Purification and characterization of an oxygen-stable form of dinitrogenase reductase-activating glycohydrolase from *Rhodospirillum rubrum*

Gary M. NIELSEN,*† Ying BAO,†‡ Gary P. ROBERTS†‡ and Paul W. LUDDEN*†§

Departments of *Biochemistry and ‡Bacteriology and †Center for the Study of Nitrogen Fixation, University of Wisconsin-Madison, WI 53706, U.S.A.

Dinitrogenase reductase-activating glycohydrolase (DRAG) is responsible for removing the ADP-ribose moiety from post-translationally inactivated nitrogenase of *Rhodospirillum rubrum*. Using DRAG purified from an overexpressing strain (UR276), further properties of this enzyme were studied, including its u.v.-visible and fluorescence spectra and its stability in air. DRAG appears to require no covalently bound inorganic cofactors for its activity or regulation. Previously, purified DRAG

was found to be rapidly inactivated in air. The air-catalysed lability originated with the presence of sodium dithionite and Mn^{2+} throughout the purification of the enzyme. This lability can be mimicked using H_2O_2 , which is known to oxidatively inactivate proteins containing bivalent metals. Implications for the regulation of nitrogenase are discussed with respect to the lack of sensitivity to air of the regulatory enzyme, DRAG.

INTRODUCTION

Bacterial nitrogen fixation is carried out by a complex of the two enzymes dinitrogenase and dinitrogenase reductase [reviewed in Burris (1991)]. In *Rhodospirillum rubrum* and other nitrogen-fixing organisms, dinitrogenase reductase is subject to a post-translational regulatory cycle in response to the availability of energy (illumination or microanaerobicity) and fixed nitrogen (Kanemoto and Ludden, 1984; Hartmann et al., 1986; Jouanneau et al., 1989). In the presence of NH_4^+ or the absence of sufficient light, dinitrogenase reductase ADP-ribosyl transferase (DRAT) inactivates the nitrogenase complex by transferring the ADP-ribose portion of NAD^+ to a specific arginine residue on dinitrogenase reductase (Pope et al., 1985; Lowery et al., 1986). Dinitrogenase reductase-activating glycohydrolase (DRAG), the complementary enzyme of this regulatory cycle, catalyses the removal of ADP-ribose from inactivated dinitrogenase reductase when NH_4^+ is depleted or the bacterium is exposed to light (Kanemoto and Ludden, 1984; Pope et al., 1986).

Nitrogenase from *R. rubrum* and other organisms is oxygen-sensitive and therefore is typically purified in degassed buffer in the presence of sodium dithionite, a scavenger for oxygen. DRAG was initially purified as a side fraction from the nitrogenase preparation and found to be oxygen-sensitive in those preparations (Ludden and Burris, 1976; Nordlund et al., 1977). DRAG activity exhibited a half-life in air of 2 min (Zumft and Nordlund, 1981). This led to the hypothesis that DRAG activity might be regulated by the oxygen or reducing status of the cell. In this report it is demonstrated that the oxygen-dependent loss of activity is an artifact of the purification of the enzyme in the presence of dithionite and Mn^{2+} . When purified in the absence of these compounds, DRAG does not lose activity in an aerobic environment over the course of a day.

It is proposed that H_2O_2 is involved in the oxygen-dependent inactivation of DRAG. H_2O_2 is formed when dithionite is exposed to air (Lambeth and Palmer, 1973), and breaks down into hydroxy anion and hydroxyl radical in the presence of a bivalent metal catalyst, as in the Fenton reaction (Cohen, 1985):

The very reactive hydroxyl radical will react in the vicinity of its formation, and substantial protein damage may result if the iron is protein bound (Kim et al., 1985; Stadtman and Oliver, 1991).

Purification of DRAG to homogeneity using deoxygenated buffers in the absence of the oxygen scavenger dithionite was successful, and DRAG activity was demonstrated in aerobic extracts. These results explain why *R. rubrum* cells grown aerobically exhibit active DRAG. The cause of aerobic inactivation of DRAG was traced to the presence of dithionite and Mn^{2+} in the purification buffers. DRAG was further physically characterized after purification from an overexpressing strain of *R. rubrum*.

EXPERIMENTAL

Materials

Dithionite was obtained from Fluka (Ronkonkoma, NY, U.S.A.). Mops was from U.S.B. (Cleveland, OH, U.S.A.). DEAE-cellulose was from Whatman (Hillsboro, OR, U.S.A.). Hydroxyapatite was from Calbiochem (La Jolla, CA, U.S.A.). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Bacterial strains and growth

The wild-type strain, UR1, is the laboratory strain of ATCC11170. UR2 is a Str^r isolate of UR1. *R. rubrum* strain UR276 contains the gene for DRAG (*draG*) on a multicopy plasmid under the control of the *nifH* promoter of *R. rubrum*, along with *draG* on the chromosome under the control of the *dra* promoter. Strain UR276 is a derivative of strain UR2 (Str^r), which carries the *draTG* genes under the transcriptional control of the *R. rubrum nifH* promoter on plasmid pUX-BF111. The replicon of this plasmid is pRK404 (Ditta et al., 1985), which replicates in *R. rubrum* at low copy number. It was constructed as follows: the 5.2 kb *Bgl*II fragment, carrying the *R. rubrum nifD'H draTG* region (Fitzmaurice et al., 1989), was ligated into the *Bam*HI site of pUC19 (Yanisch-Perron et al., 1985), creating

Abbreviations used: DRAG, dinitrogenase reductase-activating glycohydrolase; DRAT, dinitrogenase reductase ADP-ribosyl transferase; DTT, dithiothreitol.

§ To whom correspondence should be addressed.

pWPF109. The 400 bp region between the genes for *nifH* and *draT* carries the divergently transcribed *nif* and *dra* promoters and there are *Bam*HI sites about 40 bp 5' from the start of *draT* and approx. 100 bp into *nifH*. pWPF109 was digested with *Bam*HI and religated, with transformants screened for those plasmids that had inverted this promoter-containing 0.46 kb *Bam*HI fragment, and this plasmid was termed pUX-BF18. This plasmid now has the *nifH* promoter, and a portion of the *nifH* gene, immediately upstream of *draTG*. This *nif-dra* fusion region was removed from this plasmid by *Pst*I digestion and cloned into the *Pst*I site of pRK404. The resulting constructs were analysed for the orientation of the *PinifdraTG* region relative to the *Plac* on the vector, and an isolate, pUX-BF101, was identified where these promoters were oppositely oriented. Finally, a *Pst*I fragment carrying the *kan* gene from pUC4K (Vieira and Messing, 1982) was ligated into the *Pst*I site adjacent to *Plac*, with the *Pkan* oriented identically to *Plac*, creating pUX-BF111. The resulting plasmid can replicate in *R. rubrum*, has Km^r and Tc^r as the selectable markers, and supports the transcription of the *draTG* region only from the *Pinif* promoter.

Under *nif*-derepressing conditions, UR276 dramatically over-expresses DRAG, but fails to accumulate significantly higher amounts of DRAT. This is apparently because the fragment of *nifH* that is 5' to *draT* is in a different reading frame and we surmise that the translating ribosomes occlude the rather poor *draT* start site (Fitzmaurice et al., 1989). Examination of the sequence suggests that the translating ribosomes encounter a stop signal about 200 bp into *draT* and we surmise that they then are able to bind and translate *draG*, leading to the observed accumulation of that protein. Consistent with this model, a subsequent derivative of this plasmid, where the ribosome binding site of *nifH* is fused directly to the start of *draT*, leads to overexpression of both DRAG and DRAT (D. Lies, unpublished work). Wild-type *R. rubrum* (strain UR1) only contains the chromosomal copy of *draG*. *R. rubrum* was grown in 100-litre batches on Ormerod's medium (Bose et al., 1961; Ormerod et al., 1961) as modified (Kanemoto and Ludden, 1984; Fitzmaurice et al., 1989). The cell paste was frozen in liquid nitrogen and stored at -80 °C until use.

Anaerobic purification of the DRAG

The protocol for purification of DRAG in the absence of the oxygen scavenger dithionite was adapted from that described previously (Saari et al., 1984), with the replacement of Tris buffer with Mops buffer (pH 7.8) and the absence of dithionite and Mn²⁺ in the extraction buffer. All buffers contained 1 mM dithiothreitol (DTT) and were sparged with nitrogen immediately before use. Approx. 500 g of frozen cell paste was mechanically sheared with a Bead Beater (Biospec Products, Bartlesville, OK, U.S.A.) in an anaerobic glove box (Vacuum Atmospheres, Hawthorne, CA, U.S.A.) in 100 mM Mops, pH 8.0. The broken cell extract was centrifuged at 54000 *g* for 7 h in a Beckman Type 19 rotor, and the resulting pellet resuspended in 100 mM Mops, pH 8.0. The resuspended pellet was again centrifuged for 7 h, and the resulting pellet resuspended in 50 mM Mops, pH 8.0, containing 500 mM NaCl. DRAG becomes soluble on treatment with the high salt concentration. After repetition of the centrifugation step, the supernatant containing DRAG was exchanged into 50 mM Mops, pH 7.8, containing 0.5 mM MnCl₂ using an Amicon (Danvers, MA, U.S.A.) concentrator equipped with a YM-10 membrane, until the NaCl concentration decreased below 25 mM. The desalted solution was applied to a DEAE-cellulose column equilibrated with 50 mM Mops, pH 7.8, containing

25 mM NaCl and 0.5 mM MnCl₂. The column was washed with the same buffer, and DRAG was eluted with 50 mM Mops, pH 7.8, 50 mM NaCl and 0.5 mM MnCl₂. The fractions containing DRAG activity were loaded on to a hydroxyapatite column equilibrated with 50 mM Mops, pH 7.8, containing 50 mM NaCl. The hydroxyapatite column was washed with equilibrating buffer and then 100 mM potassium phosphate, pH 7.8. DRAG was eluted near the end of a 100–300 mM potassium phosphate gradient. At this stage of the purification, DRAG was recovered routinely at a purity of greater than 98% as measured by laser densitometry. Pure DRAG could be obtained by rechromatography on DEAE-cellulose or gel filtration. The reactive red affinity chromatography step employed by Saari et al. (1984) was not necessary to achieve homogeneity.

DRAG assay coupled with nitrogenase

DRAG activity was measured by coupling the activity of dinitrogenase reductase with the reduction of acetylene by dinitrogenase (Burris, 1972). A partially purified mixture of dinitrogenase and ADP-ribosylated dinitrogenase reductase used in the assay of DRAG was purified from wild-type *R. rubrum* as described previously (Saari et al., 1984). The reaction mix for the DRAG was as described previously (Saari et al., 1984) with the following modifications: 1 mg/ml BSA was added to the mixture, Tris buffer was replaced with 40 mM Mops (pH 7.5), and the dithionite concentration was decreased to 5 mM. DRAG from strain UR276 was diluted with anaerobic 50 mM Mops (pH 7.5) containing 1 mM DTT before assay to ensure that increasing the amount of DRAG caused a linear increase in the amount of ethylene formed. DRAG activity is defined as the μ mol of ethylene formed/min per mg of DRAG in a 15 min acetylene-reduction assay with excess *R. rubrum* nitrogenase proteins.

Stability of DRAG

Purified DRAG (200 μ l; 0.085 mg/ml) was incubated with 200 μ l of anaerobic buffer (50 mM Mops, pH 7.5) containing various compounds. This mixture was slowly stirred in a 2 ml conical vial either open to the air or sealed in an atmosphere of 100% nitrogen. Samples (3 μ l) containing DRAG were removed at intervals over 30 min to test for activity. DRAG activity was monitored by coupling with nitrogenase as described above. The samples, at a dilution in excess of 100-fold, had no direct effect on the assay for DRAG activity.

In experiments using iodoacetamide, the volume of purified DRAG was reduced to 100 μ l (nominally containing 1 mM DTT) and the volume of buffer to 300 μ l, thereby reducing the consumption of iodoacetamide by the DTT.

Other methods

Metal analysis was conducted by the Soil & Plant Analysis Laboratory, University of Wisconsin-Madison/Extension, using an inductively coupled plasma spectrometer. DRAG was separated by SDS/PAGE on a gel containing 11% total acrylamide and 5% bisacrylamide cross-linker. Proteins were immunoblotted to nitrocellulose paper as previously described (Liang et al., 1991), and incubated with antiserum to DRAG purified from wild-type *R. rubrum*. Immunoreactive material was visualized using alkaline phosphatase anti-IgG. Fluorescence emission studies were conducted using a Jobin Yvon JY3 fluorimeter. A Shimadzu UV-160 spectrophotometer was used to record u.v.-visible spectra.

Table 1 Purification of DRAG from wild-type and strain UR276 of *R. rubrum*

Values are either total units of activity (nmol of ethylene produced/min) recovered from 10 g of cell paste or, for values in parentheses, specific activity (nmol of ethylene produced/min per mg of DRAG protein). The data for UR1 + dithionite are from Saari et al. (1984).

	UR1		UR276	
	Anaerobic	+ Dithionite	Anaerobic	+ Dithionite
Crude extract	2400	2450	138 000	45 600
Salt wash of membranes	1400	1520	96 700	31 600
DE-52 pool	—	859	—	—
Hydroxyapatite	—	293 (8090)	6900 (138 000)	14 800 (56 600)
Final purity	—	31 (40100)	—	—

Figure 1 Equivalent mobility of DRAG from wild-type and overexpressing strains

Samples of DRAG from wild-type and strain UR276 were subjected to SDS/PAGE and immunoblotting. Lanes 1 and 2, immunoblotting with antibody raised against wild-type DRAG; lanes 3 and 4, silver-stained gel. Lane 4, molecular-mass standards; lanes 1 and 3, DRAG from strain UR276; lane 2, DRAG from wild-type *R. rubrum*. DRAG samples from strain UR276 were purified through the hydroxyapatite column step, anaerobically but without dithionite. The arrow to the left of lane 1 shows the position of DRAG.

Figure 2 Fluorescence emission spectrum of DRAG from strain UR276

DRAG (0.085 mg/ml, in 300 mM potassium phosphate buffer, pH 7.8, containing 1 mM DTT) was excited at 277 nm. The fluorescence spectrum was recorded in a 1 cm pathlength quartz cuvette from 290 to 440 nm. Maximum intensity is at 340 nm.

Figure 3 U.v.-visible spectrum of DRAG from strain UR276

The spectrum was corrected for a blank consisting of 300 mM potassium phosphate buffer, pH 7.8, and 1 mM DTT. The spectrum of DRAG (0.085 mg/ml, in 300 mM potassium phosphate buffer, pH 7.8, containing 1 mM DTT) was recorded in a 1 cm pathlength quartz cuvette.

RESULTS

Purification of DRAG from UR1 and UR276 strains of *R. rubrum* under different conditions of anaerobicity

DRAG activity could be recovered from UR276 from anaerobic preparations with or without the presence of 1 mM dithionite (Table 1). The specific activity of DRAG from the overexpressing strain was threefold greater than that previously reported for DRAG purified from wild-type in the presence of dithionite (Saari et al., 1984). This difference is attributed to the presence of non-active DRAG in the earlier preparation.

Attempts to purify DRAG using aerobic buffers were partially successful. DRAG activity was stable aerobically in extracts from both wild-type and strain UR276 of *R. rubrum* (results not shown). However, aerobic DEAE-cellulose chromatography resulted in rapid loss of activity. To maintain enzyme stability during DEAE-cellulose chromatography, the matrix was made anaerobic by equilibration with nitrogen-sparged buffers containing Mn^{2+} (but lacking dithionite).

Physical characteristics of the overexpressed enzyme and equivalence with wild-type enzyme

Samples of DRAG from strain UR276 and wild-type were subjected to SDS/PAGE and immunoblotting. No differences in mobility or antigenicity between normally expressed DRAG from wild-type or overexpressed DRAG from UR276 were observed (Figure 1). The fluorescence emission spectrum showed only the expected pattern for tyrosine (Figure 2). The u.v.-visible spectrum was silent in the visible region and showed the characteristic absorption pattern for proteins in the near-u.v. region (Figure 3). Inductively-coupled-plasma-emission analysis of DRAG rechromatographed on DEAE-cellulose using metal-free buffers revealed no metals associated with the enzyme (results not shown). With this method the limit of detection for Mn^{2+} was 0.003 p.p.m. (0.05 μM), and the DRAG samples were at concentrations of 2.5 μM and 10 μM .

Stability of DRAG to air

DRAG from strain UR276, which had been purified anaerobically in the absence of dithionite, was exposed to air after dilution into buffer containing DTT and various concentrations of dithionite (Figure 4). When dithionite was absent and the solution was exposed to air, DRAG activity did not change

Figure 4 Effect of air on solutions of DRAG containing different concentrations of dithionite

Samples of DRAG (0.085 mg/ml) in 200 mM potassium phosphate, pH 7.8, containing 1 mM DTT were diluted with an equal volume of dithionite-containing solution in 50 mM Mops, pH 7.8, containing 2 mM DTT. The samples were exposed to air and portions of the diluted DRAG solutions were removed at the indicated times for determination of DRAG activity. ●, No dithionite; ○, 1 mM dithionite; ■, 3 mM dithionite; □, 5 mM dithionite; ◆, 5 mM dithionite and 0.1% BSA.

Figure 5 Effect of air on DRAG in solutions containing different bivalent cations and dithionite

A solution of DRAG (0.085 mg/ml) in 200 mM potassium phosphate, pH 7.8, containing 1 mM DTT was diluted with an equal volume of 50 mM MOPS, pH 7.8, containing 2 mM DTT and various cations and/or dithionite. A sample of the diluted DRAG solution was removed at the indicated times for determination of DRAG activity. ●, 0.5 mM $MnCl_2$; ○, 0.5 mM dithionite; ■, 0.5 mM dithionite and 1 mM $CaCl_2$; □, 0.5 mM dithionite and 5 mM $MgCl_2$; ◆, 0.5 mM dithionite and 1 mM $FeCl_2$; ◇, 0.5 mM dithionite and 0.5 mM $MnCl_2$; ▲, 5 mM dithionite and 0.5 mM $MnCl_2$.

during the 30 min time course. If dithionite was present during the exposure to air, DRAG activity decreased with time. This decrease in activity could not be prevented by a large excess of BSA. The rate of activity loss increased with increasing dithionite

Figure 6 Effect of H_2O_2 on DRAG activity in aerobic solutions

A solution of DRAG (0.085 mg/ml) in 200 mM potassium phosphate, pH 7.8, containing 1 mM DTT was diluted with an equal volume of an anaerobic solution containing H_2O_2 in 50 mM Mops (pH 7.8)/2 mM DTT. A portion of the diluted DRAG solution was removed at the indicated times for determination of DRAG activity. ●, 0.5 mM $MnCl_2$; ○, 1 mM H_2O_2 ; ■ and □, 0.5 mM $MnCl_2$ and 0.5 mM H_2O_2 ; ◆, 0.5 mM $MnCl_2$ and 5 mM H_2O_2 ; ◇, 0.5 mM $MnCl_2$ and 9 mM H_2O_2 .

concentration. Exposure of the dithionite solution to air for 1 day removed the ability of the dithionite solution to affect DRAG activity (results not shown).

DRAG stability was determined in the presence of numerous bivalent cations (Figure 5). Mn^{2+} increased the rate at which DRAG activity was lost. In addition to its use for DRAG activity assays *in vitro* (Ludden and Burris, 1976; Saari et al., 1984), Mn^{2+} appears to make DRAG more susceptible to inactivation by dithionite in air. Both Mg^{2+} and Fe^{2+} are able to substitute for Mn^{2+} in the activation of nitrogenase by DRAG (Nordlund et al., 1978; Yoch, 1979; Guth and Burris, 1983; Nordlund and Norén, 1984), but neither of these accelerated the inactivation of DRAG in the presence of dithionite. Ca^{2+} , which inhibits DRAG, also had no effect on the rate of loss of DRAG activity in air in the presence of dithionite.

Sensitivity of DRAG to H_2O_2

Because oxidation of dithionite in air yields H_2O_2 (Lambeth and Palmer, 1973), dithionite was replaced by H_2O_2 in the DRAG-stability assay. After exposure to H_2O_2 , DRAG activity declined (Figure 6). These results mimicked the effect on DRAG of dithionite in air. H_2O_2 at the same concentration as dithionite affected DRAG activity to a similar degree. With the addition of Mn^{2+} , DRAG became much more labile in the presence of H_2O_2 . Breakdown of dithionite also generates bisulphite and thio-sulphate (Lambeth and Palmer, 1973; Mayhew, 1978), neither of which affected DRAG activity (results not shown).

The loss of DRAG activity in the presence of H_2O_2 and Mn^{2+} could be prevented by ADP-ribose, an inhibitor of DRAG activity (Figure 7). This effect was not caused by chelation of Mn^{2+} by ADP-ribose, as twofold more Mn^{2+} than ADP-ribose did not cause inactivation (results not shown). ADP, which does not inhibit the hydrolysis of N-glycosidic bonds by DRAG, did not prevent inactivation.

Figure 7 Effect of ADP-ribose on DRAG activity in the presence of Mn^{2+} and H_2O_2 in aerobic solutions

A solution of DRAG (0.085 mg/ml) in 200 mM potassium phosphate, pH 7.8, containing 1 mM DTT was diluted with an equal volume of a solution containing ADP-ribose and H_2O_2 in 50 mM Mops (pH 7.8)/2 mM DTT. A sample of the diluted DRAG solution was removed at the indicated times for determination of DRAG activity. All solutions contained 0.5 mM H_2O_2 and 0.5 mM $MnCl_2$, with the addition of 1 mM ADP-ribose (●) or 1 mg/ml BSA (○). ■, No addition.

DISCUSSION

Overexpressed DRAG from strain UR276 has the same physical properties as the enzyme purified from wild-type *R. rubrum*. Enzymes from both sources activate dinitrogenase reductase, although the overexpressed enzyme has been purified to a higher specific activity. Their antigenic responses and mobilities on SDS/PAGE are indistinguishable. DRAG from strain UR276 exhibits the same requirement for Mn^{2+} for activation, and the addition of Mn^{2+} with dithionite renders the overexpressed enzyme susceptible to aerobic inactivation as with wild-type. We ascribe the differences in specific activity of the purified enzymes (Table 1) to the variability of DRAG inactivation during purification.

Originally, overexpressed DRAG was purified in the presence of Mn^{2+} and dithionite in an attempt to observe spectroscopically a difference between the native and air-exposed enzyme. Although no differences were observed, controls to verify that aerobic manipulations destroyed DRAG activity were unsuccessful (results not shown). Further experiments showed that a concentrated solution of DRAG in dithionite- and Mn^{2+} -containing buffer lost only part of its DRAG activity on exposure to air, whereas diluted solutions of DRAG in the same buffer were completely inactivated on air treatment (results not shown). These results led to the observation that inactivation of DRAG is dependent on the ratio of the concentrations of DRAG and dithionite.

DRAG, purified in the absence of dithionite, is stable to air, and a solution to DRAG exposed to air for 22 h at 4 °C retains its original activity. Under strict anaerobic conditions, a solution of DRAG in dithionite-containing buffer at 4 °C only exhibits a partial loss of activity. DRAG activity declines if dithionite is present during the exposure to air, and this decrease in activity is dependent on the dithionite concentration. However, the rate of activity loss is slower than the previously observed 2 min half-life in air (Zumft and Nordlund, 1981). To attain a 2 min half-life

for DRAG activity, it must be in a solution containing both dithionite and Mn^{2+} before exposure of the solution to air. Other bivalent metals are unable to replace Mn^{2+} , and the rate of inactivation with these other metals approximates the rate with dithionite alone. It is surprising that Fe^{2+} does not confer susceptibility to air because Fe^{2+} does substitute for Mn^{2+} in the nitrogenase-activation assay (Nordlund and Norén, 1984).

A number of possible dithionite degradation products were added to an assay measuring DRAG stability. Only H_2O_2 was able to inactivate DRAG. The rate of inactivation with peroxide was enhanced greatly in the presence of Mn^{2+} . DRAG might be inactivated by a mechanism similar to the Fenton reaction, with the bound Mn^{2+} replacing Fe^{2+} as the catalyst. In this manner, DRAG would always experience the deleterious effects of the hydroxyl radical, as that radical would be formed at the binding site of Mn^{2+} . Alternatively, the damage might be caused by generation of Mn^{3+} at the active site of the enzyme. Evidence that DRAG binds Mn^{2+} is shown in the requirement for Mn^{2+} in the DRAG activity assay and the inability of ADP-ribosylated dinitrogenase reductase, the substrate for DRAG, to bind Mn^{2+} (Guth and Burris, 1983). Attempts to prevent inactivation by adding trapping agents, such as catalase or 0.1 % BSA, were unsuccessful, as would be expected if inactivation of DRAG was occurring by a 'caged' Fenton reaction. All attempts at restoring activity were also unsuccessful (results not shown). Although failure to restore DRAG activity could occur for many reasons, this fact is also consistent with the localized destruction of the Mn^{2+} -binding site. ADP-ribose, an inhibitor of DRAG activity (Pope et al., 1986; Saari et al., 1986), was able to prevent the inactivation of DRAG mediated by H_2O_2 and Mn^{2+} . Taken together, inhibition of DRAG activity by ADP-ribose and the prevention of inactivation of DRAG by ADP-ribose suggests that the observed inactivation of DRAG occurs at the catalytic site. Identification of the affected residues may allow identification of amino acids at or near the active site of the enzyme.

Care must be taken when using dithionite or other reducing substances, especially with enzymes that are activated by metals, as the reductant may generate reactive oxygen metabolites that will specifically inactivate the protein at metal-binding sites. In these cases it may be advisable to use metal-free buffers and anaerobic conditions. This course of action has been taken for glutamine synthetase purified from *R. rubrum* (Nordlund et al., 1985). This enzyme was twice as active when purified anaerobically than when purified aerobically.

We gratefully acknowledge Dr. V. K. Shah for his helpful discussions. We also thank R. Allen, J. Fox and S. Ross for their critical reading of the manuscript. This work was supported by NSF Grant no. MCB-8821820 (to P.W.L.). Y.B. was supported by USDA Grant no. 93-37305-9237 (to G.P.R.).

REFERENCES

- Bose, S. K., Gest, H. and Ormerod, J. G. (1961) *J. Biol. Chem.*, **PC13**–PC14
- Burris, R. H. (1972) *Methods Enzymol.* **24**, 415–431
- Burris, R. H. (1991) *J. Biol. Chem.* **266**, 9339–9342
- Cohen, G. (1985) in *CRC Handbook of Methods for Oxygen Radical Research* (Greenwald, R. A., ed.), pp. 55–64. CRC Press, Boca Raton, FL
- Ditta, G., Schmidhauser, T. and Jakobson, E. (1985) *Plasmid* **13**, 149–153
- Fitzmaurice, W. P., Saari, L. L., Lowery, R. G., Ludden, P. W. and Roberts, G. P. (1989) *Mol. Gen. Genet.* **218**, 340–347
- Guth, J. H. and Burris, R. H. (1983) *Biochem. J.* **213**, 741–749
- Hartmann, A., Fu, H. and Burris, R. H. (1986) *J. Bacteriol.* **165**, 864–870
- Jouanneau, Y., Roby, C., Meyer, C. M. and Vignais, P. M. (1989) *Biochemistry* **28**, 6524–6530
- Kanemoto, R. H. and Ludden, P. W. (1984) *J. Bacteriol.* **158**, 713–720
- Kim, K., Rhee, S. G. and Stadtman, E. R. (1985) *J. Biol. Chem.* **260**, 15394–15397
- Lambeth, D. O. and Palmer, G. (1973) *J. Biol. Chem.* **248**, 6095–6103

- Liang, J., Nielsen, G. M., Lies, D. P., Burris, R. H., Roberts, G. P. and Ludden, P. W. (1991) *J. Bacteriol.* **173**, 6903–6909
- Lowery, R. G., Saari, L. L. and Ludden, P. W. (1986) *J. Bacteriol.* **166**, 513–518
- Ludden, P. W. and Burris, R. H. (1976) *Science* **194**, 424–426
- Mayhew, S. G. (1978) *Eur. J. Biochem.* **85**, 535–547
- Nordlund, S. and Norén, A. (1984) *Biochim. Biophys. Acta* **791**, 21–27
- Nordlund, S., Eriksson, U. and Baltscheffsky, H. (1977) *Biochim. Biophys. Acta* **462**, 187–195
- Nordlund, S., Eriksson, U. and Baltscheffsky, H. (1978) *Biochim. Biophys. Acta* **504**, 248–254
- Nordlung, S., Kanemoto, R. H., Murrell, S. A. and Ludden, P. W. (1985) *J. Bacteriol.* **161**, 13–17
- Ormerod, J. G., Ormerod, K. S. and Gest, H. (1961) *Arch. Biochem. Biophys.* **94**, 449–463
- Pope, M. R., Murrell, S. A. and Ludden, P. W. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3173–3177
- Pope, M. R., Saari, L. L. and Ludden, P. W. (1986) *J. Biol. Chem.* **261**, 10104–10111
- Saari, L. L., Triplett, E. W. and Ludden, P. W. (1984) *J. Biol. Chem.* **259**, 15502–15508
- Saari, L. L., Pope, M. R., Murrell, S. A. and Ludden, P. W. (1986) *J. Biol. Chem.* **261**, 4973–4977
- Stadtman, E. R. and Oliver, C. N. (1991) *J. Biol. Chem.* **266**, 2005–2008
- Vieira, J. and Messing, J. (1982) *Gene* **19**, 259–268
- Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene* **33**, 103–119
- Yoch, D. C. (1979) *J. Bacteriol.* **140**, 987–995
- Zumft, W. G. and Nordlund, S. (1981) *FEBS Lett.* **127**, 79–82

Received 24 January 1994/29 March 1994; accepted 7 April 1994