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Reactions of Spinach Ribulose-1,5-bisphosphate Carboxylase with Tetranitromethane†

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ABSTRACT: Tetranitromethane [TNM, $C(NO_2)_4$] rapidly inactivated spinach ribulosebisphosphate carboxylase (RuBPCase). The extent of inactivation was increased by preincubation of the enzyme with Mg^{2+} and bicarbonate. Activity was substantially protected by 6-phosphogluconate, a competitive inhibitor of carboxylation, by ribulose 1,5-bisphosphate, a substrate, and by *p*-(chloromercuri)benzoate, which was used to covalently block enzyme sulfhydryl groups. Sulfhydryl titration with Ellman's reagent showed that over one-third of the titratable cysteine residues were lost upon complete inactivation. The losses of activity and of sulfhydryl groups titratable in nondenatured enzyme occurred with similar kinetics. Approximately half the sulfhydryl groups that were lost upon TNM-induced inactivation were restored by mercaptoethanol and dithiothreitol (DTT). High concentrations of DTT, however, did not restore more than 20% of the activity lost. Amino acid analysis revealed that about one-third of the modified sulfhydryls had been irreversibly oxidized. Both amino acid analysis and difference spectroscopy showed that little or no tyrosine modification occurred. These results suggest that TNM inactivates spinach RuBPCase by modification of cysteine sulfhydryls. This indicates that the inactivation of RuBPCase associated with SH modification does not depend solely on the presence of bulky groups attached to the modified sulfur. This conclusion differs from that obtained in similar studies using the RuBPCase of *Rhodospirillum rubrum*, in which TNM modified a single tyrosine per dimer of catalytic subunits [Robison, P. D., & Tabita, F. R. (1979) *Biochem. Biophys. Res. Commun. 88*, 85].

The enzyme D-ribulose-1,5-biphosphate carboxylase/oxygenase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] (RuBPCase[1]) is bifunctional. It catalyzes the CO_2-fixation step in the C-3 photosynthetic carbon-reduction cycle, which converts a molecule each of D-ribulose 1,5-bisphosphate (RuBP) and CO_2 to two molecules of D-3-phosphoglycerate (3-PGA) (Calvin et al., 1955). The enzyme also catalyzes the reaction of RuBP with O_2 to yield a molecule each of 3-PGA and 2-phosphoglycolate, a substrate of photosynthetic carbon oxidation (Ogren & Bowes, 1971).

In all higher plants, the enzyme is composed of nonidentical large (M_r 56 000) and small (M_r 14 000) subunits (Siegel et al., 1972). Small subunits are not found in RuBPCase from the purple, nonsulfur bacterium *Rhodospirillum rubrum*. This enzyme consists of a dimer of large (M_r 56 000) subunits. RuBPCases in higher plants show great structural homology, especially in the large subunits, which bear the catalytic site (McIntosh et al., 1980; Zurawski et al., 1981). Amino acid composition data (Takabe & Akazawa, 1975a) and partial sequence data (Stringer et al., 1981; Herndon et al., 1982) suggest that only limited sequence homology exists between RuBP carboxylase from a higher plant, spinach, and that from *R. rubrum*.

Nevertheless, affinity-labeling techniques have indicated conservation of primary structure in the active sites of carboxylases from these two sources (Herndon et al., 1982). In both enzymes, lysine residues are essential for catalysis (Paech & Tolbert, 1978; Schloss et al., 1978a; Robison et al., 1980; Lorimer, 1981b) and for the binding of activating CO_2 and Mg^{2+} (O'Leary et al., 1979; Lorimer, 1981a). Arginine (Schloss et al., 1978b; Chollet, 1981) and histidine (Saluja & McFadden, 1982) may also be important in catalysis by the spinach enzyme. Recently, Fraij & Hartman (1982) suggested that methionine is located at the active site of the *R. rubrum* enzyme. Numerous studies have shown sulfhydryl

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[1] Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; RuBPCase, ribulosebisphosphate carboxylase; PCMB, *p*-(chloromercuri)benzoate; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; PGA, 3-phosphoglycerate; TNM, tetranitromethane, $C(NO_2)_4$; 6-PGluA, 6-phosphogluconate; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SCM-cysteine, *S*-(carboxymethyl)cysteine; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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reagents to inactivate spinach RuBP carboxylase (Rabin & Trown, 1964; Akazawa et al., 1968; Takabe & Akazawa, 1975b; Schloss et al., 1978a). However, it is believed that cysteine is not involved directly in catalysis, since there are no structural homologies between cysteine-containing peptides of the spinach and *R. rubrum* enzymes (Stringer et al., 1981; Herndon et al., 1982). Robison & Tabita (1979) proposed that tyrosine is essential in catalysis by *R. rubrum* RuBPCase, on the basis of their observation that tetranitromethane (TNM) inactivates the enzyme and modifies one tyrosine per large subunit dimer. TNM has been shown to nitrate tyrosine residues in certain proteins (Sokolovsky et al., 1966) but has also been found to oxidize cysteine to a variety of products (Riordan & Christen, 1968; Sokolovsky et al., 1969; Lane & Dekker, 1972). A specific active site tryptic peptide isolated from spinach RuBP carboxylase with affinity labels contains lysine, arginine, tyrosine, and cysteine (Schloss et al., 1978a).

Grebanier et al. (1978) demonstrated inactivation of higher plant RuBP carboxylase using TNM. In that study, the cross-linking activity of the reagent was used to probe for conformational changes in spinach and pea leaf RuBP carboxylase. However, the cross-linking effects per se were not considered responsible for the inactivation.

The object of the present study was to characterize the inactivation of spinach RuBP carboxylase by TNM. Tyrosine modification in spinach RuBP carboxylase was checked because this amino acid had been suggested to play a role in catalysis by the *R. rubrum* enzyme. Our results indicate, however, that in the spinach enzyme, TNM acts primarily by oxidizing thiols.

Materials and Methods

Materials. Spinach leaves were obtained from local markets. Cysteic acid, *S*-(carboxymethyl)cysteine, 3-nitro-L-tyrosine, RuBP, 6-PGluA, DTT, iodoacetic acid, DTNB, TNM, PCMB, and Bicine buffer were obtained from Sigma Chemical Co. $NaDodSO_4$ and a Coomassie Blue protein determination kit were obtained from Bio-Rad. Sodium [^{14}C]bicarbonate (1 mCi/mL, 50 mCi/mmol) was obtained from New England Nuclear. Methanesulfonic acid (4 N) (containing 0.2% tryptamine), standard amino acid mixtures, and the Pico buffer system II were obtained from Pierce Chemical Co. Constant-boiling HCl (approximately 6 N) was obtained by distillation of reagent-grade HCl.

Isolation of Spinach Ribulosebisphosphate Carboxylase. The isolation procedure described previously (Roy et al., 1978) was used with the following modifications: The homogenization buffer contained 25 mM Tris-HCl (pH 7.8), 50 mM 2-mercaptoethanol, 1 mM Na_2EDTA, and 1 mM PMSF. Sucrose-gradient buffers were of the same composition as the homogenization buffer, but PMSF was omitted. Bio-Gel A5M and DEAE-Sephadex A-50 column steps were eliminated in the present procedure. Newly isolated ribulosebisphosphate carboxylase yielded a specific activity of 1.5 μmol/(min·mg) and retained ca. 50% activity for 4 weeks when stored in liquid nitrogen as a 50% saturated $(NH_4)_2SO_4$ precipitate. Enzyme was judged to be >95% pure by the presence of only minor amounts of protein bands other than large and small subunits on $NaDodSO_4$–polyacrylamide gels.

Protein Determination. Concentration of purified ribulosebisphosphate carboxylase in milligrams per milliliter was determined by multiplying the A_{280}, measured in a 1-cm cell, by 0.61. Where the protein was modified by *p*-(chloromercuri)benzoate (PCMB) or when it was mixed with 2-mercaptoethanol or sodium dodecyl sulfate, a Coomassie Blue dye binding method was used (Bradford, 1976).

Assay for Ribulosebisphosphate Carboxylase Activity. The radiotracer assay for carboxylase activity was performed as reported earlier (Grebanier et al., 1978) with the exception that 25 mM Tris-HCl (pH 8.2, 25 °C), 1 mM Na_2EDTA, and 50 mM 2-mercaptoethanol was the standard buffer used. All activation and assay steps, except where noted, were performed at 30 °C in the presence of 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, which were required for maximum activation of the carboxylase (Lorimer et al., 1976). Each 0.2-mL reaction contained 0.5 μCi of $NaH[^{14}C]O_3$. Mercaptoethanol was omitted from both control and experimental samples whenever TNM was employed. Two-minute assay times were employed unless noted otherwise. Catalysis was confirmed to be linear over this period.

Modification by Tetranitromethane. The procedure was similar to that used previously (Grebanier et al., 1978). Concentrations of enzyme and TNM, however, were lowered to prevent irreversible cross-linking and to obtain measurable rates of inactivation.

After removal of mercaptoethanol from stored enzyme by gel filtration or dialysis, the enzyme was either activated for 30 min at 30 °C in the presence of 10 mM $MgCl_2$ and 10 mM $NaHCO_3$ or left in its inactive state, where noted. To begin inactivation, TNM solutions in 95% ethanol were diluted 100-fold into the enzyme solutions. TNM action was later quenched with 2-mercaptoethanol, except where noted. To study inactivation kinetics, it was desirable to quench TNM and initiate carboxylation simultaneously. Thus, 0.01 mL of enzyme containing TNM was diluted into 0.19 mL of the reaction mixture described previously containing 10 mM mercaptoethanol and 0.5 mM RuBP. Controls verified that the levels of mercaptoethanol used were sufficient to stop TNM action before significant catalysis could take place (J. Patrizio, unpublished results). In certain experiments, where noted, 2-mercaptoethanol was not used to quench TNM reactions.

In cases where inactivation was performed in the absence of Mg^{2+} and HCO_3^-, enzyme was incubated with these following TNM treatment and prior to assay, to ensure activation of any unmodified enzyme. Thus, TNM effects could be considered to be related to the activation steps, the catalytic steps, or both. No attempt was made to distinguish these in the present study.

Protection by 6-PGluA and RuBP. The ability of 6-PGluA, a modulator of activity, to protect against TNM inactivation was tested in both the presence and absence of Mg^{2+} and HCO_3^- by employing high concentrations of 6-PGluA (see Table I for details). For studies of RuBP protection, enzyme was not activated prior to TNM addition (details in Table I).

Protection by PCMB. The method of Paech & Tolbert (1978) was used. Stored enzyme was dialyzed to remove mercaptoethanol; then, sulfhydryl groups were covalently blocked with PCMB prior to the addition of TNM (details in Table V). TNM inactivation was quenched with 10 mM mercaptoethanol.

Spectroscopy. Spectra were obtained for TNM-inactivated and control RuBP carboxylase at pH 9.0, using a Perkin-Elmer 552 spectrophotometer. The concentration of 3-nitrotyrosine can be measured by its characteristic absorbance at 428 nm (Sokolovsky et al., 1966). We confirmed that the molar extinction of authentic 3-nitrotyrosine is 4200 L/(mol·cm).

Amino Acid Analysis. Control and TNM-inactivated RuBPCase were either carboxymethylated or allowed to stand, followed by hydrolysis under two different conditions: for tyrosine determination, 24-h hydrolysis in 4 N methanesulfonic acid–0.2% tryptamine was employed (Simpson et al., 1976);

for determination of cysteine and its derivatives, sulfhydryls were carboxymethylated as described by Norton et al. (1975) except that mercaptoethanol was omitted during the reaction with thrice-recrystallized iodoacetic acid. The hydrolysis was carried out in sealed evacuated vials with constant-boiling 6 N HCl at 109 °C for 24 h. The amino acids were determined with the amino acid analyzer described by Bullock & Myer (1978) equipped with Durrum DC-4 resin and the Pico buffer system II.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out as previously described (Grebanier et al., 1978).

Sulfhydryl Determination. Titrations with Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), were performed on both denatured and nondenatured enzyme to detect the loss of sulfhydryl residues upon TNM inactivation. In addition, restoration of DTNB-titratable sulfhydryls was tested by titrating enzyme after both incubations with thiols and without.

Two methods were used: (1) The reaction was quenched with 10 mM 2-mercaptoethanol followed by incubation of the enzyme on ice in either the presence or absence of 0.1 M mercaptoethanol, followed by dialysis to remove reductant and titration in either the native or denatured state (see Table III for details); or, (2) the reaction was terminated by precipitation with 80% saturated ammonium sulfate, and then the enzyme was reprecipitated with 50% saturated ammonium sulfate, resuspended in Tris–EDTA buffer, denatured by addition of solid urea to 8 M, and incubated 3.5 h at 25 °C in the presence of 2.5 mM DTT or held on ice. DTT was later removed by gel filtration over a Bio-Gel P2 column (1.4 × 28 cm), and the protein was immediately titrated with DTNB.

To titrate nondenatured enzyme, 0.5 mL of enzyme at 0.9 mg/mL in Tris–EDTA buffer (pH 8.2) was mixed with 0.2 mL of 14.1 mM DTNB in 0.05 M Na_2HPO_4 (pH 7.0). To titrate in the denatured state, enzyme at about 0.25 mg/mL (either in 2.5% $NaDodSO_4$ or 8 M urea) was mixed with the same proportions of DTNB. Optical density at 412 nm was read 45 min later when the titration rate matched that of DTNB in buffer. Reaction times of 45 min showed ca. 98% completion with respect to reaction times of 120 min. Neither the presence of thiols to quench TNM, the order of the denaturation and reduction steps, nor the use of dialysis vs. gel filtration to remove thiols affected the final titration results.

Results

Inactivation by TNM. RuBP carboxylase was inactivated rapidly by low concentrations of TNM (Figure 1). The conditions used in these experiments were much milder than those previously shown to cause irreversible inter- and intrasubunit cross-linking (Grebanier et al., 1978). The absence of irreversible cross-linking in the present experiments was confirmed by the presence of only minor amounts of polypeptide species other than monomeric large and small subunits during polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ and mercaptoethanol. L_x, a polypeptide species believed to result from an irreversible intrasubunit cross-link in the large subunit (Grebanier et al., 1978), was also absent in the present experiments. Control experiments confirmed that higher TNM concentrations lead to the cross-linked products reported earlier.

The inactivation data in Figure 1 did not fit pseudo-first-order kinetics or any simple scheme as shown by attempted computer fit to various kinetic equations. Complicated kinetics were also observed during TNM inactivation of RuBP carboxylase from *R. rubrum* (Robison & Tabita, 1979). These

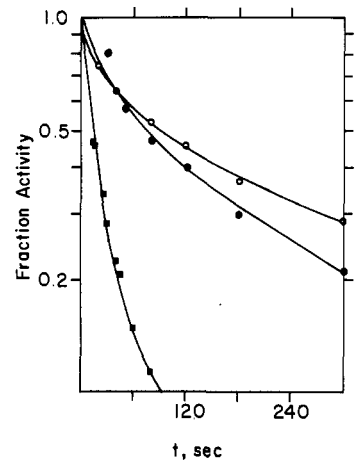

FIGURE 1: Inactivation of RuBPCase by TNM. Enzyme activated as described under Materials and Methods was exposed to TNM at 30 °C unless noted otherwise. After the times specified on the abscissa, the samples were quenched by the addition of 10 mM 2-mercaptoethanol and assayed for activity. (●) 0.01 mg/mL enzyme and 11 μM TNM at 28 °C; (○) 0.09 mg/mL enzyme and 11 μM TNM; (■) 0.9 mg/mL enzyme and 110 μM TNM. The data for each curve represent the mean for three experiments. Control activity was 1.0 μmol/(min·mg of protein).

authors found a 50% activity loss in 4 min when the bacterial enzyme at 9.7 μM was inactivated by 336 μM TNM (a 17-fold excess with respect to 19.4 μM catalytic subunit). In contrast, we found a 50% activity loss in only 10 s when the spinach enzyme at 1.6 μM was inactivated with 110 μM TNM (8.5-fold excess with respect to 13 μM catalytic subunit). Inactivation occurred much more rapidly with the spinach enzyme even though the activation, buffer, pH, and temperature were similar to those used in the *R. rubrum* study.

Protection against TNM Inactivation. To evaluate TNM effects on catalytic-site residues, the differential effects of Mg^{2+}, HCO_3^-, 6-PGluA, and RuBP on TNM-induced inactivation were examined. Mg^{2+} and HCO_3^- have been found to potentiate inactivation of pea RuBP carboxylase by TNM (Grebanier et al., 1978). Mg^{2+} and CO_2 activate RuBPCase (Lorimer et al., 1976) and appear to cause a conformational change (Grebanier et al., 1978). Mg^{2+} stabilizes the binding of carboxyarabinitol bisphosphate, a transition-state analogue, to the enzyme (Pierce et al., 1980). This behavior suggests that Mg^{2+} increases the accessibility of catalytic sites to substrates or other reagents. 6-PGluA operates as a modulator of activity by binding to the catalytic site (Badger & Lorimer, 1981).

As shown in Table I, preincubation of enzyme with Mg^{2+} and HCO_3^- allowed an 80% loss of activity upon subsequent exposure of the enzyme of TNM. In the absence of Mg^{2+} and HCO_3^-, only a 44% loss was observed. 6-PGluA at 50 mM partially protected activity in the presence of Mg^{2+} and HCO_3^- (58% activity loss). A much more substantial protection by 50 mM 6-PGluA was seen when enzyme had not been exposed to Mg^{2+} and HCO_3^-, however. In this case only a 13% loss of activity was seen. This represents 69% protection of the activity in the absence of Mg^{2+} and HCO_3^- when 6-PGluA was present.

Protection by RuBP was tested in a separate experiment. Grebanier et al. (1978) demonstrated nearly complete protection against TNM inactivation of pea RuBP carboxylase by 0.5 mM RuBP. Similarly, protection by 0.5 mM RuBP in the absence of Mg^{2+} and HCO_3^- was observed with spinach RuBP carboxylase (Table I, experiment 2). In this experiment, TNM alone led to 93% inactivation. TNM in the presence

Table I: Effects of Prior Additions of Ligands on TNM-Induced Inactivation

prior additions	fraction of activity remaining after TNM inactivation (±0.5 range): TNM inactivated	control without TNM
Experiment 1[a]		
10 mM $MgCl_2$, 10 mM $NaHCO_3$	0.20 (±0.08)	1.00 (±0.04)
none	0.49 (±0.07)	0.88 (±0.11)
10 mM $MgCl_2$, 10 mM $NaHCO_3$, 50 mM 6-PGluA	0.28 (±0.16)	0.67 (±0.04)
50 mM 6-PGluA	0.65 (±0.33)	0.75 (±0.05)
Experiment 2[b]		
none	0.07 (±0.06)	1.00 (±0.20)
0.5 mM RuBP	0.54 (±0.13)	1.00 (±0.20)

[a] Experiment 1: 0.09 mg/mL enzyme either was first activated by $MgCl_2$ and $NaHCO_3$ as described under Materials and Methods or received no additions as noted. 6-PGluA incubations, if performed, were then conducted for 15 min at 30 °C. 11 μm TNM (final) was used to inactivate for 5 min, after which the enzyme was diluted 100-fold into reaction mixtures containing 10 mM 2-mercaptoethanol (as described under Materials and Methods) but without RuBP. All solutions were incubated 30 min in the reaction mixture to activate those samples that had not previously received Mg^{2+} and bicarbonate. RuBP (0.5 mM) was then added to initiate carboxylation. Activities were compared to controls that were treated identically except that ethanol was added instead of TNM. 6-PGluA (0.5 mM) was present during assay after 100-fold dilution into the reaction mixture. This inhibited activity. A correction for this inhibition was made in order to calculate % protection: corrected activity = 0.65(0.88)/0.75 = 0.76. The % protection by 6-PGluA in the absence of activating salts was calculated as % protection = (0.76 − 0.49)/(0.88 − 0.49) × 100. The data represent the means (±0.5 range) of three measurements from one experiment. [b] Experiment 2: Enzyme at 0.9 mg/mL was incubated with 0.5 mM RuBP at 30 °C for 2 min, followed by 110 μM TNM inactivation for 5 min. After quenching with 30 mM mercaptoethanol followed by gel filtration over a Sephadex G-25 column (1.0 × 14 cm) equilibrated with Tris-EDTA buffer, the enzyme was adjusted to 0.09 mg of protein/mL and activated by $MgCl_2$ and $NaHCO_3$. Carboxylase assays were initiated by introduction of enzyme into reaction mixtures containing 0.05 mM RuBP. Activities were compared to a control receiving no TNM. The data represent the mean (±0.5 range) of four measurements.

of RuBP led to just 46% inactivation. This constituted 51% protection by RuBP.

Spectroscopy of TNM-Inactivated vs. Control RuBP Carboxylase. Spectra were obtained in order to detect the possible formation of 3-nitrotyrosine, a frequently observed modification product of TNM in proteins (Sokolovsky et al., 1966; Christen et al., 1971). Spectra were nearly identical for the control and inactivated enzyme (Figure 2a). To quantitate any small differences in the visible region, very high protein concentrations were employed. A typical difference spectrum for the visible region from 350 to 475 nm is shown in Figure 2b. A small difference is observed over the complete region scanned. The entire difference at 428 nm, the maximum absorbance wavelength of authentic 3-nitrotyrosine, yielded a calculation of 0.76 nitrotyrosyl residue out of 220 tyrosines per holoenzyme (Siegel et al., 1972) or 0.095 out of the 19 tyrosines per catalytic subunit deduced from sequencing data (Zurawski et al., 1981; Martin, 1979). Therefore, even if all the absorbance at 428 nm is attributed to 3-nitrotyrosine, the data indicate that less than one such modified amino acid could have been present per eight catalytic sites.

Amino Acid Analysis. The cysteine and tyrosine contents of control and TNM-inactivated RuBP carboxylase were compared to characterize any changes of these specific amino

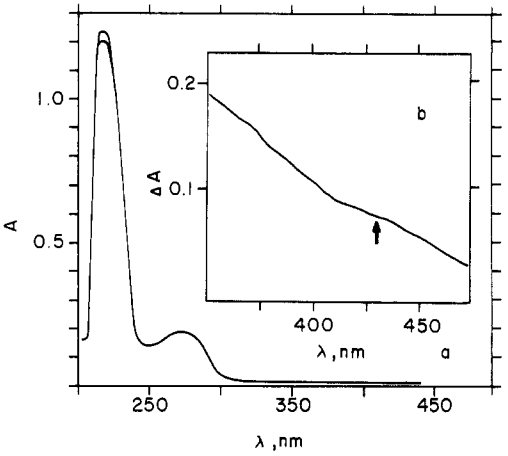

FIGURE 2: Spectroscopy of TNM-inactivated vs. control RuBPCase. Activated enzyme at 0.9 mg/mL was inactivated by 110 μM TNM for 5 min, concentrated vs. solid sucrose, dialyzed, and adjusted to 13.15 mg/mL and pH 9.0. The spectra shown were obtained by scanning 100-fold-diluted samples in the region from 220 to 440 nm with a Perkin-Elmer 552 spectrophotometer. Except at 225 nm, where the inactivated sample showed a slightly higher absorbance, the spectra were superimposable (a). The inset (b) shows the difference spectrum (inactivated − control) from 350 to 475 nm, at 13.15 mg/mL, using an expanded scale. Arrow denotes maximum absorbance of 3-nitrotyrosine.

Table II: Amino Acid Analysis of Cysteine Derivatives in S-Carboxymethylated Control and TNM-Inactivated and Control RuBPCase[a]

amino acid	mol of residues/mol of enzyme (±0.5 range): control	TNM-inactivated
tyrosine	234 (±2)	230 (±10)
3-nitrotyrosine	0	0
3,5-dinitrotyrosine	0	0
SCM-cysteine	102 (±16)	60 (±5)
cysteic acid	trace	14 (±1)
half-cystine	trace	21 (±8)

[a] Activated enzyme at 1.0 mg/mL was inactivated by 98% with 110 μM TNM for 5 min at 30 °C. Controls received an equal amount of 95% ethanol. After precipitation from 80% saturated ammonium sulfate, the sample was redissolved, reprecipitated in 50% saturated ammonium sulfate, and subjected to gel filtration on a Sephadex G-25 column (1 × 30 cm) to remove reaction products. For the tyrosine derivatives, no carboxymethylation was performed, and the elution conditions were optimized for resolution of these amino acids. For cysteine and its derivatives, the enzyme was S-carboxymethylated as described under Materials and Methods, exhaustively dialyzed against distilled water, lyophilized, and hydrolyzed in constant-boiling HCl. Hydrolysates were analyzed by the method of Bullock & Myer (1978), which uses several amino acids as base line for computing amino acid contents. The amino acid standards used were mock hydrolysed to control for differential stability of different amino acids during hydrolysis. The sequencing data of Zurawski et al. (1981) and Martin (1979) were then used to calculate the molar production of each derivative in the RuBPCase hydrolysates.

acid residues (Table II). No significant loss of tyrosine was observed, and no 3-nitrotyrosine or 3,5-dinitrotyrosine was observed above the limits of detection. From these data it can be calculated that no more than one molecule of either of these derivatives had been produced per mol of TNM-modified RuBPCase. TNM oxidation of cysteine in proteins has been reported to lead to formation of cysteic acid and disulfide bonds. Products of the reactions between TNM and thiols can also lead to formation of cysteine sulfinic acid (Sokolovsky et al., 1969; Riordan & Christen, 1968; Lane & Dekker, 1972). To distinguish the possible cysteine derivatives in

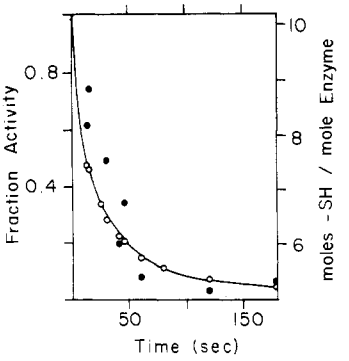

FIGURE 3: Kinetics of activity and sulfhydryl-group loss in nondenatured RuBPCase. 0.9 mg/mL activated enzyme was inactivated for the specified times with 110 μM TNM before quenching with 10 mM mercaptoethanol. After dialysis, activity and DTNB-titratable sulfhydryl groups of the nondenatured enzyme were measured as described under Materials and Methods. (○) Fraction activity remaining relative to control; (●) number of titratable sulfhydryls present per holoenzyme. The data represent the means of three experiments.

RuBPCase, the modified enzyme was carboxymethylated in the absence of reductants, hydrolyzed, and subjected to analysis. Cysteine residues that had not reacted with TNM would be recovered as *S*-(carboxymethyl)cysteine, while any disulfides should not be carboxymethylated under these conditions. The irreversibly oxidized derivative cysteic acid is highly acidic and would elute first upon amino acid analysis. As shown in Table II, the cysteic acid content was higher in hydrolysates of the TNM-treated enzyme (14 mol/mol of enzyme) than in hydrolysates of control enzyme (essentially 0 mol); moreover, a significant fraction of the cysteine (at least 20%) was rendered insensitive to carboxymethylation by TNM treatment. Apparently, cysteine is partly converted to disulfides and partly to more oxidized form, under the conditions used in these studies (see discussion).

In other experiments (data not shown), there were no significant effects of TNM treatment on the content of aspartate, serine, threonine, glutamate, glycine, alanine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine, arginine, or tryptophan.

Sulfhydryl Titration. Reactions of sulfhydryls with TNM were monitored by titration with DTNB. Although the data reported here came from experiments where 0.9 mg/mL enzyme was inactivated by 110 μM TNM, experiments using 0.09 mg/mL enzyme and 11 μM TNM yielded essentially the same results. When nondenatured enzyme was used, between four and six sulfhydryl residues were lost upon 5-min TNM treatments. In Figure 3, the kinetics of carboxylase activity loss and sulfhydryl loss in nondenatured enzyme are compared. Activity loss and sulfhydryl loss occur with similar kinetics. This further supports the view that the TNM-induced inactivation is due to sulfhydryl modification. The number of sulfhydryls detected in this experiment is smaller than that detected by amino acid analysis (Table II). This was considered to be due to steric hindrance of DTNB in nondenatured enzyme. Accordingly, enzyme was titrated both before and after denaturation. In the nondenatured enzyme, six sulfhydryl groups out of 16 were lost upon TNM treatment (Table III). This represented a 38% loss of titratable residues. As expected, a greater number of sulfhydryls, 56, were titratable in Na$DodSO_4$-denatured enzyme. In this case, however, 19 sulfhydryls were lost. This represents 34% or approximately the same fraction lost as was detected in nondenatured enzyme. Similar percentage losses were detected by amino acid analysis (36–41%, Table II). The detection of the same fraction of sulfhydryls lost regardless of the conformation of the enzyme suggested that TNM may have modified "buried" as well as "exposed" sulfhydryl residues.

Table III: DTNB-Titratable Sulfhydryl Groups of TNM-Inactivated and Control RuBPCase

	no. of DTNB-titratable sulfhydryls/ holoenzyme (±0.5 range)		
		TNM inactivated	
	control	3 h, 0 °C	3 h, 25 °C, 0.1 M mercapto-ethanol
nondenatured	16 (±1)	10 (±1)	14 (±2)
Na$DodSO_4$ denatured	56 (±5)	37 (±3)	44 (±9)

[a] Activated enzyme at 0.9 mg/mL was inactivated (by 98%) with 110 μM TNM for 5 min at 30 °C, followed by addition of 10 mM mercaptoethanol to quench TNM. To test for reversibility of sulfhydryl loss, portions of the control and TNM-inactivated samples were placed either on ice or in 0.1 M mercaptoethanol for 3 h at 25 °C. All samples were dialyzed exhaustively to remove mercaptoethanol and then titrated with DTNB both in the native state and after denaturation in 2.5% Na$DodSO_4$ as described under Materials and Methods. Samples of control enzyme that had been stored on ice in 10 mM mercaptoethanol or incubated in 0.1 M mercaptoethanol were both titrated. Sulfhydryl content did not differ between these controls in the native or denatured state. The control reported is a sample of enzyme held on ice. Molar extinction of the 3-carboxylato-4-nitrothiophenolate anion was determined to range from 13 600 to 14 000 L/(mol·cm) by titration of stock mercaptoethanol solutions. For nondenatured enzyme, the data represent the means of four experiments. For Na$DodSO_4$-denatured enzyme, the data represent the mean of triplicate titrations in one experiment.

It was of interest to see if the loss of sulfhydryls was reversible. TNM-inactivated enzyme was incubated with 25 mM DTT. The data in Table III show that a greater number of sulfhydryls were titratable in those inactivated samples that were incubated with DTT (14 in native, 44 in denatured enzyme) than those not incubated (10 in native, 37 in denatured enzyme). The data agree with the amino acid analyses, which indicated that only some of the sulfhydryls were converted to disulfides by TNM. To eliminate the possibility that disulfide bonds were inaccessible to reducing agent in the nondenatured enzyme, experiments were also performed in which inactivated enzyme was first denatured in 8 M urea and then reduced with 2 mM DTT. The results were the same as those shown in Table III (data not shown).

In enzyme that had been completely inactivated and then treated with 2.5 mM DTT, 16–21% of the activity was restored (Table V, lines 2 and 3). To test whether partially inactivated enzyme could be reactivated to a greater extent, we added DTT to several samples that had been inactivated for different periods of time (Table IV). On the average, only 20% of the lost activity could be recovered.

Protection against TNM Inactivation by PCMB. The data so far indicate that thiols are the sensitive target for TNM in spinach RuBPCase. Therefore, PCMB, a reagent that covalently blocks sulfhydryl residues, was used to demonstrate protection of the enzyme from the effects of TNM. Of course, PCMB treatment in itself inactivates RuBPCase; but, reductive removal of the alkylating agent from the enzyme restores activity and should allow measurement of the extent of TNM inactivation occurring during PCMB blockage. As seen in line 2 of Table V, in enzyme that was not first treated with PCMB, none of the activity survived TNM. DTT restored 16–21% of the activity of TNM-modified enzyme. The PCMB-treated sample, however, upon removal of PCMB after TNM exposure, retained 66–92% of the carboxylase activity

Table IV: Limited Reactivation of TNM-Inactivated RuBPCase by DTT[a]

time after TNM addition (s)	initial activity remaining: before 25 mM DTT incubation (%)	initial activity remaining: after 25 mM DTT incubation (%)	reactivation (% ±SD)
8	54	62	17
20	36	62	41
30	29	43	25
40	31	40	13
60	22	39	22
90	25	32	9
150	16	26	12
			20.0 (±10.9)[b]

[a] Enzyme at 0.9 mg/mL was inactivated by 110 μM TNM and quenched at various times by addition of 10 mM mercaptoethanol and assayed immediately. The activity remaining is expressed as a percent of that shown by a control that recieved the same compounds but in reverse order. All solutions were then incubated with 25 mM DTT for 3 h at 30 °C, activated with $MgCl_2$ and $NaHCO_3$ as described under Materials and Methods, and assayed. Untreated enzyme retained its activity over this time period. The data represent the means of triplicate assays. % reactivation = (activity after DTT − activity before DTT)/(control activity − activity before DTT) × 100. [b] Mean reactivation.

as compared to a control that was similarly PCMB treated but not exposed to TNM before incubation with DTT. This constituted from 57 to 91% protection by PCMB against TNM inactivation (see Table V).

Discussion

The data presented here demonstrate that the inactivation of spinach RuBPCase by TNM is accompanied by substantial modification of sulfhydryl groups. This inactivation is partly reversible, which may be related to the fact that some of the sulfhydryl modification can also be reversed. The data indicate that SCM-cysteine, cystine, and cysteic acid were present in the carboxymethylated TNM-modified enzyme. This observation is consistent with the suggestion of Sokolovsky et al. (1969) that a sulfenyl nitrate intermediate produced by TNM reaction could lead to formation of cysteic acid or disulfides. Other derivatives could also be formed, including cysteinesulfenic acid and cysteinesulfinic acid. Although cysteinesulfenic acid has been inferred to be present in some proteins (Little & O'Brien, 1969; Parker & Allison, 1969), it is unstable in the presence of oxygen and would yield cysteinesulfinic acid under the present conditions. Cysteinesulfinic acid decomposes in 6 N HCl at 110 °C (Calam & Waley, 1962) and would not be detected. However, since the recovered derivatives of cysteine account for all the cysteine in RuBPCase, we conclude that little cysteinesulfinic acid was present before hydrolysis.

Because the inactivation is partially protected by active site reagents like 6-phosphogluconate and RuBP, it is likely that at least some of the modified sulfhydryls are located at or near the active site. Many previous studies have shown that sulfhydryl modification leads to inactivation of RuBPCase (Rabin & Trown, 1964; Takabe & Akazawa, 1975b; Schloss et al., 1978a). Although it is not believed that cysteine plays a role in catalysis per se (Stringer et al., 1981; Herndon et al., 1982), the general belief is that the SH groups play some structural role (Takabe & Akazawa, 1975a,b). The modification of these groups by iodoacetate or other alkylating agents certainly must lead to substantial structural changes locally and has been reported to lead to extensive conformational changes (Chollet & Anderson, 1977). In contrast to the alkylating agents, tetranitromethane results in quite small structural changes on a local scale such as those required by the extra oxygen atoms in oxidized cysteine derivatives or those resulting from stabilization of the disulfide bonds. Nevertheless, the inactivation is substantial and at least part of it is due to the conversion of cysteinyl to cystine residues. This suggest that the role of cysteine is something more than just to provide a place where alkylating agents can disrupt the structure of the enzyme.

The studies of Grebanier et al. (1978) showed that tetranitromethane inactivates pea and spinach RuBPCase and that RuBP can protect against inactivation. The concentration of TNM used in that study was great enough to cause substantial irreversible cross-linking. The inactivation of the enzyme, however, does not require such high concentrations of TNM. Thus, the inactivation observed here is not due to irreversible cross-linking.

Robison & Tabita (1979) found that tetranitromethane modifies one tyrosine per large subunit dimer upon 50% inactivation of RuBPCase from the photosynthetic bacterium *R. rubrum*. This modification occurred in the absence of any irreversible changes in sulfhydryl content. The behavior of TNM with spinach RuBPCase is clearly quite different as

Table V: Protection of RuBPCase Activity against TNM Using PCMB[a]

incubations (in order): PCMB	TNM	DTT	fraction activity after DTT incubation (±0.5 range): 5 h	6.5 h	av
−	−	+	1.00 (±0.10)	1.00 (±0.02)	1.00 (±0.02)
−	+	−	0.00	0.00	0.00
−	+	+	0.16 (±0.06)	0.21[b]	0.17 (±0.07)
+	−	−	0.00	0.00	0.00
+	−	+	0.63 (±0.05)	1.15 (±0.10)	0.89 (±0.36)
+	+	+	0.58[b]	0.76 (±0.01)	0.70 (±0.12)

[a] 1.8 mg/mL enzyme was fully activated in 0.1 M Bicine buffer (pH 9.0), 10 mM $MgCl_2$, and 10 mM $NaHCO_3$. To 4 mL of this solution was added 1 mL of 0.73 mM PCMB in 0.1 M Bicine (pH 8.0). A control sample received 1 mL of buffer lacking PCMB. After 68 min at room temperature, both samples were precipitated by addition of ammonium sulfate to 50% saturation. The precipitated enzyme was dissolved and dialyzed against 0.1 M Bicine–10 mM $MgCl_2$ buffer to remove unbound PCMB and then adjusted to approximately 0.65 mg/mL and 10 mM $NaHCO_3$ in the same buffer and incubated at 30 °C for 30 min. This would maximally activate any noninactivated enzyme present prior to TNM exposure. Both enzyme solutions were split, and half of each was exposed to 110 μM TNM for 5 min at 30 °C. All four solutions then received 10 mM mercaptoethanol to quench TNM where present. [This low concentration of mercaptoethanol was sufficient to quench TNM and did not restore activity to enzyme exposed to PCMB (not shown).] All solutions were then incubated with 2.5 mM DTT for 5 or 6.5 h to remove bound PCMB and assayed for activity. The data presented are the means of duplicate determinations. The fraction activities are expressed relative to the control in line 1. Activity in the PCMB-treated control was partially to fully restored (line 5) after DTT incubation to that of non-PCMB, non-TNM treated enzyme. This necessitated correction of the fraction activity in the PCMB, TNM treated control [example: 0.76(1.00)/1.15 = 0.66] in order to calculate % protection, e.g., av % protection of 6.5-h samples = (0.66 − 0.21)/(1.00 − 0.21) × 100. [b] A single measurement was performed.

might be expected from the comparative sequence data that have become available recently (Stringer et al., 1981; Herndon et al., 1982).

The DNA sequence of the spinach large subunit gene (Zurawski et al., 1981) and the amino acid sequence of the small subunit (Martin, 1979) show that there are 80 cysteine residues per holoenzyme in spinach RuBPCase. This differs from the 96 SH groups reported by Takabe & Akazawa (1975b) and the 95 by Siegel et al. (1972) on the basis of PCMB titration and amino acid analysis, respectively. Chollet & Anderson (1977) reported 65 SH groups in tobacco RuBPCase as determined by PCMB titration and 55 as determined by DTNB titration. They attributed the high estimates of Takabe & Akazawa (1975a,b) to the incorrect use of bovine serum albumin as a standard in Lowry protein assays of RuBPCase concentration. Our results indicate that about 50–60 sulfhydryls can be routinely titrated with DTNB. This 30% underestimate is not due to oxidation of the enzyme, as shown by the absence of cystine in carboxymethylated RuBPCase, nor is it due to air oxidation during the preparation of samples for titration, since both relatively prolonged dialysis and rapid gel filtration give the same results. It is not due to an overestimate of protein concentration as a result of any hypothetical ultraviolet-absorbing contaminants in the sample, which is electrophoretically homogeneous and has a clean protein spectrum. It is conceivable that the bulky DTNB is not completely free to react with all the cysteine residues even in the "random-coil" state (8 M urea) of RuBPCase. In any case, our data agree with the DTNB titration data of Chollet and Anderson, who used the tobacco enzyme. In view of the strong conservation of sequence in the large subunits of RuBPCase and the fact that 75% of the mass of the protein is represented by large subunits, we consider it likely that DTNB titration cannot detect all the SH groups in higher plant RuBPCase. For that reason, the DTNB titration data can be taken to indicate only a statistical sampling of the general behavior of SH groups in RuBPCase.

A large proportion of RubPCase sulfhydryls are accessible to TNM, and most of those that are involved in stabilizing activity of the enzyme are also accessible to PCMB. These observations support the view that numerous sulfhydryl groups are on the surface or near the surface of the molecule. Paech (1982) recently suggested that RuBPCase may be part of a transient multienzyme complex in the stroma of the chloroplast. If that is correct, then the role of sulfhydryls in regulating the stability of such a complex would become an important question. It might be of interest to determine the effects of the relatively mild TNM-induced modification of sulfhydryls on the aggregation behavior of RuBPCase.

Added in Proof

Bhagwhat (1982) reported that TNM nitrates tyrosine in spinach RuBPCase. By contrast, our data show that TNM can inactivate RuBPCase without affecting tyrosine residues.

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Registry No. Ribulose-1,5-bisphosphate carboxylase, 9027-23-0; tetranitromethane, 509-14-8; 6-phosphogluconic acid, 921-62-0; D-ribulose 1,5-bisphosphate, 24218-00-6; *p*-(chloromercuri)benzoic acid, 59-85-8.

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Asparaginase II of *Saccharomyces cerevisiae*: Comparison of Enzyme Stability in Vivo and in Vitro†

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ABSTRACT: Asparaginase II of *Saccharomyces cerevisiae* is a cell wall mannan containing glycoprotein. Recent studies have demonstrated that asparaginase II activity increases in exponentially growing cell cultures and then decreases as the cells enter the stationary phase. Enzyme inactivation has been attributed to a Zn^{2+}-dependent protease which is synthesized de novo during the late exponential phase [Pauling, K. D., & Jones, G. E. (1980) *J. Gen. Microbiol. 117*, 423–430; Pauling, K. D., & Jones, G. E. (1980) *Biochim. Biophys. Acta 616*, 271–282]. We have investigated the mechanism of asparaginase II inactivation using both whole cell suspensions and highly purified enzyme. Our data indicate that the rate of asparaginase II inactivation in cell suspensions is primarily influenced by pH changes that occur as a consequence of cell growth and glucose fermentation and that enzyme inactivation is not dependent on Zn^{2+} or on de novo protein synthesis. Also, in vitro studies with purified enzyme show kinetics of inactivation that are similar to those observed in vivo. Consequently, involvement of a yeast protease in the inactivation process is relatively unlikely.

Asparaginase II is a phosphomannan protein which is secreted into the cell wall of *Saccharomyces cerevisiae* (Dunlop & Roon, 1975; Jones, 1977a,b; Dunlop et al., 1976, 1978, 1980a). Synthesis of the enzyme is regulated by the nitrogen supply (Dunlop & Roon, 1975; Dunlop et al., 1980a,b; Kang et al., 1982). Asparaginase II activity can vary over 100-fold, with very low levels being observed in cell growing on a readily metabolized nitrogen source such as glutamine and high levels being found in cells starved for nitrogen or grown on a poor nitrogen source such as proline. Recently, it was reported that during exponential growth of *Saccharomyces* of minimal ammonia medium, asparaginase II is synthesized in high levels and then is inactivated during the time of transition to the stationary phase (Pauling & Jones, 1980a,b). This biphasic pattern of enzyme synthesis and inactivation has been confirmed by studies in our laboratory (Roon et al., 1982).

In studying the inactivation of asparaginase II, Pauling & Jones (1980a,b) found that enzyme inactivation was prevented by the addition of a protein synthesis inhibitor (cycloheximide), metal chelator (EDTA),[1] or sulfhydryl reagents (pOHMB, iodoacetate). Also, the rate of asparaginase II inactivation was decreased in cells treated with a protease inhibitor, TPCK. Furthermore, the addition of Zn^{2+} to cells treated with EDTA stimulated the rate of enzyme inactivation. On the basis of these data, Pauling and Jones suggested that asparaginase II is degraded by a Zn^{2+}–dependent protease which is synthesized de novo during the late exponential phase of growth.

The possibility that asparaginase II is degraded by a proteolytic system is an intriguing one for at least two reasons. First, the elucidation of such an inactivation system could shed further light on the mechanisms whereby enzymes of nitrogen catabolism are regulated. Proteolysis could provide a novel physiological mechanism for the (down) regulation of asparaginase II when its catalytic function (ammonia production) is no longer necessary. Second, proteolysis of asparaginase II might serve as a model for the turnover of other yeast exoenzymes such as invertase and acid phosphatase (Schekman, 1982). It would be of considerable interest to understand the mechanism whereby a proteolytic enzyme was able to degrade asparaginase II, an exoenzyme which is presumably entrapped within the mannan/glucan matrix of the yeast cell wall. Exoenzymes such as asparaginase II are secreted into the yeast cell wall in the bud portion of growing cells perhaps within a periplasmic-like space (Tkacz & Lampen, 1973; Field & Schekman, 1980). The exoenzymes are accessible to exogenous substrates of low molecular weight. However, it is presently unclear to what extent these exoenzymes would be accessible to freely diffusible extracellular proteases or to proteases secreted into the yeast cell wall subsequent to exoenzyme deposition. Thus, a convincing demonstration of the protease-dependent turnover of asparaginase II within the yeast cell wall would raise many questions concerning the physical characteristics of this cellular structure and the turnover of the exoenzymes entrapped therein.

The present report documents our studies on the inactivation of asparaginase II with whole cell suspensions and highly purified enzyme preparations. Although in many respects we have confirmed the experimental results of Pauling & Jones (1980a,b), a detailed comparison of the kinetics of asparaginase II inactivation in vivo and in vitro does not support their proteolysis model. Rather, our data suggest that asparaginase II is denatured as the result of the decrease in pH which occurs

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[1] Abbreviations: TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid; YNB, yeast nitrogen base (without amino acids and ammonium sulfate); pOHMB, *p*-(hydroxymercuri)benzoate.

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