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Oxygen-Dependent Inactivation of Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase in Crude Extracts of *Rhodospirillum rubrum* and Establishment of a Model Inactivation System with Purified Enzyme

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Ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RuBPC/O) was inactivated in crude extracts of *Rhodospirillum rubrum* under atmospheric levels of oxygen; no inactivation occurred under an atmosphere of argon. RuBP carboxylase activity did not decrease in dialyzed extracts, indicating that a dialyzable factor was required for inactivation. The inactivation was inhibited by catalase. Purified RuBPC/O is relatively oxygen stable, as no loss of activity was observed after 4 h under an oxygen atmosphere. The aerobic inactivation catalyzed by endogenous factors in crude extracts was mimicked by using a model system containing purified enzyme, ascorbate, and $FeSO_4$ or $FeCl_3$. Dithiothreitol was found to substitute for ascorbate in the model system. Preincubation of the purified enzyme with RuBP led to enhanced inactivation, whereas Mg^{2+} and HCO_3^- significantly protected against inactivation. Unlike the inactivation catalyzed by endogenous factors from extracts of *R. rubrum*, inactivation in the model system was not inhibited by catalase. It is proposed that ascorbate and iron, in the presence of oxygen, generate a reactive oxygen species which reacts with a residue at the activation site, rendering the enzyme inactive.

Evidence has been presented that aerobic inactivation of ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (RuBPC/O) in *Rhodospirillum rubrum* proceeds by a two-step process: the enzyme is first inactivated by an alteration or modification, and then the inactive protein is proteolytically degraded (2). This is very similar to well-described systems, such as glutamine synthetase (GS) and glutamine phosphoribosylpyrophosphate aminotransferase, in which oxidative inactivation precedes the actual degradation of the modified protein (11, 21). With regard to GS, inactivation may be mediated by a variety of enzymatic [cytochrome P-450, glucose oxidase, horseradish peroxidase, NAD(P)H oxidase, and xanthine oxidase] and nonenzymatic [ascorbic acid, dihydroxyfumaric acid, and NAD(P)H plus menadione] systems (14). Inactivation was found to result in the loss of 1 of 16 histidine residues, with the subsequent formation of 1 carbonyl group per GS subunit (8). Levine has suggested that a mixed-function oxidation system generates a reactive oxygen species which reacts with a histidine residue to introduce a carbonyl moiety at the active site of the enzyme (9, 10). The oxidatively modified form of GS then becomes increasingly vulnerable to intracellular and exogenous proteases (16–18).

A number of other procaryotic and eucaryotic enzymes are also susceptible to oxidative modification. Pyruvate kinase, creatine kinase, lactate dehydrogenase (14), 3-phosphoglycerate kinase (3), superoxide dismutase (5), and tyrosinase (7) are all susceptible to oxidative inactivation. Thus, loss of catalytic activity due to oxidative modification appears to be a common method of enzymatic regulation. In this study, evidence is presented that RuBPC/O is also susceptible to oxidative modification in vitro.

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MATERIALS AND METHODS

Materials. Dithiothreitol (DTT) and bovine liver catalase were purchased from Sigma Chemical Co. (St. Louis, Mo.). RuBP was synthesized enzymatically from ribulose 5-phosphate by the method of Horecker et al. (6). All other chemicals were of reagent grade.

Purification of RuBPC/O. *Rhodospirillum rubrum* was grown autotrophically under 1.5 to 2% CO_2 as described previously (19). Cells were disrupted by two passages through a French pressure cell at 12,000 lb/in^2. Unbroken cells and membranes were removed by ultracentrifugation at 100,000 × *g* for 60 min in a Beckman 70 Ti rotor. The enzyme was purified through the DEAE-cellulose step as described previously (20). This preparation was greater than 90% pure as judged by sodium dodecyl sulfate (SDS) gel electrophoresis. A portion of this highly pure preparation (75 U) was further purified by separation on a green A agarose column (Amicon Corp., Lexington, Mass). The enzyme was dialyzed against TEMMB buffer (20 mM Tris-sulfate [pH 7.2], 1 mM EDTA, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 50 mM $NaHCO_3$) to remove the NaCl and then loaded onto the column. The enzyme did not stick to the column under these conditions and was eluted in the void volume. The RuBPC/O was pure, as judged by SDS gel electrophoresis. Enzyme was stored at −70°C in TEMMB buffer with 16% glycerol.

Purified RuBPC/O from spinach was a gift of Melinda Martin (The University of Texas at Austin). Purified *Rhodobacter sphaeroides* form I and form II RuBPC/O was a gift from Florence Waddill of this laboratory.

Preparation of crude cell extracts. Cultures were harvested and washed once in TEMMB buffer. Cells were broken by two passages through a French pressure cell or by sonication as described previously (2). Unbroken cells and membranes were removed by ultracentrifugation for 1 h with a Beckman

LS-75B centrifuge at a speed of 100,000 × *g* in the 70 Ti rotor. No attempts were made to exclude oxygen from the extracts until in vitro inactivation experiments were begun.

In vitro enzyme inactivation. Incubations were performed in 23-ml vials or 25-ml Erlenmeyer flasks fitted with serum stoppers. Where indicated, the vials were preflushed with argon for 10 to 20 min to provide an anaerobic environment; otherwise, vials were fitted with stoppers to prevent evaporation, but the environment was left aerobic. Purified enzyme (approximately 0.2 mg/ml) or crude *R. rubrum* extract (approximately 2 to 3 mg of total protein per ml) was added with a syringe. Both the purified enzyme and the crude extract were prepared in TEMMB buffer and were subsequently dialyzed against 20 mM Tris sulfate, pH 7.5. Incubations were performed in this buffer unless specified differently, and cultures were incubated for the indicated length of time at 30°C on a gyrotory shaker (150 rpm). These conditions provided good aeration.

For inactivation by ascorbate, the reaction was initiated by the addition of ascorbate and $FeSO_4$ or $FeCl_3$ to a final concentration of 15 to 30 mM and 0.1 mM, respectively. Ascorbate was prepared fresh daily by neutralizing L-ascorbic acid (Baker) with 2.5 M NaOH. The ascorbate solution was stored at 4°C. For inactivation by DTT, the reaction was initiated by the addition of DTT and $FeSO_4$ to final concentrations of 5 to 10 mM and 0.1 mM, respectively. At the indicated time, samples were removed and placed on ice or immediately frozen to stop the inactivation process.

RuBP carboxylase assays. RuBP carboxylase activity was determined by previously described methods (20) except that DTT was omitted from the assay buffer. To determine the activity of a crude extract or purified enzyme which had been dialyzed against Tris-sulfate buffer (pH 7.5), it was necessary to first activate the enzyme with HCO_3^- and 10 mM $MgCl_2$. The assay was initiated by adding the activated enzyme to the reaction mix containing 20 mM $NaH^{14}CO_3$, 10 mM Mg^{2+}, and 1.6 mM RuBP. One unit of RuBPC/O activity is defined as the amount of enzyme required to catalyze the RuBP-dependent fixation of 1 μmol of CO_2 into product in 1 min at 30°C.

RESULTS

In vitro inactivation of RuBPC/O in crude extracts. When crude cell extracts of *R. rubrum* were exposed to air, RuBP carboxylase activity steadily diminished in a time-dependent manner (Fig. 1). In this particular experiment, 76% of the activity was lost after 6 h of exposure to oxygen. The amount of enzyme activity that was lost was found to vary somewhat with different extracts. Typically, however, between 40 and 70% of the RuBP carboxylase activity was lost after 4 h of incubation at 30°C. The decrease in activity was oxygen dependent; in an inert atmosphere (argon), no loss of activity occurred (Table 1). Indeed, a slight stimulation of activity (3 to 10% of the original) was frequently seen after 4 h of incubation under argon. When the crude extract containing aerobically inactivated RuBPC/O was subsequently returned to anaerobic conditions, enzyme activity was not restored (data not shown). Apparently, the oxygen-mediated inactivation seen in vitro is not reversible.

In contrast to the loss of activity seen in crude extracts, purified *R. rubrum* RuBPC/O was relatively stable to oxygen (Table 1). In some cases, activity increased slightly following 4 h of incubation under air, although slight losses in activity were often observed after prolonged incubation at 30°C. Other sources of RuBPC/O were also only slightly affected by exposure to oxygen. For example, the activity of spinach RuBPC/O decreased to 86% of its original activity within the 4-h time period. As was the case for the purified *R. rubrum* enzyme, the decrease in activity of the spinach enzyme was slight compared with the loss of activity seen in crude extracts of *R. rubrum*.

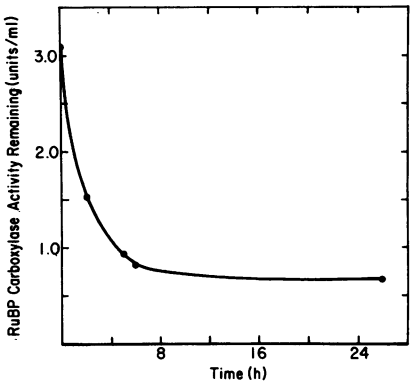

FIG. 1. Inactivation of RuBP carboxylase in crude cell extracts of *R. rubrum*. Crude extract was prepared from a culture of *R. rubrum* grown in butyrate-bicarbonate medium to the stationary phase. A 0.4-ml sample of crude extract (prepared in TEMMB buffer) was incubated under aerobic conditions for 26 h at 30°C. Samples were removed, and RuBP carboxylase activities were determined by the method described in the text at the indicated time points.

Purified *R. rubrum* RuBPC/O was inactivated when incubated with crude extracts of *R. rubrum* (Table 1), again indicating that RuBPC/O is not inherently oxygen labile. Clearly, the crude cell extracts must contain an endogenous factor(s) that is crucial to the inactivation process; subsequent purification of RuBPC/O separated the enzyme from this factor(s). The in vitro assay can be used as a crude but simple means to identify the factor(s) necessary for the oxygen-mediated inactivation. In vitro inactivation appeared to be dependent on a heat-labile component, since addition

TABLE 1. In vitro inactivation of purified RuBPC/O by a component(s) of crude extracts of *R. rubrum*[a]

Sample	RuBP carboxylase activity (U/ml)		% of original activity remaining after 16 h of exposure to air
	Original	Final	
Purified *R. rubrum* RuBPC/O	2.0	2.0	100
Purified *R. rubrum* RuBPC/O[b]	1.58	1.28	81
Crude extract	1.42	0.34	24
Crude extract[b]	1.45	0.36	25
Purified *R. rubrum* RuBPC/O + crude extract	1.56	0.36	23
Purified *R. rubrum* RuBPC/O + crude extract[b]	1.78	0.37	21
Purified *R. rubrum* RuBPC/O + boiled crude extract	2.0	2.5	125
Dialyzed crude extract[b,c]	2.0	1.6	80
Purified spinach RuBPC/O[b]	0.58	0.50	86

[a] In vitro inactivation assays were performed as described in Materials and Methods. Incubations were performed in TEMMB buffer; extracts were incubated at 30°C and shaken at 150 rpm on a gyrotory shaker.

[b] Sample was incubated under argon (original activity) or air (final activity) for 4 h.

[c] Extract was dialyzed against TEMMB buffer for 33 h prior to aerobic incubation. The extract was incubated for 4 h at 150 rpm on a gyratory shaker.

TABLE 2. Addition of reduced pyridine nucleotides and flavin nucleotides to crude extracts and dialyzed crude extracts

Sample tested	RuBPC/O activity			
	Undialyzed		Dialyzed[a]	
	U/ml	% of original activity[b]	U/ml	% of original activity
Original	0.226	100	0.183	100
After incubation				
No addition	0.118	52	0.166	91
+6.3 mM NADH	0.199	88	0.150	82
+6.3 mM NADPH	0.172	76	0.150	82
+6.3 mM flavin adenine dinucleotide	0.15	66	0.055	10
+6.3 mM flavin mononucleotide	0.064	28	0.038	21

[a] Crude extract of *R. rubrum* was dialyzed for 16 h against 50 mM Tris sulfate, pH 7.5.

[b] Percentage of original activity remaining following a 5.5-h incubation under an aerobic atmosphere at 30°C.

of boiled extract to pure RuBPC/O did not result in inactivation (Table 1).

The in vitro inactivation may also depend on a dialyzable component. Crude extracts were dialyzed at 4°C, without attempting to remove oxygen, for 33 h against TEMMB buffer and then aerated for 15 h. A dialyzed crude extract retained 80% of the original RuBP carboxylase activity, compared with only 25% activity remaining in the undialyzed crude extract (Table 1). These results indicate that a low-molecular-weight compound is necessary for inactivation.

The ability of reduced pyridine nucleotides and flavin nucleotides to stimulate the inactivation or replace the essential dialyzable component was investigated (Table 2). Crude extracts were incubated for 5.5 h in the presence of one of the nucleotides (final concentration, 6.3 mM). In this experiment, 48% of the activity was lost after a crude extract was incubated for 5.5 h under aerobic conditions without any exogenous nucleotides. Only 9% of the activity was lost when a dialyzed crude extract was exposed to air for the same length of time, confirming that the in vitro activation was dependent on a dialyzable factor(s) (Table 1). Addition of NADH or NADPH did not stimulate inactivation; indeed, with undialyzed crude extracts, reduced pyridine nucleotides appeared to protect somewhat against the inactivation process. These results are significantly different from previous results with *Klebsiella pneumoniae*, in which an NAD(P)H-dependent monooxygenase was shown to catalyze the oxidative modification of GS. In the absence of exogenous NAD(P)H, no inactivation of GS is seen (11).

Flavin nucleotides did stimulate the inactivation process considerably. In dialyzed crude extracts, flavin adenine dinucleotide and flavin mononucleotide enhanced the inactivation of RuBPC/O, possibly replacing the dialyzable component. Interestingly, addition of 6.3 mM flavin adenine dinucleotide to undialyzed crude extracts did not have a significant effect. Perhaps an endogenous factor found in the soluble fraction of *R. rubrum* extracts serves to protect against the deleterious effects of the flavin.

The effects of the reductants DTT and ascorbate on the inactivation process were investigated. The addition of 15 mM ascorbate to the crude extract resulted in less inactivation under aerobic conditions. Under anaerobic conditions, the activity was actually higher after 4 h of incubation with

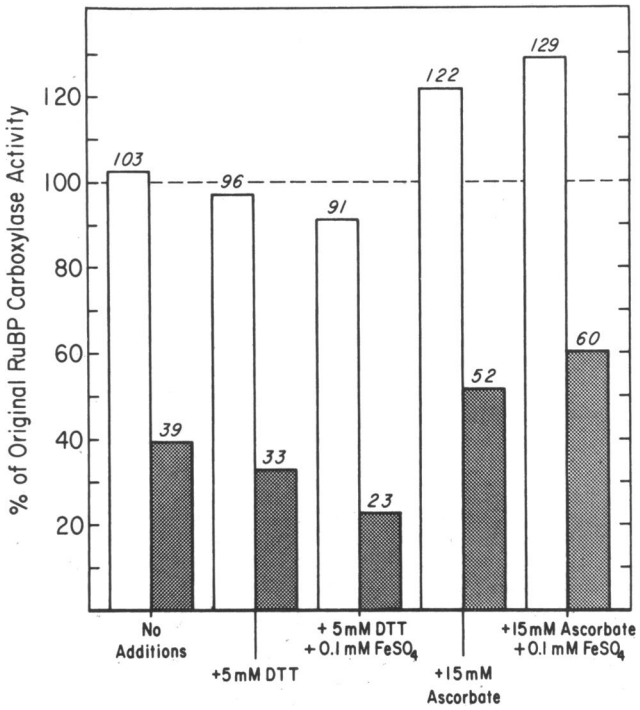

FIG. 2. Effects of DTT and ascorbate on the inactivation of RuBPC/O in extracts of *R. rubrum*. Shaded bars represent RuBP carboxylase activity in extracts incubated for 4 h under aerobic conditions. Open bars are results for extracts incubated under argon for 4 h. The dashed line is the initial activity of the extract, which was 1.9 U/ml, with a specific activity of 0.24 U/mg of protein. Thus, values below the line indicate inactivation, and values above the line indicate protection from inactivation. The numbers above the bars represent the percentage of RuBP carboxylase activity remaining.

ascorbate (Fig. 2). The addition of DTT stimulated inactivation; inclusion of 0.1 mM $FeSO_4$ further stimulated the inactivation.

DTT, together with iron, is thought to generate activated oxygen species which react readily with proteins (5, 9, 10, 14). RuBPC/O was very labile in the presence of 1 mM H_2O_2; only 7% of the original activity in crude extracts remained after 4 h of exposure to H_2O_2 (Fig. 3). As expected, catalase protected against the H_2O_2-mediated inactivation. Likewise, catalase protected against the inactivation mediated by endogenous factors in the presence of O_2, indicating the involvement of hydrogen peroxide. However, catalase did not protect against the DTT-$FeSO_4$-mediated inactivation. This is not conclusive evidence that DTT plus $FeSO_4$ does not inactivate the enzyme via the formation of H_2O_2, since it is conceivable that a molecule of H_2O_2 could be generated at a site close to the enzyme which would be somewhat sequestered from catalase. Indeed, H_2O_2 has both reversible and irreversible effects on the activity of spinach RuBPC/O (1).

Inactivation of purified RuBPC/O in a model system. In order to gain insight into the molecular nature of the oxidative inactivation of RuBPC/O, it was necessary to develop a model system with purified RuBPC/O. Purified RuBPC/O was stable in the presence of oxygen and did not lose activity when exposed to air for 30 min at 30°C. Addition of 25 mM ascorbate and 0.1 mM $FeSO_4$ resulted in a decrease in activity to 60% of the original level (Fig. 4). The presence of either 25 mM ascorbate or 0.1 mM $FeSO_4$ alone resulted in small losses of enzymatic activity (4 and 10%, respectively);

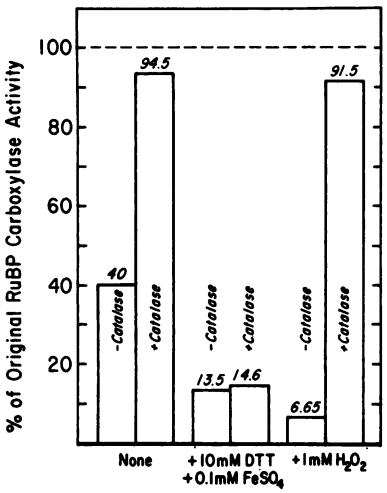

FIG. 3. Effects of catalase on the inactivation of RuBP carboxylase activity in crude extracts. Extracts were prepared from cells grown in butyrate-bicarbonate medium as described in Materials and Methods. Values are the average of two separate assays and indicate the percentage of the original RuBP carboxylase activity remaining after incubation under aerobic conditions for 4 h. The original activity (before oxygen treatment) was 2.4 U/ml and is represented by a dashed line at 100%. The numbers above the bars represent the percentage of RuBP carboxylase activity remaining.

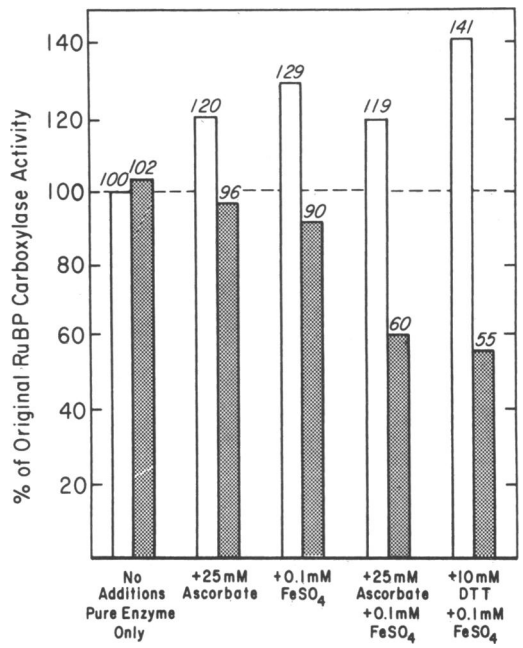

FIG. 4. Nonenzymatic model systems for the aerobic inactivation of purified RuBPC/O. RuBPC/O was purified to electrophoretic homogeneity from *R. rubrum* as described in Materials and Methods. In vitro inactivation assays were performed in TEMMB buffer. The reaction was terminated after 30 min of incubation. Shaded bars are the results for enzyme solutions incubated under aerobic conditions. Open bars are the results for enzyme solutions incubated under anaerobic (argon) conditions. Each bar represents the percentage of original activity remaining after treatment, with the original activity (before treatment) represented by a dashed line at 100%. The original activity was 0.23 U/ml, with a specific activity of 1.5 U/mg of protein.

DTT (10 mM) could substitute for ascorbate in this model system. The loss of RuBP carboxylase activity was oxygen dependent, since no inactivation was seen under an inert atmosphere. Indeed, a significant stimulation of catalytic activity was frequently seen following incubation with DTT or ascorbate under the argon atmosphere for 30 min at 30°C (Fig. 4). When this stimulation was accounted for, ascorbate and DTT, in the presence of 0.1 mM $FeSO_4$, caused 50 and 61% loss of activity, respectively, under aerobic conditions. As in the crude extract system, 1 mM H_2O_2 completely inactivated RuBPC/O (results not shown). Catalase protected against the H_2O_2-mediated inactivation but not the DTT-mediated inactivation, similar to results obtained with crude extracts (Fig. 3).

The loss of RuBP carboxylase activity in the model system occurred by a time-dependent process, and from the kinetics of inactivation (not shown), 30 min was taken as a convenient inactivation time. Interestingly, the presence of RuBP and other phosphorylated metabolites (data not shown) slightly stimulated the rate of inactivation, but preincubation with Mg^{2+} and HCO_3^- afforded protection from inactivation (Table 3). The optimally protective concentrations of Mg^{2+} and HCO_3^- were found to be 10 and 40 mM, respectively. Under these conditions, the *R. rubrum* and *R. sphaeroides* form I and form II enzymes were protected from oxidative modification in the ascorbate-DTT-iron system (from 85 to 103% of original activity remaining). The spinach enzyme was also substantially protected from inactivation by incubation with Mg^{2+} and HCO_3^-. Inactivation was found to be irreversible, since removal of ascorbate and $FeSO_4$ by dialysis did not result in recovery of RuBP carboxylase activity (Table 4).

DISCUSSION

RuBPC/O was inactivated in crude extracts by endogenous factors. The purified enzyme was relatively oxygen stable, and dialysis of crude extracts appeared to remove a factor(s) necessary for the inactivation process. The inactivation process was absolutely dependent on oxygen and was prevented by catalase, implicating an oxidative modification.

In order to gain a better understanding of the mechanism of oxidative inactivation of RuBPC/O, a model system was established which mimics the inactivation by crude extracts. RuBPC/O was inactivated in the presence of oxygen, DTT, or ascorbate and small amounts of iron. Unlike the inactivation by crude extracts, the DTT-$FeSO_4$-mediated inactivation of pure enzyme was not inhibited by catalase. It is conceivable that a reactive oxygen species may be generated at a site near the enzyme which is relatively sequestered from the protective effects of catalase.

Oxidative modification of proteins is a well-documented means of enzyme regulation. Many enzymes are susceptible to oxidation by any one of several mixed-function oxidation systems. Generally, such oxidation increases the protein's vulnerability to attack by intracellular proteases (9, 11, 14, 17). The oxidation of GS in enteric bacteria has been studied in the greatest detail. GS can be inactivated by a variety of mixed-function oxidation systems (10), and it is believed that an activated oxygen species (probably H_2O_2) is created enzymically by cytochrome P_{450} or NADPH oxidase or nonenzymatically with ascorbate or DDT and iron. This activated oxygen species then reacts with an essential histidine residue, introducing a carbonyl group into that histidine residue. This modification causes the loss of catalytic activity and the increased susceptibility to proteases (8, 10).

The inactivation of RuBPC/O certainly bears resemblance

TABLE 3. Protection and stimulation of oxidative inactivation of purified RuBPC/O from various sources

RuBPC/O[a]	Preincubations[b]		CO_2 fixed (nmol/min)	% of original activity
	60 min	30 min		
R. rubrum	None	None	4.04	100
	Mg^{2+}	Ascorbate, $FeCl_3$	2.00	49.5
	HCO_3^-	Ascorbate, $FeCl_3$	1.50	37.1
	RuBP	Ascorbate, $FeCl_3$	1.17	30.0
	RuBP + Mg^{2+}	Ascorbate, $FeCl_3$	1.13	28.0
	Mg^{2+} HCO_3^-	Ascorbate, $FeCl_3$	3.42	84.7
	None	Ascorbate, $FeCl_3$	1.67	41.3
R. sphaeroides				
Form I	None	None	14.49	100
	None	Ascorbate, $FeSO_4$	9.37	64.7
	RuBP	Ascorbate, $FeSO_4$	6.70	46.2
	Mg^{2+} + HCO_3^-	Ascorbate, $FeSO_4$	14.91	102.9
Form II	None	None	12.31	100
	None	Ascorbate, $FeSO_4$	6.83	55.5
	RuBP	Ascorbate, $FeSO_4$	4.84	39.3
	Mg^{2+} + HCO_3^-	Ascorbate, $FeSO_4$	10.69	86.8
Spinach	None	None	2.19	100
	None	Ascorbate, $FeSO_4$	0.77	35.2
	RuBP	Ascorbate, $FeSO_4$	0.71	32.4
	Mg^{2+} + HCO_3^-	Ascorbate, $FeSO_4$	1.57	71.7

[a] RuBPC/O from each source was purified to homogeneity. Before use, the enzyme was dialyzed for 17 h against 20 mM Tris sulfate, pH 7.5.
[b] Enzyme was incubated for 60 min with the indicated ligand and then for 30 min with ascorbate and either $FeCl_3$ or $FeSO_4$. The concentrations used were 10 mM Mg^{2+}, 20 mM HCO_3^-, 1 mM RuBP, 26 mM ascorbate, and 0.1 mM $FeSO_4$ or $FeCl_3$.

to the oxidative inactivation of GS and other enzymes. In both systems, the enzyme is inactivated in vitro by some modification or alteration of the enzyme, followed by degradation. Cell extracts rapidly inactivate the enzyme in vitro. In dialyzed extracts of *Klebsiella aerogenes*, inactivation of GS is absolutely dependent on the addition of exogenous NAD(P)H (11). However, in dialyzed crude extracts of *R. rubrum*, addition of reduced pyridine nucleotides did not restore the ability to inactivate RuBP carboxylase. Addition of flavins to dialyzed extracts of *R. rubrum* resulted in significant losses of RuBP carboxylase activity.

The oxygen-ascorbate-iron or oxygen-DTT-iron model system for the inactivation of RuBPC/O is similar to the well-characterized GS model system (9). In the GS system, exposure to 25 mM ascorbate for 10 min resulted in a 40 to 45% loss of activity. Thus, the kinetics of inactivation of GS and RuBPC/O are comparable. However, the inactivation of RuBPC/O was dissimilar to the inactivation of GS in several respects. GS is remarkably resistant to H_2O_2 and shows no loss of activity when exposed to concentrations as high as 50 mM for 15 min (9). In contrast, RuBPC/O is sensitive to H_2O_2 (1). The ascorbate-mediated inactivation of GS was prevented by catalase, while catalase had no effect on the ascorbate-mediated inactivation of RuBPC/O. Finally, the ascorbate-mediated inactivation of GS was completely inhibited by the chelating agent EDTA, whereas EDTA stimulated the inactivation of RuBPC/O (results not shown). The different responses of GS and RuBPC/O to additions to the system suggest differences in the mechanism of inactivation. Indeed, the oxidative inactivation of RuBPC/O appears to bear more resemblance to the inactivation of creatine kinase, in which ascorbate-induced inactivation is absolutely dependent on O_2 but is not prevented by catalase (9). The addition of 1 mM EDTA stimulates the oxidative inactivation of creatine kinase by 150%, comparable to the stimulation of inactivation of RuBPC/O.

TABLE 4. Irreversible inactivation of *R. rubrum* RuBPC/O by ascorbate and iron

Treatment of enzyme[a]	CO_2 fixed (nmol/min)	% of original activity
None	6.13	100
Dialysis for 5 h	6.27	102.3
Ascorbate + $FeCl_3$[a]	3.14	51.2
Ascorbate + $FeCl_3$, dialysis for 5 h	2.95	48.1
Ascorbate + $FeCl_3$, dialysis for 5 h, second ascorbate + $FeCl_3$ addition	1.16	18.9

[a] *R. rubrum* RuBPC/O was incubated with 25 mM ascorbate and 0.1 mM $FeCl_3$ for 30 min where indicated. Following incubation, 20 μl of enzyme was assayed.

In the case of RuBPC/O, the oxidative modification probably occurs at the activation and not the catalytic site, as evidenced by the protection against inactivation by Mg^{2+} and HCO_3^-, ligands required for carbamylation of a lysine residue at the activation site (12). All of the enzymes which have been studied that are susceptible to this particular type of inactivation have a histidine residue at their active site, and all require divalent metal cations for catalysis (9, 10). RuBPC/O requires Mg^{2+} for both activation and catalysis (12), and His-44 has been shown to be selectively modified by an affinity label in the *R. rubrum* enzyme (4). Although site-specific mutagenesis studies rule out the importance of at least His-291 in catalysis (13) and His-44 does not appear to be essential because it is not conserved in the spinach RuBPC/O (4), additional amino acids may be candidates for oxidative modification.

The physiological role of this inactivation is not known. However, certain facts lend credence to the theory that RuBPC/O is oxidatively modified in vivo. For example, exposure of anaerobically grown cells to oxygen leads to

inactivation, and this appears to be due to some form of irreversible modification or alteration of the enzyme in vivo (2). In crude extracts, the modification does not result in any obvious change in the protein that is detectable by two-dimensional gel electrophoresis (2). Additional studies are in progress with purified enzyme. Furthermore, in the present study, it is apparent that crude extracts of *R. rubrum* contain an endogenous factor(s) that is involved with the inactivation of RuBPC/O. All these facts are consistent with the theory that RuBP carboxylase activity is modulated under aerobic conditions in vivo by oxidative modification, rendering the enzyme inactive. Current studies are directed at elucidating the site of inactivation and the relationship between in vivo and in vitro oxidative modification.

ACKNOWLEDGMENT

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