris was purchased from Schwarz/Mann. Calf thymus DNA (type I, highly polymerized), diethyl pyrocarbonate (DEP), and EDTA were products of Sigma. All other biochemicals were of the highest purity obtained commercially.

Preparation of RPase and Subunits. The DNA-dependent RPase was purified from *E. coli* MRE 600 cells (Grain Processing, Muscatine, IA) according to the method of Burgess and Jendrisak (1977). Enzyme was further purified by glycerol gradient ultracentrifugation (Burgess & Travers, 1971). The protein concentrations of RPase holoenzyme and subunits were determined either by the $\epsilon^{1\%}_{280nm} = 6.2$ M^{-1} cm^{-1} (Lowe et al., 1979) or by the Coomassie Brilliant Blue dye binding assay (Bio-Rad protein assay; Spector, 1978). The enzyme was at least 98% pure as judged by $NaDodSO_4$–polyacrylamide gel electrophoretic analysis. RPase enzyme activity was assayed as described earlier (Wu & Wu, 1973). One unit of enzyme activity is defined as 1 nmol of ^{3}H-labeled ribonucleoside monophosphate incorporated into the acid-insoluble material in 20 min at 37 °C with calf thymus DNA as template. The core RPase ($\alpha_2\beta\beta'$) and σ subunit were separated by Bio-Rex 70 column chromatography (Burgess & Travers, 1971). The subunit complex $\alpha_2\beta$ and β' subunits were separated from core RPase by Affi-Gel Blue column chromatography (Wu et al., 1977; Chatterji & Wu, 1982a). The apo (Zn-free) β' subunit and subunit complex, $\alpha_2\beta$, were prepared, modified, and reconstituted as shown in Scheme I.

Spectroscopic Measurements. UV/visible spectra were recorded with a Perkin-Elmer Lambda 3B, with a 3600 data station. The concentration of DEP was determined by reacting an aliquot (10 μL) of DEP with 3 mL of 10 mM imidazole (pH 6.0) at room temperature and following the absorbance of *N*-carbethoxyimidazole at 230 nm ($\epsilon_{230nm} = 3000$ M^{-1} cm^{-1}). The increase in absorbance is rapid and quantitative (Melchior & Fahrney, 1970).

Scheme I: Preparation of *E. coli* Apo (Zn-Free) $\alpha_2\beta$ and β' Subunits and Reconstitution with Zn

native $\alpha_2\beta/\beta'$ subunits (1–2 μM)

↓

dialysis, buffer I [10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 nM KCl, 0.1 mM DTT, and 5% glycerol], 2 h, 25 °C, then 2 h, 5 °C

↓

dialysis, buffer II (buffer I without 10 mM EDTA), 4 h, 5 °C

↓

apo $\alpha_2\beta/\beta'$

↓

dialysis, 100 mM phosphate buffer (pH 6.0), 2 h, 5 °C

↓

modification with DEP (DEP/protein = 500), 20 min, room temp

↓

dialysis, buffer III (buffer II with 10^{-5} M $ZnCl_2$), overnight, 5 °C

↓

dialysis, buffer II, 2 x 4 h, 5 °C

↓

reconstitued subunits $\alpha_2\beta/\beta'$

The carbethoxylation of RPase or subunits was carried out by incubating the protein with DEP in 100 mM phosphate buffer, pH 6.0, at room temperature. The extent of inactivation was determined by measuring the residual enzyme activity of an aliquot removed from the reaction mixture at 37 °C. The number of histidine residues being modified was estimated by the molar absorption coefficient for *N*-carbethoxyhistidine, $\epsilon_{240nm} = 3200$ M^{-1} cm^{-1} (Ovadi et al., 1967).

Hydroxylamine Treatment of Inactivated RPase. RPase (2 mg/mL) was treated with a 250-fold molar excess of DEP at pH 6.0 and room temperature. After 1, 2, and 5 min, the enzyme solution was rapidly diluted with 100 mM phosphate buffer containing 0.5 M hydroxylamine (pH 6.0) and incubated at 25 °C for 30 or 60 min. The enzyme activity was then assayed as described earlier. The enzyme without DEP treatments in phosphate buffer or hydroxylamine solution was used as control.

Zn Determination. Zn content was determined by using a Perkin-Elmer Model 4000 atomic absorption spectrophotometer equipped with an HGA graphite furnace. All labware were acid washed and rinsed with deionized, distilled water before use. Prior to measurement, protein (0.1–0.5 mg/mL) was dialyzed for at least 16 h at 4 °C against 10 mM Tris-HCl (pH 8.0) buffer containing 10 mM EDTA, 0.1 mM DTT, 0.1 M KCl, and 5% glycerol. The Zn content was calculated from the standard curve constructed by using Zn standard solution (Fisher) with deionized, distilled water and the same buffer as diluents.

Preparation of pAR1435 Plasmid DNA. Plasmid pAR1435, a gift from Dr. W. Studier and Dr. A. H. Rosenberg of the Biology Department, Brookhaven National Laboratory, Upton, NY, was isolated and purified from *E. coli* HB101 cells by the method of Maniatis et al. (1982). DNA

Table I: Reversibility of the DEP Modification of RPase by Incubation with 0.5 M Hydroxylamine[a]

		enzyme act. (units/mg)[c]					
		in 0.1 M phosphate buffer for		in 0.5 M NH_2OH for		% act. recovered by NH_2OH in	
expt	group[b]	30 min[d]	60 min	30 min	60 min	30 min	60 min
1	A	808	760				
2	B			905	701		
3	C	265	245	643	721	71	102
4	D	197	255	799	682	88	97
5	E	187	119	731	575	80	82

[a] RPase (4 μM) was first modified by a 250-fold molar excess of DEP at room temperature for different times (see *b*) followed by incubation with 0.5 M hydroxylamine in 100 mM phosphate buffer (pH 6.0) for 30 and 60 min at room temperature. The enzyme activity was then determined as described under Materials and Methods. [b] Group A, control RPase without DEP in 0.1 M phosphate buffer (pH 6.0). Group B, control RPase without DEP in 0.5 M NH_2OH (pH 6.0). Groups C, D, and E, RPase modified for 1, 2, and 5 min, respectively. [c] One unit of enzyme activity is defined as 1 nmol of [^{3}H]UMP incorporated into acid-insoluble materials in 20 min at 37 °C. [d] Incubation time of 30 and 60 min.

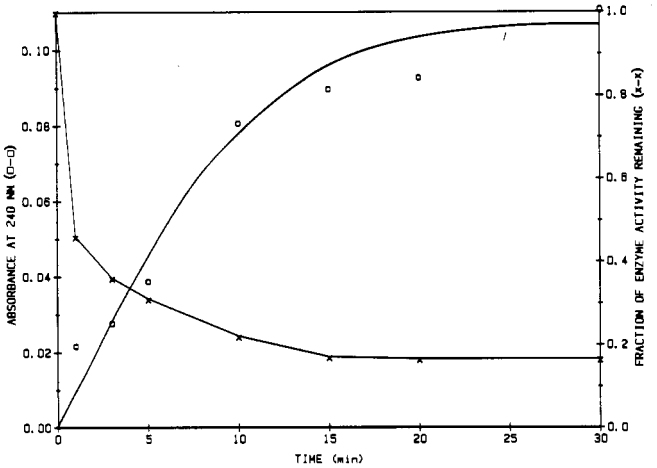

FIGURE 1: Time course of the inactivation of *E. coli* RPase with DEP. RPase (2 μM) in 1 mL of 0.1 M phosphate buffer, pH 6.0, was modified with 0.48 mM DEP at pH 6.0 and room temperature. The reaction was followed spectrophotometrically at 240 nm (□). An aliquot (10 μL) of reaction mixture was withdrawn for assay of RPase activity at the specified time interval (×). The percent error in the spectroscopy measurements and enzyme activity assays is ca. ±5%.

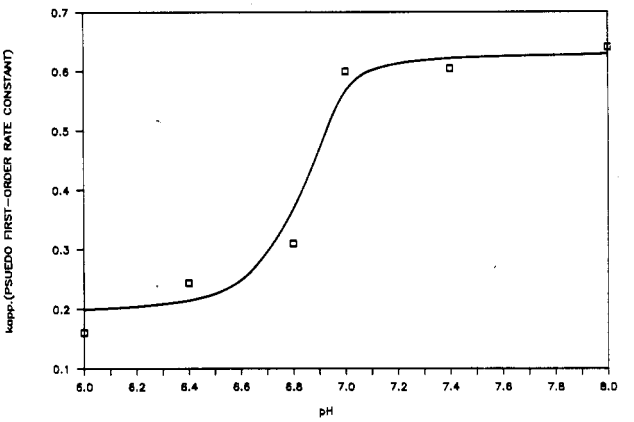

FIGURE 2: pH dependence of the pseudo-first-order rate constant (k_{app}) for the DEP inactivation of RPase. RPase (1 μM) in 1 mL of 0.1 M phosphate buffer at different pHs (6.0, 6.4, 6.8, 7.2, 7.4, 7.6, and 8.0) was modified with a 500-fold molar excess of DEP. An aliquot (10 μL) of reaction mixture was withdrawn at different time intervals (20, 40, 60, 80, 100, and 120 s) for assay of enzyme activity. For each pH, the value of k_{app} was determined by using the semilogarithmic plot as described previously.

concentration was determined by the $\epsilon^{1\%}_{260nm}$ = 200 M^{-1} cm^{-1}.

Statistical Analysis. The statistical analysis of kinetic data was carried out by using the Lotus 1-2-3 program on an IBM personal computer equipped with an 8087 math coprocessor. The best fit was obtained based on R^2 values.

RESULTS

Inactivation of RPase by DEP. Incubation of *E. coli* RPase with DEP in 0.1 M phosphate buffer at pH 6.0 resulted in a time-dependent loss of enzyme activity and a concomitant increase in absorbance at 240 nm as shown in Figure 1. Prolonged incubation (>20 min) with DEP resulted in complete inactivation of enzyme. The time course of inactivation displays a fast and slow phase, when the reagent was in great excess over enzyme. The fast phase approximated pseudo-first-order kinetics at any fixed concentration of DEP (the ratio of DEP/RPase varied from 50 to 500). The pseudo-first-order rate constant for inactivation (k_{app}) for the fast phase was obtained from the slope of the semilogarithmic plot of percent residual enzyme activity vs. time, after the contributions of the slow phase were subtracted. In other words, we have extrapolated the slow-phase line to zero time, subtracted the values of log percent inactivation for the slow phase from that of the total log percent inactivation, and replotted the fast-phase residual activity against time. The second-order rate constant (k) of inactivation of RPase, 0.97 ± 0.14 mM^{-1} min^{-1}, was then obtained from the slope of the linear plot of k_{app} against DEP concentration (figure not shown). The reaction order with respect to DEP concentration was determined by using the equation $k_{app} = kM^l$ where l is the reaction order of the modifier, M, reacting with the residues in the enzyme. The plot of $-\log k_{app}$ vs. $-\log M$ was linear, giving rise to a slope (l) of 0.7 (figure not shown). Thus, the reaction order of inactivation is approximately 1 for holoenzyme.

The enzyme activity of RPase modified with a 250-fold molar excess of DEP for 1 and 2 min could be fully recovered by incubation with 0.5 M hydroxylamine at pH 6.0 and room temperature for 1 h as shown in Table I. As the time of modification increased to 5 min, the recovery of enzyme activity decreased to 80%.

Effect of pH on RPase Inactivation by DEP. The pH dependence of the DEP inactivation of RPase was studied in 0.1 M potassium phosphate buffer between pH 5.8 and 8.0. The apparent first-order rate constant (k_{app}) of inactivation increased with pH. The plot of k_{app} for RPase inactivation vs. pH gave a typical titration curve which was drawn as a best fit of experimental points (Figure 2) with a pK value of 6.9. This pK value was within the expected range for histidine.

Effect of DEP Modification on the Structure and Conformation of RPase. When RPase was inactivated by DEP to 10% of the original enzyme activity and chromatographed on a Sephadex G-200 column, the inactivated enzyme was eluted as a single peak at the position expected for the native RPase (data not shown). The DEP-modified RPase showed no changes in content and mobility of subunits by NaDodSO$_4$-polyacrylamide gel electrophoresis as compared to the native enzyme. Resembling the native RPase, the RPases

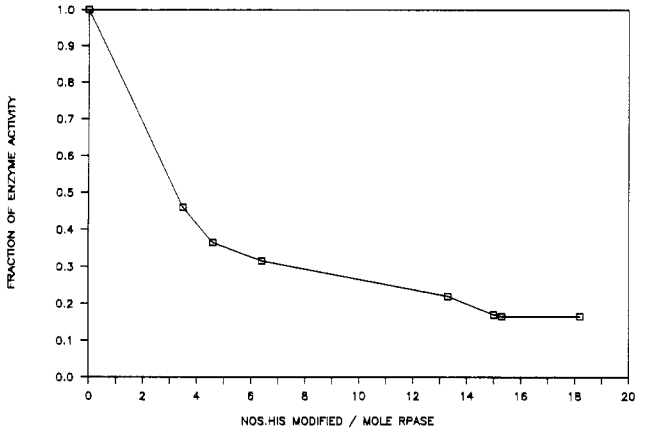

FIGURE 3: Plot of the fraction of enzyme activity remaining against the number of histidines being modified per mole of RPase. RPase (2 μM) in 1 mL of 0.1 M phosphate buffer, pH 6.0, was modified with 0.48 mM DEP. An aliquot (10 μL) of reaction mixture was withdrawn at different time intervals (3, 5, 10, 20, and 30 min) and was assayed for RPase activity. The concentration of *N*-carbethoxyhistidine formed was determined on the basis of the absorbance at 240 nm (ϵ_{240nm} = 3200 M^{-1} cm^{-1}).

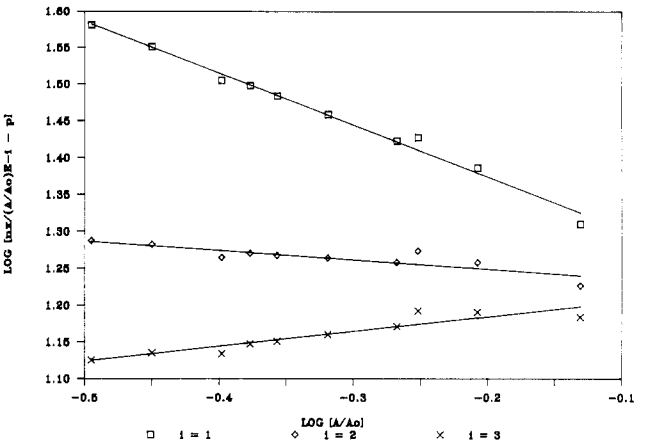

FIGURE 4: Analysis of the data for diethyl pyrocarbonate inactivation of RPase by Tsou's statistical method. RPase (1 μM) in 1 mL of 0.1 M phosphate buffer, pH 6.0, was modified with 0.11 mM DEP at room temperature and spectrophotometrically followed by A_{240nm}. At time intervals of every 10 s, an aliquot (10 μL) of reaction mixture was removed for assay of enzyme activity. The data were plotted according to eq 2 using n = 20, p = 7, and i = 1, 2, and 3 with R^2 = 0.98, 0.73, and 0.55, respectively. The value of α was obtained from the slope of the linear plot.

modified with 250- as well as a 1100-fold molar excess of DEP still fluoresced at 340 nm when excited at 280 nm. Furthermore, the UV/visible spectra of the modified RPase exhibited the same spectral characteristics possessed by the native RPase with the exception of an additional peak at 240 nm due to the formation of *N*-carbethoxyhistidine. These results indicated that the inactivation of enzyme by DEP did not induce gross conformational change of the enzyme.

Rate of N-Carbethoxyhistidine Formation of RPase. Upon incubation with DEP, the absorption spectrum of RPase showed the appearance of a peak at 240 nm, a characteristic of *N*-carbethoxyhistidine as depicted earlier (Miles, 1977). On the basis of ϵ_{240nm} = 3200 M^{-1} cm^{-1}, one can calculate the concentration of *N*-carbethoxyhistidine formed at a given stage of modification which in turn represents the number of histidinyl residues being modified per mole of RPase.

The pseudo-first-order rate constant of *N*-carbethoxyhistidine formation (k_f) was obtained from the semilogarithmic plot of the percent fraction formed vs. time (figure not shown). The percent fraction of *N*-carbethoxyhistidine formed was calculated on the basis of the maximum number, 20, of histidines modified in RPase as shown in Figure 3. The slope of the plot gave a value of 0.93 ± 0.05 mM^{-1} min^{-1} for the second-order rate constant (k') of formation. Furthermore, the reaction order of *N*-carbethoxyhistidine formation with respect to DEP concentration was determined to be 0.9 (~1) from the slope of the linear plot of −log k_f vs. −log DEP concentration (figure not shown). Thus, the rate constant and reaction order of DEP inactivation and *N*-carbethoxyhistidine formation are essentially identical. This strongly supports the contention that histidine modification is responsible for the inactivation of RPase.

Determination of the Number of Essential Histidines. Inactivation of RPase by DEP can be correlated to the number of histidine residues modified per RPase molecule, as shown in Figure 3. The plot is nonlinear, indicating that not all the histidinyl residues reacted at the same pseudo-first-order rate. The shape of the curve suggests that some of the histidines were more accessible to the reagent than the others. Extrapolation of the fraction of enzyme activity remaining from the fast-phase reaction to zero activity indicated that about seven histidines were modified. As pointed out previously (Tsou, 1962; Horiike & McCormick, 1979), such an extrapolation rarely provides an accurate estimate of the number of essential residues. Therefore, the statistical method of Tsou (1962) was used to calculate the number of essential histidinyl residues responsible for the enzyme activity of RPase. In this method, one considers that the enzyme has n modifiable residues of type x, among which p of the residues react with the reagent at a pseudo-first-order rate connstant, k_1, and $n - P$ residues react at a pseudo-first-order rate constant, k_2 ($=\alpha k_1$). Of the p residues, i are essential for enzyme activity. Assuming that the modification of any one of the essential residues results in complete loss of activity, the fraction of those molecules remaining that have full activity, denoted by A/A_0, will be equal to the fraction of activity remaining; the number of residues modified per molecule, m, will be given by (Horiike & McCormick, 1979)

$$m = n(1 - x) = (n - p)(A/A_0)^{1/i} - (n - p)(A/A_0)^{n/i} \quad (1)$$

Equation 1 can be rewritten in the form

$$\log [nx/(A/A_0)^{1/i} - p] = \log (n - p) + (\alpha - 1/i) \log (A/A_0) \quad (2)$$

Figure 4, the plot of the left-hand component in eq 2 against log (A/A_0), was obtained by using the values of n = 20, p = 7 and varying the values of i. As shown in Figure 4, only when i = 1 did we obtain the best linear fit (R^2 = 0.98). Thus, the linear regression analysis of the above data indicated that one histidinyl residue (since i = 1) is critical for catalytic function. The value of α ($=k_2/k_1$) estimated from the slope indicated a 7-fold difference between the rate constant of the modification for the essential histidinyl residue and that for the nonessential histidinyl residue.

Effect of Preincubation of Substrates or Template on RPase Inactivation. It can be seen from Figure 5 that the preincubation of substrate ATP, 0.11 and 1.14 mM, with RPase afforded ~25% and ~95% protection, respectively, against inactivation of RPase by DEP. This effect was quite pronounced in the fast phase of the DEP modification reaction. On the other hand, the template pAR1435 DNA did not protect RPase from inactivation by DEP while it slightly accelerated the process. These results are consistent with the essential histidinyl residues being located at (or near) the substrate binding site.

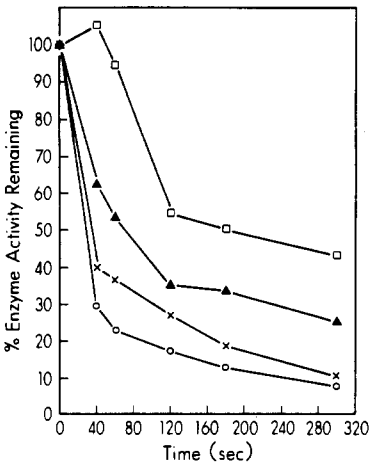

FIGURE 5: Effect of preincubation of substrate ATP or template with RPase on the modification of RPase by DEP. RPase (1.13 μM) was preincubated with 113 μM (▲) and 1.14 mM (□) ATP or 1 μM pAR1435 DNA (○) for 5 min at room temperature followed by modification with a 250-fold molar excess of DEP. At different time intervals, an aliquot (10 μL) of reaction mixture was withdrawn for assay of enzyme activity. (×) represents RPase alone.

Table II: Zn Content of the Unmodified and DEP-Modified $\alpha_2\beta$ and β' Subunits of *E. coli* RPase

subunits	Zn content[a] (mol/mol of protein) control[b]	modified[c]
$\alpha_2\beta$	0.70 ± 0.09	0.26 ± 0.12
β'	0.71 ± 0.03	0.12 ± 0.11

[a] The subunit was dialyzed extensively against buffer II (see Scheme I) after reconstitution with Zn. Zn content was determined by atomic absorption spectrometry. [b] Subunit was first made Zn-free by Scheme I and then reconstituted with Zn. [c] Apo subunit was modified with DEP in molar ratio of 1/500 (protein/DEP) for 20 min at room temperature in 100 mM phosphate buffer (pH 6.0). After modification, the subunit was reconstituted with Zn.

Modification of Apo (Zn-Free) $\alpha_2\beta$ and β' Subunits with DEP. The subunits were rendered Zn free by dialysis against Tris-HCl buffer (pH 8.0) containing 10 mM EDTA as described in Scheme I. These subunits were then modified with a 500-fold molar excess of DEP followed by reconstitution with Zn. The metal content of the reconstituted subunits with or without DEP treatment as determined by atomic absorption spectrometry is shown in Table II. The results showed that Zn could not be incorporated into the $\alpha_2\beta$ subcomplex and β' subunit once the subunits were modified with DEP.

DISCUSSION

We have confirmed the earlier report (Rozovskaya & Bibilashvili, 1979) that *E. coli* RPase was inactivated by DEP. The DEP-modified RPase showed no alterations in the UV/visible and fluorescence spectra as compared to the native RPase. It also retained the native hydrodynamic and physical properties as evidenced by the comparative studies of the modified and native RPase by gel filtration and NaDodSO$_4$–polyacrylamide gel electrophoretic analysis. Thus, the modification of RPase by DEP did not cause gross changes in the secondary, tertiary, or quaternary structure of enzyme. The inactivation of RPase appeared to be attributable to the modification of essential amino acid residue(s). Though DEP is specific for histidine at pH 6.0 (Muhlrad et al., 1967; Dann & Britton, 1974), other nucleophilic residues such as tyrosine, serine, lysine, and cysteine might also be modified in addition to histidine (Muhlrad et al., 1967; Melchior & Fahrney, 1970; Miles, 1977). O-Carbethoxylation of tyrosyl residues can be detected by a decrease in absorbance at 278 nm, while modification of serine residues by DEP is readily reversible in neutral aqueous solution. Both effects were not observed in the DEP-inactivated RPase. Modification of amino groups could lead to the inactivation of RPase that is not reversed by treatment with NH_2OH. Since we have observed the reversibility of DEP-inactivated RPase by NH_2OH, the interaction of an essential amino group of RPase with DEP can be ruled out. The possible modification of cysteine residues by DEP can be tested by titration of the sulfhydryl residues in the native and modified enzymes by Ellman's reagent (Habeeb, 1970). Most importantly, the reversibility of the DEP-inactivated *E. coli* RPase by hydroxylamine, which occurs solely by decarbethoxylation of the modified histidine(s) (Miles, 1977), confirmed that histidine was modified. This conclusion is further supported by the pH dependence of DEP inactivation which showed a titratable group with a pK value of ~6.9, the approximated pK value for histidine in proteins. The pH titration data are not consistent with the fact that sulfhydryl group is responsible for the inactivation of RPase.

Hydroxylamine can fully restore the enzyme activity of *E. coli* RPases which have been inactivated by lower concentrations (<0.5 mM) of DEP (having approximately six to nine histidines modified). The modification of RPase at higher DEP concentrations (>0.5 mM) (having more than nine histidines modified) led to the refraction of enzyme reactivation by hydroxylamine (Rozovskaya & Bibilashvili, 1979). The latter phenomenon may be attributed to the dicarbethoxylation of histidines, which occurs at high concentration of modifier (Miles, 1977). In our studies, DEP concentrations were usually kept at <0.5 mM. Furthermore, our observation that the reaction order of *N*-carbethoxyhistidine formation is 0.7 contradicts the dicarbethoxylation of histidines. Another possibility is that at higher DEP concentrations it might modify other residues such as sulfhydryl or amino groups which have been reported earlier (Rozovskaya & Bibilashvili, 1979).

Inactivation of RPase by DEP and *N*-carbethoxyhistidine formation both followed the same first-order kinetics. The fact that the second-order rate constants (k) of inactivation and formation of *N*-carbethoxyhistidines were found to be essentially the same (~0.9 mM^{-1} min^{-1}) supports our earlier contention that only one kind of amino acid residue, histidine, reacted with DEP, leading to the inactivation of RPase. Treatment of our kinetic data of inactivation using Tsou's statistical method revealed the presence of one essential histidinyl residue for enzyme activity which reacted about ~7 times faster than the other histidinyl residues. Using radioactively labeled DEP, one can verify and identify the histidine. Such a study is in progress. The preincubation of RPase with a lower concentration (0.11 mM) of substrate ATP gave partial (~25%) protection against DEP inactivation, while a higher ATP concentration (1.14 mM) offered almost total (~90%) protection against DEP inactivation, which is consistent with the dissociation constant (~200 μM) for ATP and RPase. This effect is significant in the fast phase of the inactivation reaction, when the active-site histidine is being protected. Inactivation still occurred in the later time points, which may be due to the modification of other residues. Our results showed that the DNA template offered no protection against DEP, concurring with the earlier report (Rozovskaya & Bibilashvili, 1979), yet it accelerated DEP inactivation slightly. These results suggest that the active-site histidine might be located at or near the substrate binding site of the enzyme. Our earlier finding that ATP is in direct coordination with the intrinsic Zn ion in *E. coli* RPase (Chatterji & Wu,

1982b) suggests that the metal moiety might be in close proximity to the essential histidine.

Modification of Zn-free subunits, β' and $\alpha_2\beta$, of RPase by DEP resulted in the formation of an *N*-carbethoxyhistidinyl derivative. It has been shown that DEP reacts with the deprotonated form of imidazole nitrogen (Holbrook & Ingram, 1973) which coordinates with the intrinsic metal ion in a number of metalloproteins. The unavailability of histidine(s) in the DEP-modified subunits for coordination with Zn during reconstitution might explain the failure of Zn incorporation into the reconstituted apo subunits. It is also possible that carbethoxylation of the imidazole nitrogen of histidine might induce a conformational change in the enzyme, leading either to steric hindrance for Zn binding or to loss of Zn binding site. Further studies are needed to rule out this possibility. By differential UV/visible spectroscopy of the native and modified subunits, we did not observe any gross conformational change induced by DEP modification. On the basis of this observation and the earlier one for the formation of Co(III)–Co(III) RPase (Wu et al., 1977), we concluded that histidine may be one of the ligands at the coordination site of Zn in both β and β' subunits of RPase. The active-site histidine which we proposed to be involved in ATP binding may or may not be involved in the ligation to Zn. This is not surprising, since in a number of Zn metalloenzymes such as aldolase (Smith & Mildvan, 1981) and carbonic anhydrate (Lindskog, 1983), more than one histidine participates in the Zn ligation. The above result and the number of ligands involved in Zn binding can be substantiated by other physical studies such as X-ray crystallography, circular dichroism, NMR, and EPR. Studies using EPR spectroscopy are under way to characterize the Zn ligands in *E. coli* RPase.

In summary, we have shown that one histidinyl residue is apparently critical for the enzyme activity of *E. coli* RPase and this residue might be in close proximity to the intrinsic metal at the initiation site of the β subunit. In addition, both Zn-containing subunits β and β' may have histidine as a ligand coordinated to Zn. Using chemical modification of histidine by diethyl pyrocarbonate, we have attempted to probe the microenvironment of the metal site in *E. coli* RPase. This technique can be a simple and useful tool to study the metal environment of other metalloenzymes.

Registry No. DEP, 1609-47-8; RPase, 9014-24-8; L-His, 71-00-1; Zn, 7440-66-6.

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