Phytochemistry, Vol. 27, No. 7, pp. 1969–1972, 1988.
Printed in Great Britain.

0031-9422/88 $3.00 + 0.00

ARGININE RESIDUE(S) AT THE ACTIVE SITE(S) OF THE NITRATE REDUCTASE COMPLEX FROM *AMARANTHUS*

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(*Received* 27 *November* 1987)

Key Word Index—Nitrate reductase; *amaranthus*; arginine residue; NADH binding site.

Abstract—Chemical modification of nitrate reductase (NR) from *Amaranthus dubious* leaves with either phenylglyoxal or 2,3-butanedione reduces the catalytic activity of the enzyme complex. The kinetics of the reaction in the presence of either reagent indicates a rapid partial inactivation followed by a slower rate of inactivation which leads eventually to completely inactive enzyme. NADH-NR and the $FMNH_2$-NR activities of the NR complex are inactivated at a faster rate as compared to the NADH dehydrogenase activity. NADH protected the slower phase of inactivation of NADH-NR and NADH dehydrogenase but the rapid phase of inactivation could not be prevented by any of the ligands tested. The inactivation of $FMNH_2$-NR could not be prevented by any compound tested.

The data suggest that NR contains active site arginine residue(s) that are involved in NADH binding site of the enzyme. Arginine modification also affects the partial activity associated with the molybdenum containing moiety, i.e. $FMNH_2$-NR.

INTRODUCTION

Although nitrate reductase (EC 1.6.6.1) from plants has been the subject of several investigations [1], many aspects of its regulation and mechanism of catalytic function remain obscure. Identification of the ionizable groups of amino acid residues of the enzyme protein participating in the binding of substrates is essential for understanding the mechanism of catalytic function of an enzyme. In the case of nitrate reductase (NR) from higher plants such studies have not been reported. Pan and Nason [2] demonstrated the presence of essential arginine residues at the active site of NR from the fungus *Neurospora crassa.*

Nitrate reductase uses NADH as one of the substrates and this substrate possesses an anionic group. Such groups often form an anionic interaction with arginine residues at the active site of an enzyme [3, 4]. In view of this it was logical to expect the presence of functional arginine residues at the catalytic site of NR. This communication reports evidence for the presence of arginine residues at the active site that on modification with either phenylglyoxal or 2,3-butanedione (both fairly specific in reacting with arginine) cause substantial reduction of the catalytic activities of NR.

RESULTS

Inactivation studies

Nitrate reductase demonstrates a physiological activity in which NADH is oxidized and NO_3^- is reduced. In addition it also shows two partial activities: one in which NADH is oxidized to reduce *in vitro* several acceptors including DCIP is designated as NADH dehydrogenase. The other activity ($FMNH_2$-NR) observed *in vitro* is the reduction of NO_3^- using artificial donors such as $FMNH_2$. In the studies described here the effect of arginine modifiers was studied on all three activities. The modification of the enzyme with phenylglyoxal and 2,3-butanedione was carried out at pH 9.0 and 0–4°.

Treatment of the enzyme with phenylglyoxal at all of the concentrations tested showed a very rapid inactivation followed by a slower phase of inactivation of all three activities (Figs 1–3). At 20 mM phenylglyoxal, NADH-NR was quickly inactivated by 60% followed by a time dependent monophasic inactivation which led to a loss of about 80% of the activity in 1 min (Fig. 1). The $FMNH_2$-NR was quickly inactivated by about 25% at equimolar concentration of phenylglyoxal followed by a linear time dependent inactivation such that over 80% inactivation of this activity occurred in one min (Fig. 2).

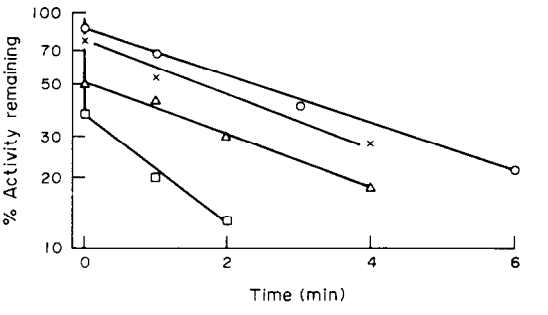

Fig. 1. Inactivation of NADH-NR by phenylglyoxal. The enzyme (10–15 μg protein) was incubated at 0–4° with 100 mM bicarbonate buffer, pH 9.0, and phenylglyoxal 2 mM (○), 5 mM (×), 10 mM (△) and 20 mM (□). Aliquots were assayed for NADH-NR activity at the indicated times.

NBRI Publication No. 337 (N.S.)
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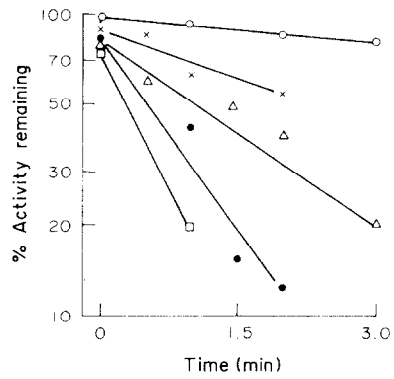

Fig. 2. Inactivation of $FMNH_2$-NR by phenylglyoxal. The enzyme (10–15 μg protein) was incubated at 0–4° with 100 mM bicarbonate buffer, pH 9.0, and phenylglyoxal 2 mM (○), 5 mM (×), 10 mM (△), 14 mM (●) and 20 mM (□). Aliquots were assayed for $FMNH_2$-NR activity at the indicated times.

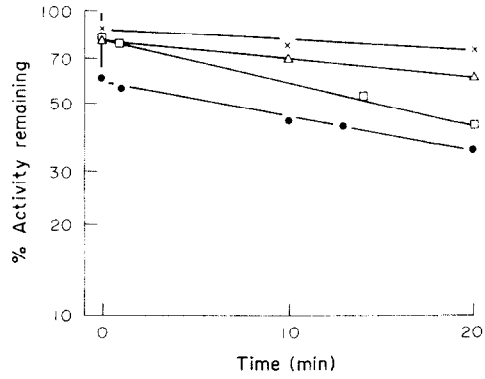

Fig. 3. Inactivation of NADH dehydrogenase by phenylglyoxal. The enzyme (10–15 μg protein) was incubated at 0–4° with 100 mM bicarbonate buffer, pH 9.0, and phenylglyoxal 5 mM (×), 10 mM (△), 20 mM (□) and 30 mM (●). Aliquots were assayed for NADH dehydrogenase activity at the indicated times.

The NADH dehydrogenase, however, was least sensitive, undergoing a quick inactivation of some 20% at 20 mM concentration followed by a very slow time dependent inactivation resulting in a loss of about 60% activity in 20 min (Fig. 3). It appears that the inhibition of NADH-NR by phenylglyoxal is primarily due to the inactivation of its $FMNH_2$-NR activity. A comparison of Figs 1 and 2 shows that at 2 mM concentration the $FMNH_2$-NR is inhibited by only 20% in 3 min whereas the NADH-NR is inactivated by over 50% during this time. For inactivation of NADH dehydrogenase by 50%, concentration of phenylglyoxal needed was 20 mM and yet it took almost 15 min. Studies using 2,3-butanedione which preferentially modifies arginine gave results which were similar to those obtained by using phenylglyoxal.

The initial quick loss of activity for individual enzyme activities was dependent on the concentrations of the modifying reagent used in both the cases.

Effect of pH on inactivation

The inactivation of the three reactions of the NR complex by 5 mM phenylglyoxal was markedly influenced by the pH at which the modification was carried out. There was a pronounced increase in the loss of the activities of NADH-NR (60–80%), NADH dehydrogenase (35–68%) and $FMNH_2$-NR (30–75%), on raising the pH from 7.5 to 9.5. A similar increase was also obtained with 2,3-butanedione. The effect of pH below pH 7.5 and above pH 9.5 was not studied due to the instability of the enzyme.

Protection by substrates against inactivation

Since the inactivation of $FMNH_2$-NR was not prevented by either NADH or NO_3^- at any of the concentrations studied (Table 1), it was decided to study the protection offered by NADH to NADH-NR, when the inactivation was brought about by 2 mM phenylglyoxal, i.e. a concentration at which $FMNH_2$-NR is inactivated by only 20% in 3 min. With 2 mM phenylglyoxal, NADH-NR activity was inhibited by about 50% in 3 min (Table 1). Preincubation with NADH provided substantial protection giving almost 80% activity. The activity (20%) which could not be protected may be associated with the loss of $FMNH_2$-NR partial activity which is inhibited by 20% in 3 min by 2 mM phenylglyoxal (see Fig. 2). Alternatively, the unprotected activity may represent the initial loss of activity (fast phase) which cannot be prevented by any of the substrates, NO_3^- gave no protection against inactivation by phenylglyoxal.

The protection by NADH suggests that arginine residues may be involved in the binding of NADH and if this is so it should also protect against the loss of NADH-dehydrogenase activity. Since NADH dehydrogenase is least susceptible to phenylglyoxal inhibition, the protection studies were carried out using 30 mM phenylglyoxal and a 10 min incubation. Under these conditions, the inactivation was almost 70 %. NADH reduces this inactivation considerably giving an activity of about 70% (Table 1).

The studies carried out with phenylglyoxal were repeated using 2,3-butanedione which also preferentially modifies arginine. Table 1 also contains information about inactivation of NADH-NR and NADH dehydrogenase using 2,3-butanedione and protection by NADH. As is evident from the Table, the data are very similar to those obtained using phenylglyoxal.

DISCUSSION

The data presented strongly suggest that nitrate reductase (NR) from amaranthus leaves possesses essential arginine residue(s) which may be involved in the catalysis of the enzyme and also in the binding of NADH to the enzyme. Arginine has often been identified as the positively charged residue providing for the binding of anions to functional sites of enzymes and other proteins [5, 6]. The present studies support such a role for the active-site arginine residue in nitrate reductase.

NR activities assayed in the presence of either phenylglyoxal or 2,3-butanedione showed an increased inactivation at higher pH. The rate changes occurred in our studies between pH 7.0 to 9.5. It is already known that

Table 1. Protective effect of substrates against inactivation of NR by phenylglyoxal

Enzyme	Inactivation reagent	Concentration (mM)	Substrate	% activity remaining
NADH-NR	Phenylglyoxal	0	None	100
		2	None	49
		2	NADH	79
		2	NO_3^-	50
	2,3-Butanedione	0	None	100
		10	None	33
		10	NADH	69
		10	NO_3^-	38
NADH-dehydrogenase	Phenylglyoxal	0	None	100
		30	None	30
		30	NADH	70
		30	NO_3^-	42
	2,3-Butanedione	0	None	100
		30	None	35
		30	NADH	65
		30	NO_3^-	37

The enzyme (10–15 μg) protein was modified with phenylglyoxal and 2,3-butanedione for 3 min in the case of NADH-NR; 20 min with phenylglyoxal and 15 min with 2,3-butanedione, in the case of NADH dehydrogenase. The enzyme was preincubated with the substrates NO_3^-/NADH for 10 min prior to the addition of the reagents. Appropriate controls were used. The reaction was assayed as described in materials and methods. $FMNH_2$-NR inactivation was not protected by any of the substrates tested (data not shown).

the pH of the inactivation medium has a dramatic effect on inactivation by both these reagents used for arginine modification [7–10].

Of the three reactions studied, NADH-NR was inactivated maximally. $FMNH_2$-NR activity was less affected whereas NADH dehydrogenase activity was least affected. This suggests that the inhibition of NADH-NR may be primarily due to inactivation of its $FMNH_2$-NR activity.

The protection experiments using NADH suggest that arginine residues may be involved in the binding of NADH. This argument is also supported by the data on protection of activity of NADH dehydrogenase. The unprotected ($\sim$ 30%) activity of this partial reaction may be due to the modification of some other arginine residues which do not participate in the binding of NADH but are still functionally important. This protection of NADH-requiring enzyme activities of NR against inactivation by NADH suggests that arginine residues may be located at the active site of the enzyme that binds NADH. The protection by NADH is afforded only against the slow secondary phase of inactivation by these reagents. The NO_3^- provides no protection against inactivation indicating that the NO_3^- binding site of the protein does not contain functional arginine residues.

Biphasic inactivation patterns have been obtained for all three activities of NR, such curves have been observed for other enzymes also [11, 12]. It is proposed that the fast initial rate of inactivation may be due to a relatively exposed location of some of the arginine residues at the periphery of the enzyme and that these residues are also important for the catalytic activity of the enzyme.

The specificity of phenylglyoxal for essential arginine residues is suggested by the observation that inactivation does not involve modification of essential -SH groups of the enzyme (β-mercaptoethanol was added to the modified enzyme which is known to reverse the modification of -SH groups). Secondly, the conditions used for modification were mild enough to prevent reaction of the reagent with other amino acids such as histidine and lysine [13]. Phenylglyoxal has already been shown to be efficient in the speed and specificity of its reaction with arginine residues as compared to other reagents.

2,3-Butanedione is also quite specific for modification of arginines in proteins. The conditions employed in our experiments were such that modification of lysine was negligible [5, 14]. Tyrosine or tryptophan residues under controlled conditions (as used by us) are not modified by this reagent [11]. Other amino acids react more slowly with this reagent in solution. However, photochemical inactivation by 2,3-butanedione is known, in which case residues other than arginine are modified [15]. This is quite unlikely in our case as the reactions were performed in the dark and for short periods only [11, 16].

The partial but definite regeneration of activity of 2,3-butanedione inactivated enzyme on the removal of reagent by dialysis with time, is an added proof for the modification of principally arginine residues under the experimental conditions [17]. We, therefore, conclude that arginine residues are present at the NADH binding site of the enzyme.

EXPERIMENTAL

The leaves from field-grown *A. dubious* were homogenized (at 0–4°) in a medium containing 50 mM K-Pi buffer, pH 8.0, 5 mM EDTA, 20 mM β-mercaptoethanol, 5 mM KNO_3 and 10% glycerol (extraction buffer). The slurry was filtered through 4 layers of muslin cloth and centrifuged at 15000 *g* for 45 min. The supernatant was passed through a column of Blue Sepharose

Table 2. Reversibility of 2,3-butanedione reaction by dialysis

Dialysis time (hr)	Reagent (mM)	% activity remaining (NADH-NR)
0	0	100
2	10	28
18	10	48
24	10	69

Enzyme containing (50 μg) protein was modified by 10 mM 2,3-butanedione, for 2 min at 4°. Dialysis was carried out at 0–4° against the medium used for extraction of enzyme.

CL-6B (1.5 × 6 cm), equilibrated with the extraction buffer. The column was washed with about 700 ml extraction buffer and eluted with 50 ml 0.3 M KNO_3 in the extraction buffer. Fractions (2 ml each) were collected and assayed for NR activity. The fractions containing high NR activity were pooled and concentrated by sucrose/glycerol.

NADH-NR was assayed in a final vol of 1 ml containing 100 mM K-Pi buffer, pH 7.5, 10 mM KNO_3 and 0.4 mM NADH and enzyme. The incubation was carried out for 30 min at 27°. The activity was estimated colorimetrically by the procedure described in ref. [18]. $FMNH_2$-NR and NADH-dehydrogenase activities were estimated by the method described in ref. [19]. The enzyme preparation had a specific activity of 2.0 μmol NO_2^- produced/mg protein/min at 27°.

Protein determinations were done as described in ref. [20]. Phenylglyoxal was purchased from Sigma and 2, 3-butanedione from Fluka. All other reagents were of highest purity available. Solns of phenylglyoxal was prepared fresh daily, 2,3-butanedione was diluted to the required concentrations with H_2O.

Inactivation experiments were carried out in small aliquots containing 10–15 μg protein in 100 mM HCO_3^- buffer pH 9.0 and the indicated amount of phenylglyoxal. When 2,3-butanedione was used, borate buffer 50 mM, pH 9.0 was taken. The incubations were performed for indicated time at 0–4° in dark to minimize light induced decomposition [11]. At designated time intervals, aliquots were withdrawn and diluted 20-fold in the assay mixture containing 5 mM β-mercaptoethanol. Control experiments have shown that in the absence of the reagent, the enzyme was stable.

REFERENCES

1. Guerrero, M. G., Vega, J. M. and Losada, M. (1981) *Annu. Rev. Plant Physiol.* **32**, 169.
2. Pan, S. and Nason, A. (1978) *Biochem. Biophys. Acta* **523**, 297.
3. Lange, L. G. III, Riordan, J. F. and Vallee, B. L. (1974) *Biochemistry* **13**, 4361.
4. Bleile, D. M., Foster, M., Brady, J. W. and Harrison, J. H. (1975) *J. Biol. Chem.* **250**, 6222.
5. Riordan, J. F. (1979) *Mol. Cell Biochem.* **26**, 71.
6. Bjerrum, P. J., Wieth, J. O. and Borders, C. L. Jr. (1983) *J. Gen. Physiol.* **81**, 453.
7. Kantrowitz, E. R. and Lipscomb, W. N. (1976) *J. Biol. Chem.* **251**, 2688.
8. DiPietro, A. and Goffeau, A. (1985) *Eur. J. Biochem.* **148**, 35.
9. Kasher, J. S., Allen, K. E., Kasamo, K. and Slyam, C. W. (1986) *J. Biol. Chem.* **261**, 10808.
10. Fürst, P. and Solioz, M. (1987) *Biochem. Biophys. Res. Commun.* **142**, 107.
11. Zakim, D., Hochman, Y. and Kennoy, W. C. (1982) *J. Biol. Chem.* **258**, 6430.
12. Dailey, H. A. and Fleming, J. E. (1986) *J. Biol. Chem.* **261**, 7902.
13. Takahashi, K. (1968). *J. Biol. Chem.* **243**, 6171.
14. Yankeelov, J. A. (1970) *Biochemistry* **9**, 2433.
15. Gripon, J. C. and Hofmann, T. (1981) *Biochem. J.* **193**, 55.
16. Ryang, H. S. and Wang, S. Y. (1978) *J. Am. Chem. Soc.* **100**, 1302.
17. Mukherji, S. and Bhaduri, A. (1985) *J. Biol. Chem.* **264**, 4519.
18. Hageman, R. H. and Reed, A. J. (1980) *Methods Enzymol.* **69**, 270.
19. Jawali, N., Sainis, J. K. and Sane, P. V. (1978) *Phytochemistry* **17**, 1527.
20. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265.