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Action of Corn and Rice-inactivating Proteins on a Purified Nitrate Reductase from *Chlorella vulgaris*[1]

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ABSTRACT

When nitrate reductase (NR) purified from *Chlorella* was incubated with NR-inactivating proteins purified from corn roots and rice cell suspension cultures or with trypsin there was a loss in NADH-NR and NADH cytochrome *c* reductase (NADH-CR) activities with time whereas the reduced methylviologen NR (MV-NR) remained active. When NADH-NR and NADH-CR activities were inactivated completely by the incubation with corn protein, the major protein band obtained by polyacrylamide gel electrophoresis shifted from an R_F value of 0.12 to an R_F of 0.25 and reduced MV-NR activity moved to the new position on the gel. When NADH-NR and NADH-CR activities were partially inactivated by the corn protein, NADH-NR activity was detected in an intermediate position (R_F value of 0.18). Incubation with trypsin also caused a change in the NR protein migration pattern (R_F value of 0.20). This protein band also had reduced MV-NR activity. Thus, the corn inactivator degrades NR in a fashion similar to but not identical with trypsin. The incubation of NR with rice inactivating protein resulted in a loss of NADH-NR but had no effect on the migration of NR protein or on the reduced MV-NR activity or mobility suggesting that the rice protein binds to *Chlorella* NR.

NR[6]-inactivating proteins which are considered to have a regulatory role on NR levels in plant tissues have been isolated from corn roots (29, 31), rice cell cultures (35, 36), and soybean leaves (10). The proteins from corn roots and rice cells were further characterized by polyacrylamide disc gel electrophoresis (34). Electrophoresis of these proteins on polyacrylamide gels with a Tris-glycine buffer system indicated that the corn protein has more basic surface charges than the rice protein. The activities of NADH-NR inactivation and hydrolysis of azocasein were associated with the same protein band after electrophoresis of the corn protein using either Tris-glycine or β-alanine-acetate buffer systems, indicating that this protein is a NR-inactivating protease (34). The rice protein, on the other hand, had no protease activity and the inactivation of rice cell NR by it could be reversed by the addition of NADH (34, 37), suggesting that the rice protein is a specific binding protein. Both proteins had a major inhibitory effect on NADH-NR and NADH-CR activities and were much less effective in reducing the $FMNH_2$-NR and reduced MV-NR activities. A purified inactivating protein from soybean leaves also had no protease activity (10). Change of the protein mobility through Sephadex G-75 in the presence of soybean leaf NR indicated that it is also a specific binding protein. However, its action on the NADH-CR component of NR was less specific than the two inactivating proteins with which we have worked.

Purification and stabilization of NR from higher plant sources have proven to be a difficult task, although there have been a few reports of success in recent years (3, 19). However, NR from *Chlorella* has been purified and characterized (22, 23) and this NR was used in the current investigation as a substrate for the corn- and rice-inactivating proteins. Changes in properties of *Chlorella* NR were investigated with polyacrylamide gel electrophoresis. The results show that the mobility of *Chlorella* NR is altered after treatment with corn-inactivating protein whereas there is no apparent change in mobility after treatment with rice-inactivating protein.

MATERIALS AND METHODS

Plant Materials. Corn seedlings (var. Wf9 × 38-11) were grown in liquid culture solution with a modified 0.1-strength Hoagland solution containing 10 mM KNO_3 and 0.08 μM molybdate for 7 days as described in the accompanying paper (34). The mature roots of the seedlings (primary root minus 0 to 1-cm tip and basal regions) were used for purification of NR-inactivating proteins. Rice cells were cultured in liquid R-2 medium for 10 days as reported before (35). Growth conditions for *Chlorella vulgaris* and the purification of NR were described previously (22, 23).

Purification of NR-inactivating Proteins. A 106-g sample of corn roots and an 85-g sample of rice cells were used for the purification of NR-inactivating proteins. The activity of the inactivators was assayed with partially purified corn leaf NR (34). The corn protein was purified 921-fold and the rice protein, 1,660-fold using standard purification procedures involving precipitation with $(NH_4)_2SO_4$, fractionation at pH 4.0, adsorption on CM-cellulose (29), and gel filtration with Sephadex G-100 column (34). Peak fractions of corn protein (2.5 ml, 25 μg of protein) and rice protein (5 ml, 80 μg of protein) from a second Sephadex G-100 gel filtration were dialyzed in 10 mM K-phosphate (pH 7.0) for 16 h and concentrated by lyophilization. The proteins were then applied to 7.5% of acrylamide gel and electrophoresed using Tris-glycine buffer system with a pH of 9.4 during the electrophoresis (5). After electrophoresis, the inactivating proteins were extracted with 2 ml of 0.1 M K-phosphate (pH 7.0) as described previously (34).

Enzyme Assays and Protein Determinations. NADH-NR was assayed as described previously (34). NADH-CR and reduced MV-NR were also assayed as described before (21) except that incubations were carried out at 28 C. One unit of NADH-NR and reduced MV-NR was defined as that amount which produced 1 nmol NO_2^- formed per min at 28 C. One unit of NADH-CR was defined as that amount which caused an *A* increase of 1.0 at 550

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[6] Abbreviations: NR: nitrate reductase; $FMNH_2$: reduced flavin adenine mononucleotide; CR: cytochrome *c* reductase; MV: methylviologen.

nm per min at 28 C. Protein was measured by the method of Lowry *et al.* (14).

Inactivation of *Chlorella* NR by the NR-inactivating Proteins. Purified NR (10 μl; about 1 mg protein per ml) stored in 0.5 M K-phosphate (pH 7.6), containing 45% of glycerol was diluted five times with 0.5 M K-phosphate (pH 7.6) with or without 0.2% of BSA before use in the inactivation assay. Purified inactivating proteins (50 μl) were incubated with the 50 μl of diluted NR for 0–5 h at 28 C. After the appropriate time intervals, 5 μl of the reaction mixture was diluted with 0.4 ml of 0.5 M K-phosphate (pH 7.6) containing 0.2% of BSA to prevent a nonspecific inactivation of NR which results from dilution. NADH-NR and associated activities were assayed with 50-μl aliquots of the diluted reaction mixture. The activity of the *Chlorella* NADH-NR was 20,000 units per mg protein, of the reduced MV-NR was 57,000 and of the NADH-CR was 1,520. The inactivating activity was calculated from the difference in the activity with or without inactivating protein. After the incubation of NR with the inactivating protein, 2 drops of glycerol were added to the reaction mixture and then subjected to polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Analytical disc gel electrophoresis of NR with the inactivating proteins was performed at 4 C using a Tris-glycine buffer system (5) or a diethyl-barbital buffer system (23). Gels (7.5% acrylamide for Tris-glycine buffer system and 6.0% for barbital system) were prepared from a stock solution of 30% acrylamide and 0.8% bis-acrylamide. Gels were 7 cm long and 6 mm in diameter. They were run at 1 mamp per tube for 30 min and then set at 2 mamp per tube until the tracking dye front was close to the bottom of the gels. After running, the gels were sliced every 2.5 mm and assayed in the slices for NADH-NR and reduced MV-NR activities as described previously (34). Protein was stained with Coomassie brilliant blue R and each gel was scanned at 550 nm with Gilford spectrophotometer type 2400.

RESULTS

Although the activity of NADH-NR purified from *Chlorella* was stable over long periods of time at concentrations of 1 mg protein per ml, it lost all of its activity upon dilution. In fact, the NADH-NR activity was lost completely during the incubation for 40 min at 28 C when the original NR preparation was diluted 150 times with 0.5 M K-phosphate (pH 7.5) (Fig. 1A). The addition of 0.2% of BSA to the diluting buffer prevented this loss, suggesting that BSA prevents the disruption of the subunit structure of NR. The effect of BSA here is similar to that reported by Sherrard and Dalling for wheat leaf NR (19). With less dilution (10 times) the NADH-NR was stable for a 5-h incubation at 28 C (Fig. 1B). Because of these results, 0.5 M K-phosphate containing 0.2% of BSA (pH 7.6) was used as the diluting buffer after treatment of the *Chlorella* NR with the inactivating proteins.

Purified *Chlorella* NR containing 11 μg of protein was incubated with 86 units of purified corn-inactivating protein or 93 units of rice cell protein. The unit of each protein was determined with corn leaf NADH-NR (34). NADH-NR and NADH-CR activities were inactivated by those inactivating proteins during the incubation (Fig. 2, A and B). The corn protein caused a 50% inactivation after a 1-h incubation and almost complete inactivation after 3 h. The rice protein caused a 65% inactivation after a 5-h incubation. Neither inactivating protein inactivated the reduced MV-NR component of *Chlorella* NR and, in fact, there was a slight increase in activity after treatment with corn protein. These effects resemble the action of trypsin on *Chlorella* NR reported earlier (9) and illustrated in Figure 3. Both the NADH-NR and NADH-CR components of NR were inactivated by incubation with 0.5 μg of trypsin, and the reduced MV-NR component was less sensitive to trypsin digestion (Fig. 3). Trial experiments showed that BSA had no influence on the inactivating activities of either inactivating proteins or trypsin. These experiments estab-

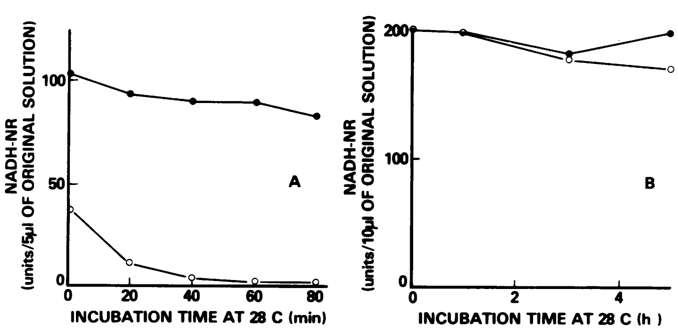

FIG. 1. Effect of BSA on stability of NADH-NR purified from *Chlorella*. A: purified *Chlorella* NR (5 μl containing 5.5 μg protein) was diluted 150 times with 0.5 M K-phosphate (pH 7.6) with (●) or without (○) 0.2% BSA. A 50-μl aliquot of diluted NR solution was incubated at 28 C. B: *Chlorella* NR (10 μl containing 11.0 μg protein) was diluted 10 times with the buffer with (●) or without (○) 40 μg BSA and was incubated at 28 C. In each case, A and B, a 5-μl aliquot was taken at each time and was diluted to 0.4 ml with buffer containing 0.2% BSA.

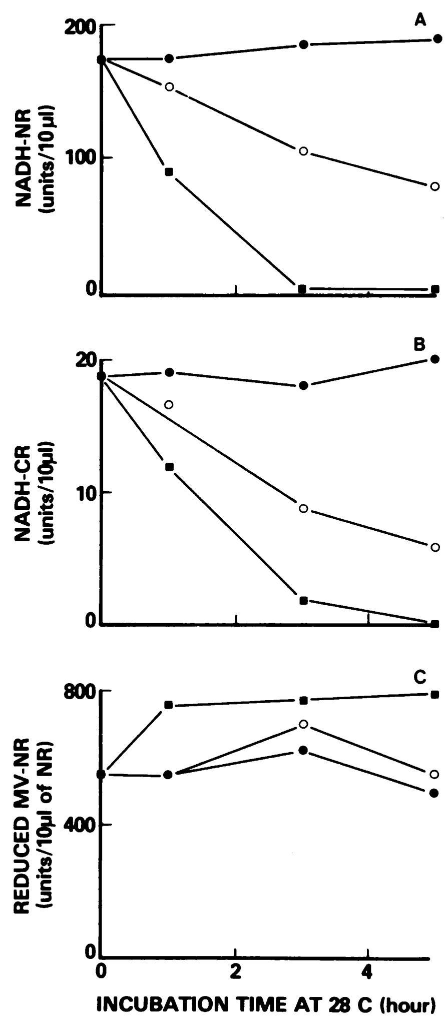

FIG. 2. Effect of NR-inactivating proteins from corn and rice cells on the activities of the NR complex from *Chlorella*. *Chlorella* NR (10 μl) was diluted five times with 0.5 M K-phosphate containing 0.2% BSA (pH 7.6) and was then incubated with 50 μl of NR-inactivating protein purified from corn root (■) or rice cells (○) or with K-phosphate alone (●). A 5-μl aliquot of the incubation mixture was taken each time, diluted to 0.4 ml with 0.5 M K-phosphate (pH 7.6) containing 0.2% BSA, and was assayed for NADH-NR (A), NADH-CR (B), and reduced MV-NR (C).

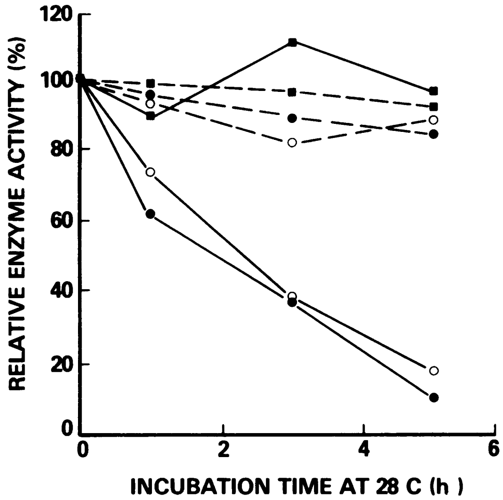

FIG. 3. Effect of trypsin on the activities of NADH-NR (●), NADH-CR (○) and reduced MV-NR (■) from *Chlorella*. *Chlorella* NR (10 μl) was incubated with 40 μl of 0.5 M K-phosphate containing 0.2% BSA (pH 7.6) with (——) or without (- - -) 0.5 μg of trypsin (50 μl). Initial activities of NADH-NR, NADH-CR and reduced MV-NR were 200, 15.2, and 570 units per 10 μl of original NR solution, respectively.

lished the incubation intervals to be used in examining the inactivation products. Five h was used in each case for a complete reaction and a 1-h incubation was used to detect products from a partial reaction.

When *Chlorella* NR was run on a 6% polyacrylamide gel with Tris-barbital buffer there was one protein band as reported previously (23). With a 7.5% polyacrylamide gel and a Tris-glycine buffer system, however, the purified NR had one major protein band with an R_F of 0.12 and two minor bands with R_F values of 0.03 and 0.06 (Figs. 4 and 5). In the control experiment NADH-NR and reduced MV-NR activities were associated with the major protein band (Fig. 6A). After a 5-h treatment, with the rice inactivator, only 42% of the NADH-NR activity remained and the position of the protein band and reduced MV-NR activity was not altered (Fig. 6C). With the corn inactivator, on the other hand, there was a shift both in the major protein band and in the reduced MV-NR activity (Fig. 6B). Both the major protein band and the reduced MV-NR activity moved toward the anode with an R_F of 0.25 and the minor bands had R_F values of 0.11 and 0.18 (Fig. 4). With shorter incubation times (1 h) the same profile of protein migration and shift in reduced MV-NR were observed but in this case the NADH-NR was in an intermediate position with a R_F value of 0.18. Trypsin digestion of NR also gave shifts in the protein bands. In this case the R_F values of the major protein band and the two minor bands were 0.20, 0.16, and 0.11, respectively (Fig. 4). The reduced MV-NR activity was also detected in the major protein band after incubation with trypsin for 5 h (Fig. 6D). After 3-h incubation the NADH-NR activity was still observed and was associated with a protein band with an R_F of 0.16.

Inactivation of rice cell NR by rice-inactivating protein was reversed by addition of NADH (34, 37). As seen earlier with rice and corn leaf NRs (34), addition of NADH inhibited NADH-NR activity in *Chlorella* (Table I). Inactivation of *Chlorella* NADH-NR by the rice inactivator was inhibited by addition of NADH during the reaction, but as with corn leaf NR (34) the inhibition was not reversed by NADH. NADH had no effect on the corn inactivator.

DISCUSSION

Protein turnover is an important mechanism for controlling the levels of specific enzymes or proteins in eukaryotic organisms. The turnover rate of specific proteins, a parameter studied more extensively in animals than plants, is a characteristic of the particular protein. It has been shown, *e.g.* to be related to mol wt, acidity of the protein, and sensitivity to heat denaturation or endopeptidases (17). In addition aberrant proteins made as a result of genetic mutation are selectively degraded (17). In a few cases,

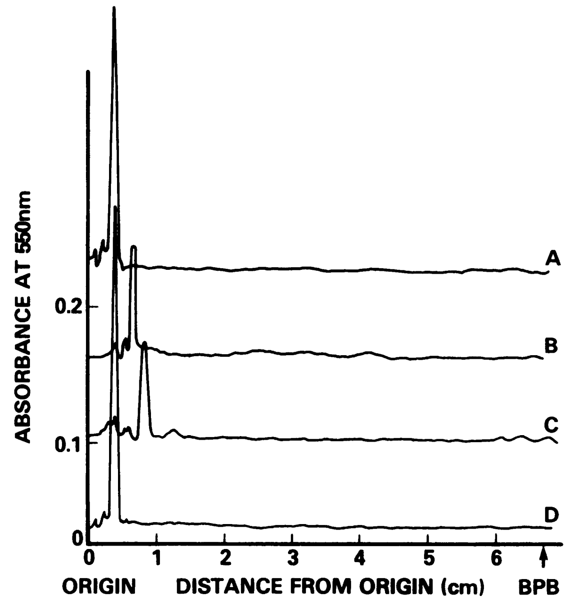

FIG. 4. Gel scan of *Chlorella* NR protein after treatment with corn- and rice-inactivating proteins and trypsin. A: NR incubated with K-phosphate (pH 7.6); B: NR incubated with 0.5 μg of trypsin; C: NR incubated with corn-inactivating protein; D: NR incubated with rice-inactivating protein. Incubation time was 5 h.

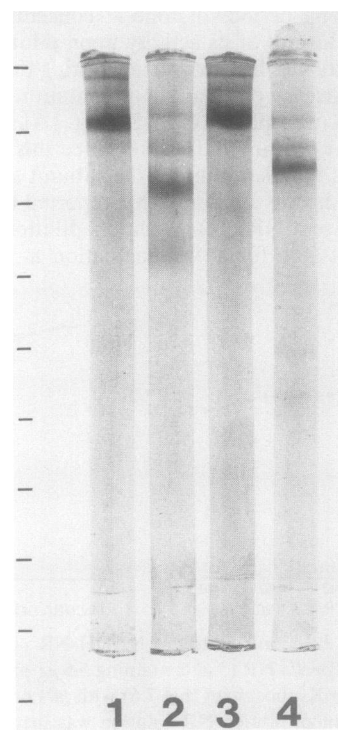

FIG. 5. Disc gel electrophoresis of *Chlorella* NR after treatment with K-phosphate (1), corn inactivator (2), rice inactivator (3), or trypsin (4). Treatment as in Figure 4.

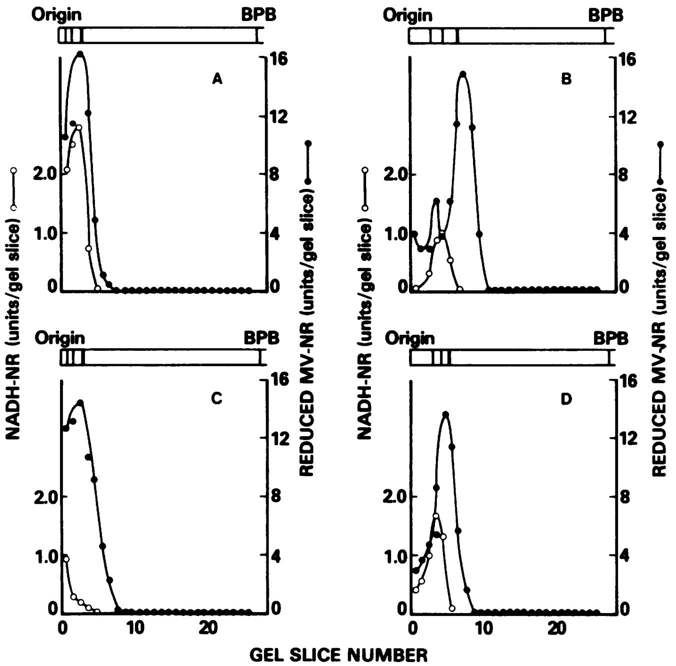

FIG. 6. Distribution of NADH-NR and reduced MV-NR activities along the polyacrylamide gels. A 50-μl sample of incubated enzyme was applied to the gel and the electrophoresis was carried out as described under "Materials and Methods." A: NR without inactivating protein; the sample was incubated for 5 h prior to application to the gel. B: NR with corn-inactivating protein; a 5-h incubation was used for the reduced MV-NR and 1 h for NADH-NR. C: NR with rice-inactivating protein; the incubation time was 5 h. D: NR with trypsin; the incubation time was 5 h for reduced MV-NR and 1 h for NADH-NR. After electrophoresis, the gels were sliced every 2.5 mm and enzyme activity was assayed in each gel slice. The assay period was 5 min for reduced MV-NR (●) and 30 min for NADH-NR (○).

Table I. *Effect of Addition of NADH on Inactivation of NADH-NR from Chlorella by Inactivating Proteins from Rice and Corn*

Chlorella NR (10 μl) was diluted to 4 ml with 0.1 M Tris-HCl buffer containing 0.2% BSA (pH 8.0). A 50-μliter aliquot of diluted NR solution was incubated with corn-inactivating protein (20 μl of Sephadex G-100 fraction containing 0.4 μg of protein) or rice protein (20 μl of Sephadex G-100 fraction containing 0.8 μg of protein) for 60 min. NADH (0.5 μmol) was added during incubation of NR with the inactivating proteins. Values in parentheses are actual units of NR left after treatment with NADH and the inactivating proteins.

Time of NADH Addition	NADH-NR Activity	Inactivating Activity	
		Rice protein	Corn protein
min	*units*	*% loss of NR*	
Control	1.15	92.1 (0.09)	81.9 (0.21)
0	0.56	4.0 (0.54)	64.3 (0.20)
30	0.63	41.3 (0.37)	73.5 (0.17)
40	0.67	59.8 (0.27)	76.4 (0.16)

additions of substrate or cofactors have been shown to protect specific proteins against inactivation (17). In a few cases there has been an active search for protein variants that are less stable either *in vivo* or *in vitro, e.g.* catalase in inbred lines of mice (7) or nitrate reductase in inbred lines of corn (33).

Filner *et al.* (6) have documented many cases where in a developmental sequence there is an increase in enzyme activity followed by a decline. The regulation of the decline in enzyme activity or content is as important to the cells' economy as is the regulation of the induction process. There are now well documented cases in the literature of a reversible inactivation of invertase (16); phenylalanine ammonia-lyase (2, 26) and NR (15, 21, 24, 37). NR from *Chlorella,* for example, is reversibly activated and inactivated *in vitro* by ferricyanide or NADH and cyanide (21, 24). In a survey employing different environmental conditions Pistorius *et al.* (15) showed that the enzyme exists *in vivo* in an active and an inactive form and that the proportion of the enzyme in the active form depended on specific cues from the environment. Recent work suggests that NR in higher plants may also be reversibly inactivated (20, 27).

NR is also one of those enzymes showing a fairly high turnover rate (6). Using density labeling Zielke and Filner (38) were able to show a half-life for the enzyme of approximately 4 h. In their system neither the presence of NO_3^- nor the age of the cell culture influenced the rate of enzyme loss. In the corn root system, the NR obtained from mature roots has a much shorter half-life than the NR obtained from 10 mm root tips (1). The reduced stability of NR in the mature root sections could result from the corn NR-inactivating protein which is also more active in the mature regions of the root (30) or on an altered primary structure or conformation of the NR protein. The *in vivo* inactivation of the enzyme (1) affects the three NR activities equally (NO_3^--induced NADH-CR, NADH-NR, and $FMNH_2$-NR) even though the inactivating protein preferentially inactivates the NADH-CR portion of the enzyme (31, 34). This observation suggests that some part of the inactivating system which is active *in vivo* has not yet been recovered for analysis *in vitro*.

When enzyme levels are determined by activity, it is difficult to distinguish between an irreversible degradation of the enzyme and a reversible inactivation. In the current experiments we have been able to use a purified NR obtained from *Chlorella* to examine the effects of macromolecular inactivators on that protein. The results show quite clearly that the inactivator obtained from corn roots acts by limited proteolysis whereas the inactivator obtained from rice cells does not. Evidence supporting the action of the corn inactivator is: (*a*) the purified corn protein characterized by gel electrophoresis hydrolyzed azocasein (34); (*b*) the major protein band of *Chlorella* NR which has reduced MV-NR activity was shifted toward the anode after incubation with the corn protein; and (*c*) NADH-NR activity which was inactivated to about 50% was detected in a different position from the reduced MV-NR activity determined after a 5-h incubation period. The fact that the rice protein had no effect on the electrophoretic mobility of the protein bands of *Chlorella* NR and the reduced MV-NR activity suggests that the rice inactivator does not act by hydrolysis. This observation supports the previous result of Yamaya and Ohira (37) which showed that rice inactivator inactivates the rice NR reversibly. We have not been able to reverse the effect of the rice inactivator on either corn leaf (34) or *Chlorella* NR (Table I).

Examples of enzyme turnover which have been examined at the molecular level suggest that a limited hydrolysis is involved initially in the degradation of a specific protein (8, 11–13, 18, 28). It was thought that specific proteases were involved in these reactions (11, 18, 28) and that specific cofactors or end products could protect the substrate protein from degradation (4, 12, 13, 18, 28). In the current study we have shown that the NR from *Chlorella* is degraded by the corn-inactivating enzyme or trypsin and more specifically that it is the NADH-CR portion of the protein that is particularly sensitive to proteolytic attack. However, according to the electrophoresis data the action of these two proteases is similar but not identical. Two NR-inactivating proteins have also been purified from *Neurospora* (25) and although the *Neurospora* NR uses NADPH rather than NADH, it is still the CR portion of the NR that is inactivated (Sorger, personal communication). Wallace (32) has examined another aspect of the specificity of the corn-inactivating protein. He has been able to demonstrate that proteases from yeast which preferentially inactivate tryptophan synthase (11) also inactivate corn root NR and that the corn inactivator, which is also fairly specific in its action (29, 31), inactivates yeast tryptophan synthase. Thus, it seems that certain proteins which have a fairly high turnover *in vivo* are selectively sensitive to proteolytic attack. Mechanistically it is easier to imagine a specific change in a particular protein which makes it accessible to degradation by a general protease rather than a specific protease for each protein and there are examples of this in the literature. Schimke *et al.* (18) were able to show, *e.g.* that tryptophan additions protected tryptophan pyrrolase from turnover *in vivo* and from tryptic digestion *in vitro*. Similarly, Howard and Solomonson (9) have shown that the inactive form of *Chlorella* NR is resistant to degradation by trypsin whereas the active form is susceptible. These results suggest that it is the conformation of the protein that confers the specificity of its inactivation rather than the action of a specific protease. A clarification of this problem will require a more detailed investigation involving: (*a*) discrete alterations in environmental conditions, and (*b*) a wider search for enzyme mutants with an altered *in vivo* stability.

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