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Oxidation of *Neurospora crassa* NADP-Specific Glutamate Dehydrogenase by Activated Oxygen Species

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The glutamine synthetase and the NADP-specific glutamate dehydrogenase activities of *Neurospora crassa* were lost in a culture without carbon source only when in the presence of air. Glutamine synthetase was previously reported to be liable to in vitro and in vivo inactivation by activated oxygen species. Here we report that NADP-specific glutamate dehydrogenase was remarkably stable in the presence of activated oxygen species but was rendered susceptible to oxidative inactivation when chelated iron was bound to the enzyme and either ascorbate or H_2O_2 reacted on the bound iron. This reaction gave rise to further modifications of the enzyme monomers by activated oxygen species, to partial dissociation of the oligomeric structure, and to precipitation and fragmentation of the enzyme. The in vitro oxidation reaction was affected by pH, temperature, and binding to the enzyme of NADPH. Heterogeneity in total charge was observed in the purified and immunoprecipitated enzymes, and the relative amounts of enzyme monomers with different isoelectric points changed with time of the oxidizing reaction.

Modification of proteins by activated oxygen species has been identified in various enzymes (5, 13, 14, 17, 20, 26–28, 30, 41, 43, 46, 48, 51). This modification is generally accompanied by changes in catalytic activity, usually inhibiting it (5, 13, 14, 17, 20, 26–28, 41, 43), but there are also examples of enzyme activation mediated by highly reactive oxygen metabolites (30, 48, 51). Several oxidation systems have been used, including neutrophil oxidative burst (34, 39), enzymatic systems such as cytochrome P-450 (17, 44), and nonenzymatic systems such as the ascorbate model system (27). Modification of proteins by mixed-function oxidation has been found in pathological-related processes associated with oxygen toxocity (45), host defense mechanisms (34), and aging (35). The physiological functions of oxidation by activated oxygen species have not been defined yet, but they may include intracellular proteolysis (8, 11, 37–40) and regulation of anaerobic and aerobic oxidation metabolism (21). We have recently suggested that oxidation of proteins by activated oxygen species is also related to developmental processes in microorganisms (W. Hansberg and J. Aguirre, J. Theor. Biol., in press).

Part of our research has focused on the regulation of nitrogen metabolism during germination and conidiation of *Neurospora crassa* (6, 7, 19). In the mycelium which forms the aerial hyphae (47), glutamine synthetase (GS) and NADP-specific glutamate dehydrogenase [GDH(NADP)] activities are lost (7). The *N. crassa* GS is modified in vitro by activated oxygen species (1) in a similar way to that of *Escherichia coli* GS, in which mixed-function oxidation has been studied in great detail (15, 17, 26–28, 32). *N. crassa* GS is also oxidized in vivo; the activity loss observed in a mycelium starved of carbon source (31) occurred only when the culture was aerated. No activity change is observed when aeration of the culture is suspended (1). Under aerating conditions, the α and β polypeptides of GS (12, 42) are modified, giving rise to more acidic polypeptides (1). The same modification of this enzyme is observed (i) when a cell extract is incubated with reduced NAD or NADP and (ii) when purified GS is incubated with hydrogen peroxide and ferrous iron (Fe^{2+}) or with ascorbate and ferric iron (Fe^{3+}) in the presence of oxygen (1). The in vitro-oxidized GS is completely degraded in a cell extract, whereas the nonoxidized enzyme is preserved. The endogenous proteolytic activity that preferentially degrades the oxidized GS is not susceptible to phenylmethylsulfonyl fluoride and is partially inhibited by Mn^{2+} or EDTA (1).

Since GDH(NADP) followed the same pattern as GS with respect to the activity loss in an aerated liquid culture without carbon source (this report) or when the mycelium was exposed directly to the air (J. Aguirre, R. Rodríguez, I. Toledo, and W. Hansberg, manuscript in preparation), we analyzed a possible oxidative modification of GDH(NADP) by activated oxygen species. Here we present data to indicate that the GDH(NADP) of *N. crassa* was remarkably stable in the presence of activated oxygen species but was rendered susceptible to modification, aggregation, and fragmentation by oxygen radicals when chelated iron was used.

MATERIALS AND METHODS

Materials. Reagents used for the oxidation reactions and bovine GDH(NADP) were from Sigma Chemical Co., St. Louis, Mo. Reagents for electrophoresis were from Bio-Rad Laboratories, Richmond, Calif., and for some gels, ampholytes were from LKB Instruments, Bromma, Sweden. All other chemicals were reagent grade from J. T. Baker Chemical Co., Xalostoc, Mexico.

Strains and culture conditions. Wild-type *N. crassa* 74A and the *am-132* strain, which is a GDH(NADP) deletion mutant (24), came originally from the Fungal Genetics Stock Center, now at the University of Kansas Medical Center, Manhattan, Kans. Both strains were donated by J. Mora of this university.

Conidia were obtained from slant cultures in minimal medium of Vogel (49) supplemented with 1.5% (wt/vol) sucrose, which were grown for 3 days in the dark at 29°C followed by 2 days in the light at 25°C. A stock of conidia was kept frozen at −70°C in distilled water.

Liquid cultures were grown for 12 h at 30°C from a

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conidial inoculum of 10^6 cells per ml of Vogel minimal medium supplemented with 1.5% sucrose. Aeration was provided by a gyratory shaker at 240 rpm. The cultures of the *am-132* strain were supplemented with 5 mM glutamate. In some experiments after 12 h of growth, the cultures were transferred into a fresh minimal medium without sucrose and divided in two parts. One part was agitated in Erlenmeyer flasks at an air-to-liquid volume ratio of 20, and the other part was agitated in closed flasks that were filled to the top to eliminate the air chamber. At different times, the cultures were filtered, dehydrated with an excess of acetone, and stored at −20°C until used.

Determination of GDH(NADP) activity. The acetone-dehydrated mycelia were ground in a mortar with dry ice, and the resulting fine powder was suspended in 10 mM phosphate buffer, pH 8. After centrifugation for 10 min in a microcentrifuge, the supernatant was used to determine the GDH-(NADP) activity by measuring the oxidation of NADPH at 340 nm, as described by Fincham (16). The purified enzyme or the cell extracts were incubated for 8 min in the assay buffer containing 2-oxoglutarate and ammonium before the reaction was started by the addition of NADPH. Specific activity is expressed as moles of oxidized NADPH per min per mg of protein. Protein was determined by the method of Lowry et al. (29).

Oxidizing reaction. Since we have found that GDH-(NADP) was activated about 40% by incubation with dithiothreitol (DTT) (J. Aguirre, R. Rodríguez, and W. Hansberg, manuscript in preparation), the purified enzyme was incubated with 25 mM DTT for 45 min at 30°C and filter dialyzed before it was used for the oxidation experiments. All reagent solutions used for the oxidizing reaction were prepared fresh at a concentration 20 times that of the final concentration. The reagent solutions were added directly to the enzyme or cell extract, always in the same order. Incubation was done in open 0.5-ml Eppendorf tubes at 30°C, unless otherwise stated. When the reaction was done in the presence of N_2 or O_2, a flux of either of these gases was passed during 5 min over 100 μl of each reagent solution in 0.5-ml Eppendorf tubes while the tube was being agitated in a vortex mixer. Tubes were then closed and incubated. After the incubation period, the samples were dialyzed by filtration through Sephadex G-50-80, unless otherwise stated, and analyzed. Filter dialysis was done in preequilibrated minicolumns packed in insulin syringes which were centrifuged half a minute at 2,500 rpm in a benchtop Beckman TJ-6 centrifuge.

Purification of GDH(NADP). The method of purification was based on the procedure of Barratt and Strickland (3), with some modifications (J. Aguirre and W. Hansberg, Fungal Genet. Newsl. **35**:5–6, 1988).

Anti-GDH(NADP) antibodies. A solution of 85 μg of the purified GDH(NADP) in complete Freund adjuvant was injected twice into an adult rabbit, with a 20-day interval. Half of each dose was given subcutaneously, and the other half was given intramuscularly. At 10 days after the dose 2, 30 ml of blood were collected. The coagulated blood was centrifuged, and total pooled immunoglobulins were obtained from the serum by three precipitations with $(NH_4)_2SO_4$, to 50% saturation. The third $(NH_4)_2SO_4$ precipitate was suspended in half the serum volume of 150 mM phosphate buffer (pH 7.2)–0.85 NaCl, dialyzed against the same buffer, and stored at −20°C.

Immunoprecipitation of GDH(NADP). Thirty or forty microliters of anti-GDH(NADP) pooled immunoglobulins and albumin, at a final concentration of 1 mg/ml of the same buffer, was added to the cell extracts in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, pH 8, containing 0.6 mg of protein. The resulting mixtures were incubated for 4 h at 4°C. Then, 100-μl samples of the incubation mixture were layered above discontinuous gradients and centrifuged for 30 min at 10,000 × *g* in a Sorvall HB4 rotor. The gradients were made in 250-μl Eppendorf tubes with 100 μl of 1 M sucrose and 50 μl of 0.5 M sucrose in the same HEPES buffer. After centrifugation, the gradients were frozen with dry ice, the tips were cut and drained, and the pellets were suspended and analyzed (36).

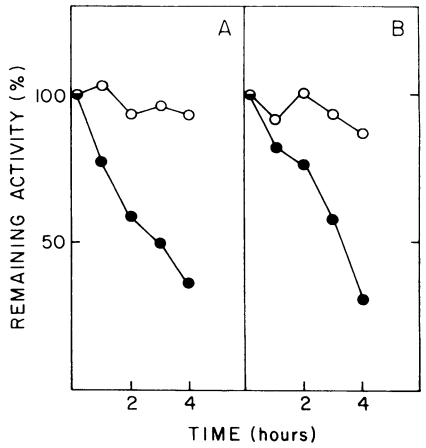

FIG. 1. GS and GDH(NADP) activities in aerated and unaerated cultures of *N. crassa* in a medium without a carbon source. A 12-h liquid culture was transferred to a fresh medium without a carbon source and incubated with (●) or without (○) aeration. GS- (A) and GDH(NADP)- (B) specific activities were determined at different times after transfer, expressed as percentage of the initial activity. The data are mean values of three different experiments.

Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was done according to the method of Laemmli (25); two-dimensional PAGE (2D-PAGE) was done according to the method of O'Farrell (33). The O'Farrell procedure for the first dimension of the 2D-PAGE was followed in the electrofocusing PAGE, but 0.75-mm-thick slab gels were made instead of tube gels. The nondenaturing electrofocusing PAGE was run as described by G. Giulian in the 1986 Hoefer Scientific Instruments catalog. Staining with Coomassie R-250 was done by following the procedure described in the same catalog. The silver staining procedure was done as reported by Wray et al. (54).

RESULTS

GDH(NADP) activity loss is related to aeration conditions. GDH(NADP) activity of *N. crassa* has been found to be dependent on the availability of a carbon source (22). When a liquid culture was starved of the carbon source, GDH (NADP) activity was almost completely lost in the course of a few hours. No growth could be sustained under these conditions, and the total protein content diminished during the incubation period. As in the case of GS activity, GDH (NADP) activity loss was observed when the culture was aerated; by contrast, the loss in enzyme activity did not occur when aeration of the culture was suspended (Fig. 1).

Inactivation of GDH(NADP) in cell extracts. The GS of *N. crassa* is inactivated in cell extracts by adding either ascorbate plus Fe^{3+} or hydrogen peroxide plus Fe^{2+} (1). By using these oxidizing mixtures, the activity of GDH(NADP) in cell extracts remained unaltered (Table 1). However, when the cell extract was incubated with ascorbate and either Fe^{2+} or Fe^{3+} in the presence of EDTA, the activity was lost over the

TABLE 1. Remaining GDH(NADP) activity after incubation of a cell extract with different oxidizing mixtures[a]

Oxidizing mixture[b] (atmosphere and temp)	Remaining activity (%)[c]
None	100
ASC + Fe^{3+}	97
H_2O_2 + Fe^{2+}	94
H_2O_2 + Fe^{2+} + EDTA	90
ASC + Fe^{3+} + EDTA	54
ASC + Fe^{2+} + EDTA	50
ASC + Fe^{3+} + EDTA (4°C)	83
ASC + Fe^{3+} + EDTA (50°C)	28
ASC + Fe^{3+} + EDTA (O_2)	36
ASC + Fe^{3+} + EDTA (N_2)	82
ASC + Fe^{2+} + *o*-phenanthroline	88
ASC + Fe^{2+} + citrate	105
ASC + Fe^{2+} + oxalacetate	107
ASC + Fe^{2+} + succinate	116
ASC + Fe^{2+} + 2-oxoglutarate	110

[a] A cell extract in 100 mM phosphate buffer, pH 6, was heated for 45 min at 50°C and centrifuged. The supernatant was incubated with the different oxidizing mixtures for 3 h at 30°C, unless otherwise stated.

[b] Final concentrations: ascorbate (ASC), 20 mM; Fe^{2+} and Fe^{3+}, 0.6 mM; EDTA and *o*-phenanthroline, 1 mM; carboxylic acids, 10 mM.

[c] Expressed as percentage of the activity of the incubated supernatant without oxidizing mixture.

course of a 6-h incubation period. This loss in enzyme activity was strictly dependent on oxygen. It was also dependent on temperature, with no activity loss at 4°C and a more pronounced loss at 50 than at 30°C. The EDTA could not be substituted for by other chelating agents such as *o*-phenanthroline or di- and tricarboxylic acids. The ascorbate, iron, and EDTA had to be present during the incubation period, since their removal by filtration through Sephadex G-50 stopped further inactivation at 30 and 50°C. Hydrogen peroxide alone or in the presence of Fe^{2+}, or Fe^{2+} plus EDTA did not have a significant effect on GDH(NADP) activity (Table 1). In all these experiments, sodium azide was added to the cell extract at concentrations which completely inactivated the endogenous catalase activity (1).

The GDH(NADP) of *N. crassa* has different conformations depending on the pH of the incubation medium. In the absence of substrates and products, the enzyme is in an active form at pH 7.8 and in an inactive conformation at pH 7.0 (52). The pK for this transition is near pH 7.2 (2). We analyzed the oxidative inactivation of the enzyme at different pHs. At the end of the incubation period inactivation of GDH(NADP) by ascorbate, Fe^{2+}, and EDTA was about 20% greater at pH 6.5 or below than at pH 7.5 or above.

Inactivation of purified GDH(NADP). To study the inactivation of GDH(NADP) by activated oxygen species in more detail, we purified the enzyme to obtain a single band in overloaded gels stained with Coomassie blue (Aguirre and Hansberg, Fungal Genet. Newsl. 1988). A tenuous band beneath the dense band (see Fig. 3, lane 1) is probably a degradation product of the enzyme (Aguirre and Hansberg, Fungal Genet. Newsl. 1988). Purified GDH(NADP) was also inactivated by ascorbate, iron, and EDTA. As shown in Table 2, 62% of the activity was lost by incubating the purified enzyme with the oxidizing mixture of 2 h at 30°C. Ascorbate, iron, or EDTA by themselves or in dual combination did not have a significant inactivation effect. As shown for the enzyme in cell extracts, the inactivation reaction of purified GDH(NADP) was dependent on the presence of all three compounds and oxygen. In contrast to the oxidation reaction in cell extracts, H_2O_2 and Fe^{2+} plus EDTA had an inactivating effect with purified GDH(NADP)

TABLE 2. Oxidative inactivation of purified *N. crassa* and bovine GDH(NADP)[a]

Oxidizing mixture[b]	Remaining activity (%)[c]	
	N. crassa GDH(NADP)	Bovine GDH(NADP)
Experiment A		
None	100	100
ASC	94	96
Fe^{3+}	100	81
EDTA	105	155
ASC + Fe^{3+}	85	55
ASC + EDTA	112	155
Fe^{3+} + EDTA	106	96
ASC + Fe^{3+} + EDTA	38	70
ASC Fe^{3+} + EDTA (O_2)	25	
ASC + Fe^{3+} + EDTA (N_2)	112	
Experiment B[c]		
None	100	
ASC + Fe^{3+} + EDTA	1 ± 1	
ASC + Fe^{2+} + EDTA	1 ± 1	
ASC + Fe^{3+} + EDTA + DTT	25 ± 2	
ASC + ferric-citrate	11 ± 4	
Ferric-citrate	107	
H_2O_2	66 ± 2	
H_2O_2 + Fe^{2+}	58 ± 22	
H_2O_2 + Fe^{2+} + EDTA	15 ± 1	
H_2O_2 + Ferric-citrate	22 ± 15	
Dehydroascorbate, 1 mM (not filtered)	16 ± 6	
Dehydroascorbate, 1 mM (not filtered) (N_2)	7 ± 1	
Dehydroascorbate, 1 mM	100 ± 7	
Dehydroascorbate, 5 mM	83	
Dehydroascorbate, 10 mM	75	
o-phenanthroline	0	

[a] The enzymes in a 10 mM phosphate buffer, pH 7, were incubated with the different oxidizing mixtures at 30°C for 2 h and filter dialyzed (unless stated differently), and the remaining activity was determined.

[b] Final concentrations: ascorbate (ASC), 10 mM; Fe^{2+} and Fe^{3+}, 0.6 mM; EDTA and *o*-phenanthroline, 1 mM; ferric-citrate, 0.5 mM; H_2O_2, 0.5 mM.

[c] Mean values of two to four experiments.

and the EDTA-iron could be substituted by ferric-citrate (*o*-phenanthroline alone inhibited the enzyme) (Table 2).

Treatment of bovine GDH(NADP) with the oxidizing mixture gave similar results, but EDTA was not required for oxidation. EDTA alone had a pronounced stimulating effect on bovine GDH(NADP) activity of 55% after 2 h of incubation at 30°C (Table 2).

The conformational state of GDH(NADP) is dependent on the presence of substrates and products in the incubation medium. The active form is found in the presence of 2-oxoglutarate, NADP or 2-oxoglutarate plus NADPH or NADP, or in the presence of glutamate (52). Binding of NADPH shifts the equilibrium to the inactive conformation of the enzyme (2). The effect of the substrates and products upon the oxidation of GDH(NADP) by ascorbate, Fe^{3+}, and EDTA is shown in Fig. 2. It can be seen that NADPH, either alone or with ammonium, 2-oxoglutarate, or ammonium and 2-oxoglutarate, had a protective effect from oxidative inactivation at pH 7. Ammonium, 2-oxoglutarate, ammonium plus 2-oxoglutarate, NADP, or glutamate did not have a significant effect.

Inactivation of purified GDH(NADP) with the oxidizing mixtures containing ascorbate could be due to the formation of mono- and dehydroascorbate. Dehydroascorbate alone inhibited the enzyme at 1 mM. Nevertheless, when treated

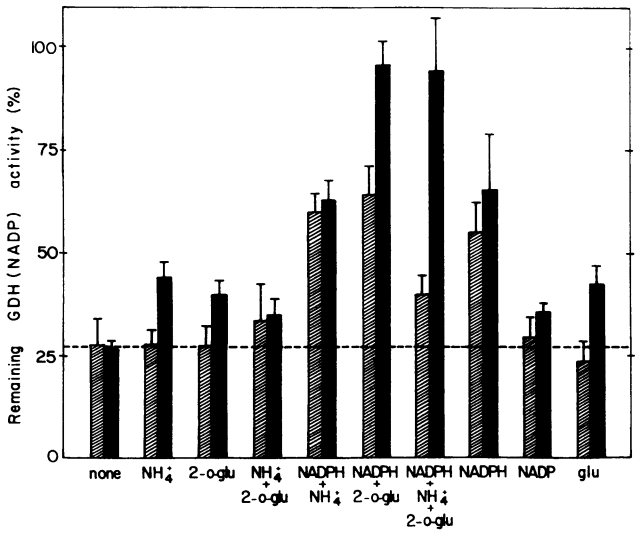

FIG. 2. Oxidative inactivation of purified GDH(NADP) in the presence of enzyme substrates and products. Purified GDH(NADP) in 100 mM phosphate buffer, pH 7.0, was incubated for 2 h at 30°C with ascorbate (10 mM), Fe^{3+} (0.6 mM), and EDTA (1 mM) plus 1 mM (▨) or 6 mM (■) final concentration of the indicated enzyme substrates or products. After incubation, the enzyme was filter dialyzed and the remaining activity determined. Abbreviations: 2-o-glu, 2-oxoglutarate; glu, glutamate.

with dehydroascorbate and then filter dialyzed, most of the activity was recovered (Table 2). Thus, inhibition by dehydroascorbate can be separated from oxidative inhibition by filter dialysis. Besides, inhibition by dehydroascorbate was independent of dioxygen (Table 2) and, in the absence of EDTA, the formation of dehydroascorbate did not significantly inhibit GDH(NADP) (Table 2).

Step-by-step oxidation of purified GDH(NADP). To minimize the formation of dehydroascorbate, a step-by-step oxidation procedure was carried out. The enzyme was incubated first with chelated iron, filter dialyzed, then incubated with either ascorbate or H_2O_2, and filter dialyzed, and the remaining activity was determined. As shown in Table 3, inactivation of the enzyme was greater when the enzyme was incubated first with chelated iron and then with either ascorbate or H_2O_2 compared with the inactivation obtained when the order of the incubations was inverted. In these experiments, H_2O_2 had a stronger inhibitory effect than ascorbate.

Fragmentation and precipitation of oxidized GDH(NADP). When incubated with ascorbate, Fe^{3+}, and EDTA for increasingly longer periods and analyzed by SDS-PAGE, purified GDH(NADP) broke down into discrete pieces. As can be seen in Fig. 3, the staining intensity with Coomassie blue of the main band diminished with longer incubation periods in the presence of the oxidizing mixture (lanes 2, 4, and 6). At the same time, many discrete bands of lower molecular weight than the GDH(NADP) monomers appeared in increasing amounts. Loss of the monomers correlates approximately with the loss of activity. A 2-h incubation with the oxidizing mixture reduced both the activity and the monomer band in the gel to about half; a 6-h incubation almost completely abolished the activity and the monomer band.

In these experiments, it was observed that part of the enzyme precipitated during the oxidizing reaction. Most of the enzyme collected by centrifugation still had a molecular weight similar to that of the monomer (Fig. 3, lanes 3, 5 and 7). Fragments of lower molecular weight are also seen in these lanes. The smear in these lanes could be due to polymerization of the enzyme polypeptides, but this explanation was excluded because the smear disappeared when the salt was not removed (Fig. 3, lane 8).

TABLE 3. Step-by-step oxidation of purified GDH(NADP)[a]

Oxidizing mixture[b]	Remaining activity (%)
Expt 1	
Fe^{3+} + EDTA → Buffer	88.6 ± 16.4
Fe^{3+} + EDTA → ASC	54.8 ± 11.6
Fe^{3+} + EDTA → H_2O_2	35.2 ± 4.1
Ferric-citrate → Buffer	75.1 ± 8.1
Ferric-citrate → ASC	65.5 ± 3.5
Ferric-citrate → H_2O_2	32.2 ± 4.3
Expt 2	
Buffer → Fe^{3+} + EDTA	100
ASC → Fe^{3+} + EDTA	94.1 ± 3.1
H_2O_2 → Fe^{3+} + EDTA	54.0 ± 5.2
Buffer → Ferric-citrate	78.6 ± 6.6
ASC → Ferric-citrate	86.0 ± 3.3
H_2O_2 → Ferric-citrate	56.5 ± 8.6

[a] Purified GDH(NADP) was incubated with the compounds of the first column, filter dialyzed, incubated with the compounds of the second column, filter dialyzed, and then activity was measured. Incubation conditions and final concentrations were the same as described in the footnotes to Table 2. Mean values of four experiments.

[b] For both experiments, the incubation time for the chelated iron mixtures was 2 h and the incubation time for buffer, ascorbate (ASC), and H_2O_2 was 1 h.

Charge heterogeneity related to oxidation of GDH(NADP). When purified GDH(NADP) was analyzed by isoelectric

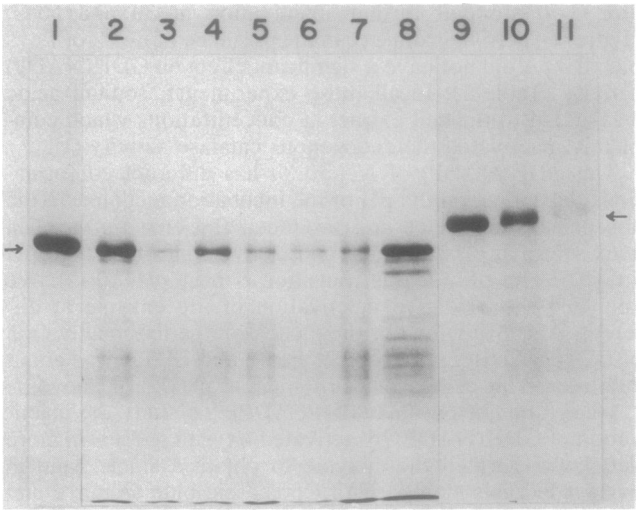

FIG. 3. Fragmentation and precipitation of oxidized *N. crassa* GDH(NADP) and fragmentation of the bovine enzyme. Samples of 25 mg of purified *N. crassa* or bovine GDH(NADP) were incubated at 30°C with ascorbate (10 mM), Fe^{3+} (0.6 mM), and EDTA (1 mM) and then centrifuged. The dialyzed supernatants and the pellets were analyzed by SDS-PAGE, and the gels were stained with Coomassie blue. Lanes 1 to 8 contained *N. crassa* GDH(NADP). Lanes: 1, incubation for 6 h without oxidizing mixture; 2, 4, and 6, supernatants incubated with oxidizing mixture for 2, 4, and 6 h; 3, 5, and 7, pellets after 2, 4, and 6 h of incubation with the oxidizing mixture; 8, total enzyme oxidized for 2 h (neither centrifuged nor dialyzed). Lanes 9 to 11 contained bovine GDH(NADP). Lanes 9, total enzyme incubated for 6 h without oxidizing mixture; 10 and 11, total enzyme oxidized for 2 and 6 h, respectively, and dialyzed. Arrows indicate bands of the enzyme monomers.

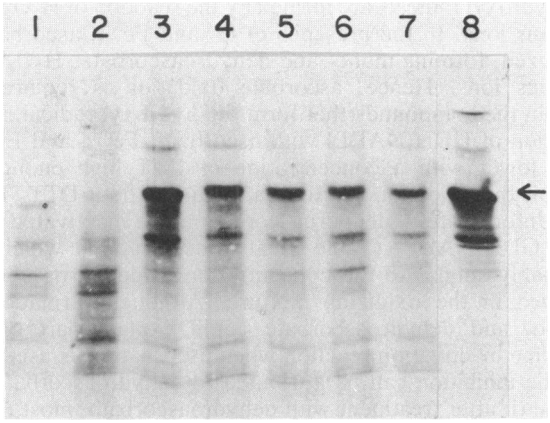

FIG. 4. Isoelectric focusing PAGE of purified GDH(NADP) precipitated by oxidation. The purified enzyme was incubated at 30°C with the following oxidation mixtures. Lanes: 1 and 2, with ascorbate, Fe^{3+}, and EDTA for 1 or 2 h, respectively; 3 and 4, with H_2O_2, Fe^{3+}, and EDTA for 1 or 2 h, respectively; 5 and 6, with ascorbate plus ferric-citrate for 1 or 2 h, respectively; 7, with H_2O_2 plus ferric-citrate for 2 h; 8, enzyme incubated for 2 h without additions. Concentrations: ascorbate, 10 mM; Fe^{3+}, 0.6 mM; EDTA, 1 mM; H_2O_2, 0.5 mM; ferric-citrate, 1 mM. Arrow indicates band of enzyme monomer.

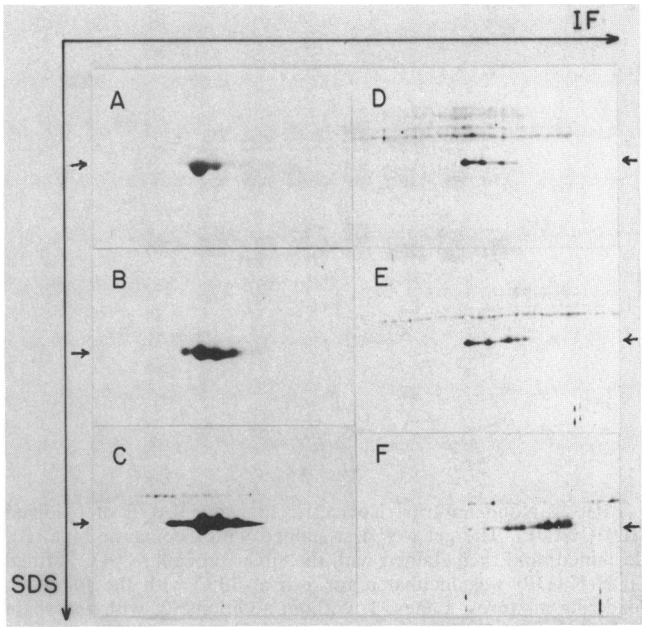

FIG. 5. Oxidized GDH(NADP) analyzed by 2D-PAGE. The purified enzyme was oxidized with ascorbate, Fe^{3+} and EDTA and analyzed by 2D-PAGE and stained either with Coomassie blue (panels A and B) or with the silver procedure (panels C through F). (A) Without treatment; (B) oxidized for 2 h; (C) same as panel A but stained with silver; (D through F) the precipitated enzyme was collected by centrifugation after 1, 2, or 6 h, respectively, with the oxidation mixture. Arrows indicate positions of the enzyme monomers.

focusing PAGE, bands with different isoelectric points were stained. Besides the intensively stained main band, six less intensively stained bands were observed, two with a more alkaline isoelectric point and four with more acidic isoelectric points than that of the main band (Fig. 4, lane 8). When collected by centrifugation after 1 or 2 h of oxidation with ascorbate or H_2O_2 in the presence of either EDTA plus Fe^{3+} or ferric-citrate, the precipitated GDH(NADP) presented the same bands although in relatively different proportions. With more incubation time, the main band decreased and the more acidic polypeptides increased in staining intensity (Fig. 4, lanes 1 to 7).

This charge heterogeneity was also observed when the purified GDH(NADP) was analyzed by 2D-PAGE. As shown in Fig. 5A and C, pure GDH(NADP) had, besides the intensively stained main spot, other less intensively stained spots, one with a more alkaline isoelectric point and two with a more acidic isoelectric point than the main spot. Purified GDH(NADP) presented a less intensively stained main spot and increased staining of the three minor spots after 2 h of incubation with ascorbate, Fe^{3+}, and EDTA (Fig. 5B).

When the precipitated GDH(NADP) was analyzed by 2D-PAGE, more acidic polypeptides than unoxidized enzyme polypeptides appeared. With time of the oxidation reaction, they increase in amount and negative charge (Fig. 5D through F).

Immunoprecipitated GDH(NADP) also presented charge heterogeneity. To be certain that the charge heterogeneity observed with the purified GDH(NADP) was not due to other polypeptides that could have been copurified with the enzyme, antibodies were raised against purified GDH (NADP) and used to detect these polypeptides in a cell extract of GDH(NADP) deletion mutant strain *am-132*. The immunoprecipitates were analyzed by 2D-PAGE and stained with silver. As shown in Fig. 6, no stained spots with a similar molecular weight as that of GDH(NADP) could be seen in the immunoprecipitate from the mutant strain, even though six times more protein was used for the mutant strain immunoprecipitate than for that of the wild-type strain. However, in the immunoprecipitated GDH(NADP) from the wild-type strain, a similar charge heterogeneity was observed as for the purified enzyme (Fig. 5C and 6A).

Charge heterogeneity in the native enzyme and dissociation of the hexamer. Active GDH(NADP) is known to be a hexamer of identical subunits (4, 18). The modified monomers in the hexameric structure should give the hexamer charge heterogeneity. Figure 7B shows the Coomassie-stained gel in which the purified GDH(NADP) was analyzed

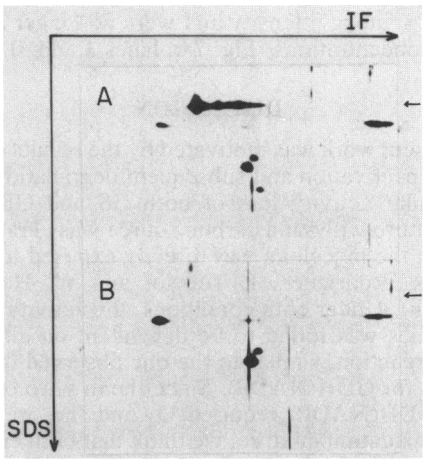

FIG. 6. Immunoprecipitated GDH(NADP) from cell extracts of the wild-type strain and the *am-132* deletion mutant strain. The immunoprecipitated GDH(NADP) from the wild-type (A) and the *am-132* (B) strains was analyzed by 2D-PAGE, and the gels were stained by the silver procedure. Arrows indicate positions of the enzyme monomers.

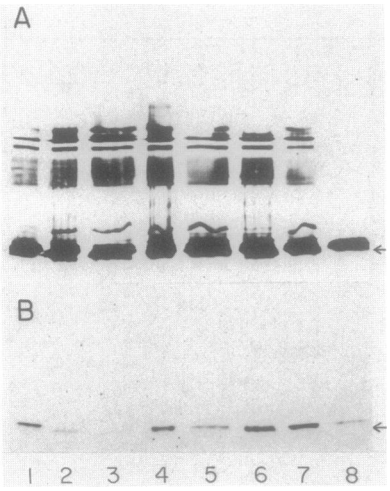

FIG. 7. Nondenaturing isoelectric focusing PAGE of oxidized GDH(NADP). The gel was first stained with Coomassie blue (B), destained, and then stained with the silver procedure (A). Purified GDH(NADP) was incubated for 1 h at 30°C with the following oxidizing mixtures. Lanes: 1, without additions; 2, with ascorbate and Fe^{2+} plus EDTA; 3, same as lane 2 but after freezing and thawing in the presence of 80 mM NaCl; 4, with H_2O_2 and Fe^{2+} plus EDTA; 5, same as lane 4 but after freezing and thawing in the presence of 80 mM NaCl; 6, with ferric-citrate and ascorbate; 7, with H_2O_2; 8, purified GDH(NADP) after freezing and thawing in the presence of 80 mM NaCl. Concentrations in the oxidizing mixtures were the same as those described in the legend to Fig. 4. Arrows indicate positions of the hexamer.

by nondenaturing isoelectric focusing PAGE. In this gel, only a very acidic band was observed that decreased in staining intensity when the enzyme was treated with the oxidizing mixtures. When the same gel was stained with silver, the single band was composed of various bands and other less-acidic bands also appeared (Fig. 7A). These less-acidic bands of the untreated enzyme diminished in staining intensity when the enzyme was frozen and thawed in the presence of high salt concentrations, conditions which favored association of the monomers into the hexameric structure (50) (Fig. 7A, lanes 1 and 8). When incubated for 2 h with the oxidizing mixtures, the less-acidic bands increased in staining intensity and were no longer affected by high salt concentrations (Fig. 7A, lanes 3 and 5).

DISCUSSION

The present work was motivated by the results obtained in oxidative inactivation and subsequent degradation of GS (1) and a similar activity loss of both GS and GDH(NADP) when a culture without a carbon source was aerated (1; Fig. 1) or when the mycelium was directly exposed to the air (J. Aguirre, R. Rodríguez, I. Toledo, and W. Hansberg, in preparation). Under both conditions, the activity loss of the two enzymes was found to be dependent on air. Thus, an oxidation reaction, similar to the one observed for GS, was postulated for GDH(NADP). Since the in vitro oxidation of GS and GDH(NADP) required O_2 and the presence of a complete oxidation mixture, we think that both enzymes are inactivated by activated oxygen species. The site-specific formation of a hydroxyl radical has been postulated for various enzymes (17, 20, 28, 41, 43). It has been observed that the hydroxyl radicals modify the amino acids of proteins and that fragmentation of the proteins occurs when dioxygen reacts on the modified residues (10).

Hydroxyl radicals are formed by the reaction of H_2O_2 with ferrous ions. In the presence of O_2 and Fe^{3+}, ascorbate is oxidized, forming mono- and dehydroascorbate, H_2O_2, and ferrous ions. Hence, ascorbate oxidation is required to obtain the compounds that form the hydroxyl radical. Inactivation of GDH(NADP) with ascorbate, Fe^{3+}, and EDTA was lower with a concentration of DTT high enough to inhibit the oxidation of ascorbate than without DTT (Table 2). Unfortunately, dehydroascorbate alone inactivated purified GDH(NADP) (Table 2) at concentrations which are probably similar to the concentration of dehydroascorbate formed in the oxidizing mixture. Although formation of mono- and dehydroascorbate could explain part of the enzyme inactivation reaction when ascorbate was used, not all the inhibition can be attributed to dehydroascorbate because (i) after treatment with dehydroascorbate, most of the enzyme activity was recovered by filter dialysis (Table 2); (ii) inhibition by dehydroascorbate was not dependent on O_2 (Table 2); (iii) dehydroascorbate formed in the oxidizing mixture without EDTA did not inhibit the enzyme to the same extent as with chelated iron, e.g., inhibition by dehydroascorbate does not explain the requirement of chelated iron in the oxidizing mixture (Tables 1 and 2); (iv) inhibition by dehydroascorbate also does not explain the step-by-step inactivation reaction (Table 3) in which dehydroascorbate probably forms in a very low concentration.

Inactivation of GDH(NADP) by activated oxygen species showed some similarities to the one observed with GS. In both enzymes (i) heterogeneity in charge was observed and the relative amounts of the polypeptides having different isoelectric points changed when the enzymes were incubated with different oxidation mixtures; (ii) besides modifications in the net charge of the monomers, inactivation by oxygen radicals also caused precipitation and fragmentation; and (iii) partial protection against inactivation by oxygen radicals was observed in the presence of some or a combination of the enzyme substrates.

Purified GDH(NADP) in 2D-PAGE showed at least four spots that differed in their isoelectric points but had no apparent differences in their molecular weights (Fig. 5A). This result indicates that some enzyme monomers were modified either in vivo or during the purification procedure. Contamination with other proteins is very unlikely, since after four different purification steps which did not include separation by molecular weight, the enzyme gave a single band after SDS-PAGE in an overloaded gel stained with Coomassie blue (Aguirre and Hansberg, Fungal Genet. Newsl. 1988) (Fig. 3). Some faint bands in the same gel and other gels were observed when stained with the silver procedure, but these had very dissimilar isoelectric points and molecular weights than those of the GDH(NADP) monomers. Moreover, by using antibodies raised against the purified GDH(NADP), the immunoprecipitated enzyme showed the same spots in 2D-PAGE with the wild-type strain cell extract but not with cell extract of the GDH (NADP) deletion mutant strain (Fig. 6).

When the purified enzyme was incubated with ascorbate, iron, and EDTA and analyzed in 2D-PAGE, the major spot in the gel decreased and the minor spots increased in staining intensity (Fig. 5A and B). The enzyme monomers which precipitated during the oxidation reaction also had similar molecular weights but were heterogeneous in charge (Fig. 5D through F).

These results indicate that (i) the GDH(NADP) in cell extracts had some monomers which were modified in their isoelectric points and (ii) the relative amounts of these modified monomers increased when the purified enzyme was

incubated with an oxidizing mixture that generated oxygen radicals.

Oxidation by activated oxygen species gave rise not only to specific modifications of the monomers but also to precipitation, dissociation, and fragmentation of the enzyme. After PAGE and staining, various bands with defined molecular weights (Fig. 3) were observed in the gel. These data indicate that fragmentation by activated oxygen species was not a random process but that there are preferential breaking points in the GDH(NADP) polypeptide. In fact, fragmentation of protein by activated oxygen species usually gives defined fragments (8, 23, 53) because of different oxidizing susceptibilities of the amino acid residues (10, 53).

GDH(NADP) was found to partially precipitate when treated with ascorbate and iron plus EDTA or with H_2O_2 Fe^{2+} plus EDTA. The precipitated enzyme analyzed by SDS-PAGE had the same molecular weight as did the enzyme without treatment (Fig. 3) but presented charge heterogeneity when analyzed in isoelectric focusing PAGE (Fig. 4) or 2D-PAGE (Fig. 5D through F). Modifications of the precipitated enzyme increased with incubation time, giving rise to polypeptides with more acidic isoelectric points than the unoxidized enzyme polypeptides. These results suggest that the monomers, when modified by activated oxygen species, change their conformation and interact in such a way as to precipitate. Some other polypeptides with small molecular weights were also observed in the gels (Fig. 3). They are probably trapped enzyme polypeptide fragments in the precipitate or enzyme fragment conglomerates that form by hydrophobic and ionic interactions (9). The same fragments are also seen in the soluble fraction (Fig. 3).

GDH(NADP) activity was less susceptible to inactivation by ascorbate, iron, and EDTA at pH 7.5 to pH 8 than at pH 6 to pH 7. It was also less susceptible when NADPH was present, alone or in combination with ammonium ions, 2-oxoglutarate, or both, than in the absence of these substrates; the enzyme products did not have a significant effect (Fig. 2). The experiments with substrates and products were done at pH 7, at which GDH(NADP) is in an inactive form in the absence of substrates or in the presence of NADPH (52). At this pH, the enzyme shifts to an active conformation in the presence of 2-oxoglutarate or 2-oxoglutarate plus NADPH (2, 52). No clear-cut conclusion could be drawn from these results to indicate that either the active or the inactive conformation of the enzyme was more liable to inactivation by activated oxygen species.

Besides the similarities mentioned with respect to the inactivation of GS and GDH(NADP) by oxygen radicals, there are some important differences in the mechanism of inactivation of the two enzymes. (i) While GS was very unstable in the presence of ascorbate and Fe^{3+} (15 min at 4°C inactivated half of the enzyme), GDH(NADP) was remarkably stable in the presence of ascorbate and iron plus EDTA (it was not inactivated at 4°C and required 1 to 2 h to inactivate half of the enzyme at 30°C) (Tables 1 and 2). (ii) Loss in GS activity was mainly due to inactivation of the enzyme, whereas with GDH(NADP), the activity loss correlated closer to the fragmentation of the enzyme polypeptides. (iii) Oxidation of GS was inhibited by EDTA, whereas oxidation of GDH(NADP) was greatly enhanced by chelating agents such as EDTA or citrate. (iv) Fe^{2+} plus H_2O_2 was the most effective oxidizing mixture with GS, whereas ascorbate and iron plus EDTA inactivated GDH(NADP) most effectively. (v) NADH or NADPH added to a cell extract oxidized GS but did not affect the GDH(NADP) activity.

The production of oxygen radicals in the oxidizing mixture was enough to inactivate GS in minutes, even at 4°C (1). Therefore, the stability of GDH(NADP) in the presence of activated oxygen species cannot be explained by the generation of a limiting amount of oxygen radicals. In order to explain the features of the GDH(NADP)-inactivating reaction by activated oxygen species, the following model is proposed. The enzyme is rendered susceptible to oxygen radicals only after a rate-limiting, site-specific oxidative reaction has taken place. For this reaction to occur, iron has to be bound to GDH(NADP); but binding of iron to the enzyme can only occur in the form of chelated iron with a chelating agent that binds to the enzyme. When the EDTA-iron or ferric-citrate is bound to GDH(NADP), the enzyme is modified by the in situ formation of activated oxygen species in the presence of H_2O_2. After this initial site-specific modification of the enzyme polypeptides, other modifications in the monomers can occur, leading both to their precipitation, due to conformational changes, and to their fragmentation, due to destruction by the oxygen radicals of certain amino acids in the enzyme polypeptides. The results in Table 3 support this model. GDH(NADP) bound EDTA-Fe^{3+} or ferric-citrate, and both ascorbate and H_2O_2 reacted on the chelated iron bound to the enzyme. Other enzymes have been reported to be only oxidized when in the presence of EDTA (27, 39, 48). Further evidence in support of this model is under current research.

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