Vol. 88, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1979 Pages 85-91

MODIFICATION OF RIBULOSE BISPHOSPHATE CARBOXYLASE FROM RHODOSPIRILLUM RUBRUM WITH TETRANITROMETHANE[1]

Peter D. Robison and F. Robert Tabita[2]

Department of Microbiology
The University of Texas at Austin
Austin, Texas 78712

Received March 12,1979

SUMMARY: Ribulose bisphosphate carboxylase from Rhodospirillum rubrum is inactivated by low concentrations of tetranitromethane. Addition of the substrate ribulose 1,5-bisphosphate and preincubation with Mg^{+2} and HCO_3^- both protect against inactivation. A spectrum of modified enzyme shows a peak at 430 nm, consistent with modification of tyrosine residues. This modified enzyme contains 1.07 nitrotyrosine residues under conditions where 49% of the activity is lost. These results provide the first evidence for an essential tyrosine residue at the active site of ribulose bisphosphate carboxylase.

INTRODUCTION

Ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39) catalyzes the transfer of CO_2 to RuBP[3] and is the initial step in the reductive pentose phosphate or Calvin cycle (1). The enzyme from the photosynthetic microorganism Rhodospirillum rubrum is a convenient tool for physical and chemical studies because of its simple quaternary structure. Whereas the R. rubrum enzyme is a dimer of large subunits and has a native molecular weight of 114,000 (2), the higher plant enzyme has a molecular weight of about 560,000 (3) and contains 8 large (catalytic) and 8 small subunits of unknown function.

Recent chemical modification studies have implicated lysine (4-7), cysteine (6) and arginine(8,9,22)residues as essential for activity. Prelim-

[1]Supported by National Institutes of Health Grant GM-24497, Robert A. Welch Foundation Grant F-691, and by NIH Postdoctoral Fellowship GM-07817 to PDR.

[2]Author to whom reprint requests should be sent.

[3]Abbreviations used are: RuBP, ribulose 1,5-bisphosphate; Tris, tris (hydroxymethyl) aminomethane; DEAE, Diethylaminoethyl; EDTA, (ethylenedinitrilo) tetraacetic acid.

0006-291X/79/090085-07$01.00/0

Copyright © 1979 by Academic Press, Inc.
All rights of reproduction in any form reserved.

inary investigations in our laboratory using 1-ethyl-3- (3-dimethylaminopropyl) -carbodiimide hydrochloride indicated an essential tyrosine might also be present (10). In this study we have examined the effect of tetranitromethane, a mild nitrating reagent that selectively modifies tyrosines (11), on RuBP carboxylase from R. rubrum. Higher levels of tetranitromethane have been previously shown to cross-link subunits of the eucaryotic enzyme from pea and spinach (12).

MATERIALS AND METHODS

RuBP carboxylase from R. rubrum was purified as in previous procedures (13) except for the elimination of the streptomycin step and the addition of a Bio Gel A 1.5 M gel filtration step after chromatography with DEAE cellulose. Sodium dodecyl sulfate gel electrophoresis (14) showed the enzyme to be >95% pure. Protein concentrations of the enzyme were calculated from the extinction coefficient at 280 nm of 0.974 Lg^{-1} (2). Enzyme activity was measured by acid stable [^{14}C] $NaHCO_3$ incorporation (15). The assay mixture (0.25 mls) contained 40 mM Tris-SO_4 (pH=8.0),10 mM dithiothreitol, 10 mM $MgCl_2$, 0.8 mM RuBP and 20 mM $NaHCO_3$ containing 2 µCi of [^{14}C] $NaHCO_3$. The assays run with 1 mM $NaHCO_3$ also contained 2 µCi of [^{14}C] $NaHCO_3$. The enzyme was activated by incubation with all assay components except RuBP for 5 minutes. The assay was then initiated with RuBP and terminated after 5 minutes by the addition of propionic acid.

Tetranitromethane solutions were made up fresh in 95% ethanol. Incubations with tetranitromethane were performed in 50 mM Tris-SO_4 (pH=8.0) at room temperature unless otherwise indicated. Concentrations of up to 10% ethanol in incubations had no effect on enzyme activity. The reaction was terminated by the addition of 25 mM dithiothreitol or 70 mM 2-mercaptoethanol. The spectrum of nitrated enzyme was run on a Cary 219 spectrophotometer operating with an automatic baseline. For spectral analysis, modified and control (incubated in the presence of the same amount of ethanol minus the tetranitromethane) enzyme were dialyzed overnight against 50 mM Tris-SO_4, pH=9.0. To determine total protein sulfhydryl content, control and modified enzyme from the spectral analysis were subsequently dialyzed overnight against 0.08 M sodium phosphate buffer (pH=8.0) containing 2% sodium dodecyl sulfate and 0.5% EDTA. The sulfhydryl content was then measured as in Habeeb (16).

RuBP was prepared enzymatically from ribose 5-phosphate (17), as described earlier (15). Tetranitromethane was from Aldrich or Sigma; samples from both sources behaved identically. [^{14}C] $NaHCO_3$ was from Amersham/Searle. All other materials were of reagent grade.

RESULTS

Incubation of R. rubrum RuBP carboxylase with low concentrations of tetranitromethane caused a rapid, concentration-dependent loss in activity (Fig. 1). A molar excess of 17 fold caused a loss of about 70% of the activity within one hour. A higher excess, 35 fold, caused the loss of about 80% of the activity. The substrate RuBP afforded significant pro-

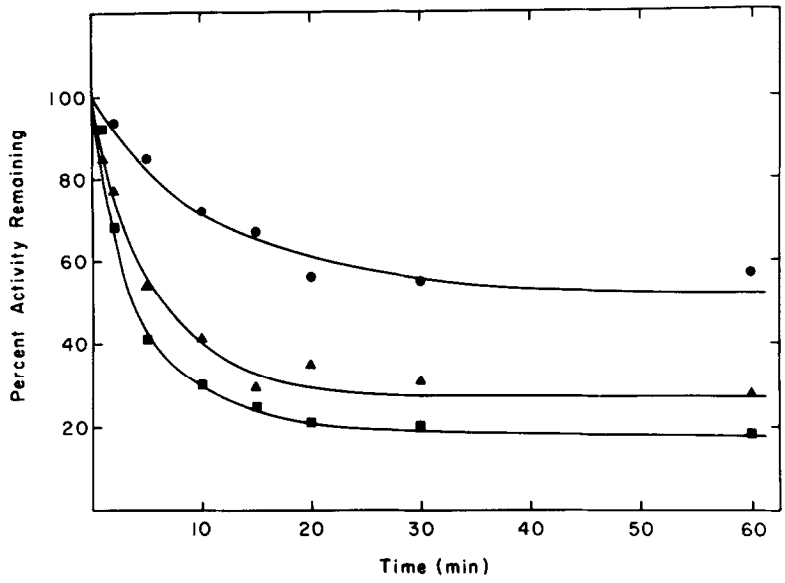

Fig. 1. Inactivation of R. rubrum RuBP carboxylase with tetranitromethane. Each incubation contained 9.7 μM enzyme. The control activity was 1.51 μmol HCO_3^- fixed/min per mg protein. ●, 84 μM tetranitromethane; ▲, 168 μM tetranitromethane; ■, 336 μM tetranitromethane.

tection against the loss of activity caused by tetranitromethane (Fig. 2). Using 17 fold excess tetranitromethane, the addition of 0.4 mM RuBP reduced the loss from 70% to about 35%. In addition, Mg^{+2} and $NaHCO_3$ protected against loss of activity (Fig. 2). The addition of 2.5 mM Mg^{+2} and 20 mM $NaHCO_3$, which were incubated with the enzyme for 5 minutes before tetranitromethane addition to ensure maximum activation, reduced the loss in activity to about 50%.

The effect of tetranitromethane on activity at low (1 mM) $NaHCO_3$ concentrations was slightly greater than the activity using 20 mM $NaHCO_3$ (Table I, Expt. 1). In contrast to the higher level, there was no protection by 1 mM $NaHCO_3$ when present with 2.5 mM Mg^{+2}. Fructose 1,6 bisphosphate, which inhibits activity at 20 mM $NaHCO_3$ and activates at 1 mM $NaHCO_3$ (10,18), afforded no protection against tetranitromethane inactivation as did 3-phosphoglycerate, the product of the carboxylase reaction (Table I, Expt. 2).

Since tetranitromethane is thought to react with the phenoxide ion of tyrosine (19), lowering the pH to 6 eliminates almost all of the nitration

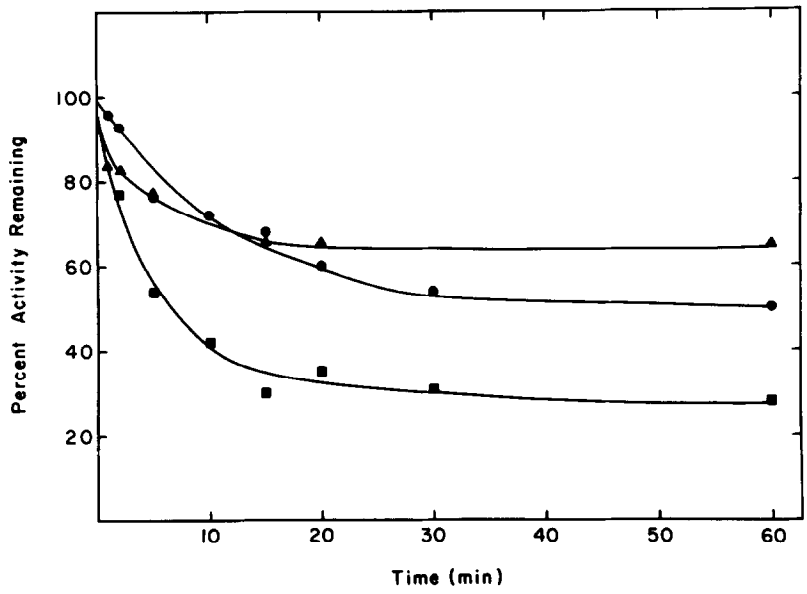

Fig. 2. Protection from tetranitromethane inactivation of RuBP Carboxylase by substrates. Each incubation contained 9.7 μM enzyme and 168 μM tetranitromethane. ▲, 0.4 mM RuBP; ●, 2.5 mM Mg^{+2} and 20 mM $NaHCO_3$; ■, no additions.

Table I. Effect of Low $NaHCO_3$ Activity, Effectors[a] and pH on Tetranitromethane Inactivation of RuBP Carboxylase

Expt.	Additions	% Activity Remaining	
		Standard Assay 20 mM $NaHCO_3$	1 mM $NaHCO_3$ Assay
1.	none	10	3
	1 mM $NaHCO_3$ + 2.5 mM Mg^{+2}	11	7
	20 mM $NaHCO_3$ + 2.5 mM Mg^{+2}	33	31
2.	none	17	-
	1 mM RuBP	63	-
	1 mM Fructose 1,6bisphosphate	15	-
	1 mM 3-phosphoglycerate	12	-
3.	pH=8.0 none	9	-
	pH=6.0 none	67	-
	pH=6.0 20 mM $NaHCO_3$ + 2.5 mM Mg^{+2}	44	-
	pH=6.0 0.5 mM RuBP	71	-

[a]Incubations were for 1 hour and contained 9.7 μM enzyme and 168 μM tetranitromethane. The pH=6.0 incubations were performed in 50 mM Tris-maleate.

of this residue. With RuBP carboxylase from R. rubrum, some inactivation by tetranitromethane was seen at pH=6, but this was far less than that seen at the higher pH (Table I, Expt. 3). This inactivation was also enhanced by the addition of 20 mM $NaHCO_3$ and 2.5 mM Mg^{+2}. This bicarbonate - Mg^{+2} enhanced inactivation is also seen when the R. rubrum carboxylase is incubated with 5,5'-dithio (bis-2-nitrobenzoic acid) (10) and indicates some oxidation of sulfhydryl groups by tetranitromethane is probably occuring at the lower pH.

Modification of the enzyme with tetranitromethane caused spectral changes which are consistent with nitration of tyrosine residues (11). A difference spectrum of modified versus control enzyme showed a broad peak centered at about 430 nm (Fig. 3). Using an extinction coefficient for nitrotyrosine of 4200 mol $liter^{-1}$ cm^{-1} at pH=9.0 (20), it was calculated there were 1.07 nitrotyrosines per enzyme molecule. This modification produced inactivation of 49% of the activity indicating nitration of two tyrosines per enzyme molecule (one per subunit) would be sufficient for elimination of all RuBP carboxylase activity. A total protein sulfhydryl determination of both control and modified enzyme showed the same amount, 2.7 sulfhydryl groups, were present in this enzyme preparation, indicating that no sulfhydryl groups were oxidized by tetranitromethane during the incubation.

DISCUSSION

Incubation of RuBP carboxylase from R. rubrum with low amounts of tetranitromethane caused a rapid and concentration-dependent loss of carboxylating activity. Addition of the substrate, RuBP, but not the product 3-phosphoglycerate or the effector fructose 1,6 bisphosphate, protected against this inactivation. Preincubation of the enzyme with $NaHCO_3$ and Mg^{+2} also protected against the loss of activity by tetranitromethane. The nitration of approximately one tyrosine residue per enzyme dimer with a subsequent loss of approximately 50% of the activity indicates that a stoichiometry of

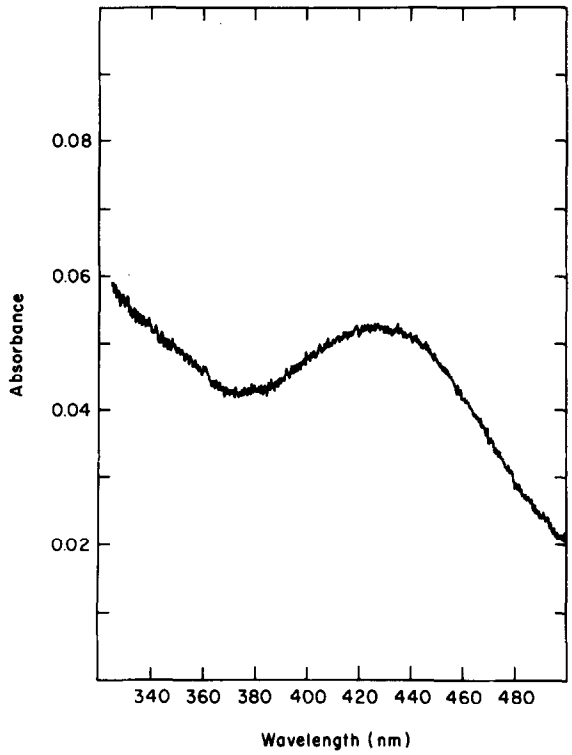

Fig. 3. Difference spectrum of tetranitromethane-modified versus control RuBP carboxylase. The modified enzyme (10.7 μM) was incubated with 168 μM tetranitromethane for 1 hour and treated as in Materials and Methods.

one per subunit is sufficient for total inactivation. This is in contrast to the stoichiometry of pyridoxal 5'-phosphate inactivation where the modification of 1 lysine residue per dimer is sufficient for loss of all R. rubrum carboxylase activity (21). Moreover, the addition of Mg^{+2} and $NaHCO_3$ stimulates rather than protects the inactivation caused by pyridoxal phosphate (4).

These results indicate that RuBP carboxylase from R. rubrum is inactivated by nitration of one specific tyrosine of the 12 present (2) on each subunit. Thus, a nucleophilic group, tyrosine, may be an important amino acid at the active site of RuBP carboxylase. Work is in progress to further characterize this tyrosine modification.

REFERENCES

1. Jenson, R.G., and Bahr, J.T. (1977) Ann. Rev. Plant Physiol. 28, 379-400.
2. Tabita, F.R., and McFadden, B.A. (1974) J. Biol. Chem. 249, 3459-3464.
3. Paulsen, J.M., and Lane, M.D. (1966) Biochemistry 5, 2350-2357.
4. Whitman, W.B., and Tabita, F.R. (1978) Biochemistry 17, 1282-1287.
5. Paech, C., and Tolbert, N.E. (1978) J. Biol. Chem. 253, 7864-7873.
6. Schloss, J.V., Stinger, C.D., and Hartman, F.C. (1978) J. Biol. Chem. 253, 5707-5711.
7. Chollet, R., and Anderson, L.L. (1978) Biochim. Biophys. Acta 525, 455-467.
8. Chollet, R. (1978) Biochem. Biophys. Res. Commun. 83, 1267-1274.
9. Lawlis, V.B., and McFadden, B.A. (1978) Biochem. Biophys. Res. Commun. 80, 580-585.
10. Whitman, W.B. (1978) Ph.D. Dissertation, University of Texas at Austin.
11. Riordan, J.F., and Vallee, B.L. (1972) in Methods in Enzymology, Vol 25, pp 515-521, Academic Press, New York.
12. Grebanier, A.E., Champagne, D., and Roy, H. (1978) Biochemistry 17, 5150-5155.
13. Tabita, F.R., and McFadden, B.A. (1974) J. Biol. Chem. 249, 3453-3458.
14. Laemmli, U.K. (1970) Nature 227, 680-685.
15. Whitman, W., and Tabita, F.R. (1976) Biochem. Biophys. Res. Commun. 71, 1034-1039.
16. Habeeb, A.F.S.A. (1972) in Methods in Enzymology, Vol 25, pp 457-464, Academic Press, New York.
17. Horecker, B.L., Hurwitz, J., and Weissback, A. (1958) Biochem. Prep. 6, 83-90.
18. Whitman, W.B., and Tabita, F.R. (1978) Fed. Proc. 37, 1426.
19. Bruice, T.C., Gregory, J.J., and Walters, S.L. (1968) J. Amer. Chem. Soc. 90, 1612-1619.
20. Sokolovsky, M., Riordan, J.F., and Vallee, B.L. (1966) Biochemistry 5, 3582-3589.
21. Whitman, W.B., and Tabita, F.R. (1978) Biochemistry 17, 1288-1293.
22. Schloss, J.V., Norton, I.L., Stringer, C.D., and Hartman, F.C. (1978) Biochemistry 17, 5626-5631.