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EFFECTS OF DENATURING AGENTS ON SPINACH NITRATE REDUCTASE

FRANCISCO F. DE LA ROSA, FRANCISCO CASTILLO and ENRIQUE PALACIÁN
Departamento de Bioquímica, Facultad de Ciencias Y C.S.I.C., Universidad de Sevilla, Spain

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; nitrate reductase; inactivation; FAD.

Abstract—The effects of various denaturing agents (temperature, pH, urea, guanidine hydrochloride and sodium dodecyl sulphate) on the 3 enzymic activities of the spinach NADH-nitrate reductase complex (NADH-nitrate reductase, NADH-diaphorase and FNH_2-nitrate reductase) have been studied. FAD showed a protecting effect against inactivation by most of these agents, the protection of NADH-diaphorase activity being especially outstanding. In the presence of FAD (20 μM), incubation with 4M urea or M guanidine hydrochloride caused inactivation of NADH-nitrate reductase and FNH_2-nitrate reductase, while NADH-diaphorase was only slightly affected By contrast, incubation with 0.03% sodium dodecyl sulphate or heating at 45° in the absence of FAD produced the preferential inactivation of the NADH-diaphorase moiety. These results indicate a difference in the structural requirements of the two functional moieties, NADH-diaphorase and FNH_2-nitrate reductase. The NADH-induced inactivation of NADH-nitrate reductase was dependent on pH: the inactivation proceeding faster in the alkaline region. A new purification procedure for spinach NADH-nitrate reductase is also described that gives a preparation with a specific activity of 45 nkat/mg.

INTRODUCTION

The enzymic complex NADH-nitrate reductase (EC 1.6.6.1) from spinach leaves catalyzes the reduction of nitrate to nitrite using NADH as the electron donor [1,2]. Two functional moieties participate sequentially in the transfer of electrons from NADH to nitrate; they can be assayed independently with artificial substrates [1,2]. NADH-diaphorase, the first functional moiety of the complex, reduces various electron acceptors (cytochrome *c*, dichlorophenolindophenol and ferricyanide) using NADH as the electron donor. FNH_2-nitrate reductase, the second functional moiety, catalyzes the reduction of nitrate using various electron donors ($FMNH_2$, $FADH_2$, reduced methyl viologen and benzyl viologen). The two functional moieties are affected independently by different treatments and inhibitors: NADH-diaphorase is inactivated by heating at 45° in the absence of FAD and by certain sulphydryl-group reagents, like *p*-hydroxymercuribenzoate (*p*HMB) [1,3,4]; while FNH_2-nitrate reductase is inactivated by cyanide, azide, NADH, NADPH and simple thiols [1–3, 5]. NADH or NADPH and cyanide cooperate synergistically in the inactivation of FNH_2-nitrate reductase [3].

The inactivation by NAD(P)H has been found with nitrate reductases from different sources and may be of physiological significance [6]. Simple thiols inactivate nitrate reductase from *Chlorella fusca* and spinach leaves in a way similar to NAD(P)H, indicating that the inactivation might be due to reduction of disulfide bridges [5,7]. The spinach nitrate reductase inactivated by NAD(P)H or thiols can be reactivated by ferricyanide [5]. In addition to ferricyanide, other oxidizing agents reactivate nitrate reductases from different sources previously inactivated by NAD(P)H [8,9].

Recently, it has been found that NAD(P)H-inactivated nitrate reductase from spinach leaves can be reactivated by FAD, FMN and riboflavin [10], and by illumination with blue light [11]. Light might cause a photooxidation of the enzyme mediated by flavins that may or may not be present as prosthetic groups.

The capacity of the spinach enzyme for NADH-induced inactivation seems to be independent of its NADH-diaphorase activity, since a *p*HMB-treated nitrate reductase that lacks detectable diaphorase activity can be inactivated by NADH as well as the untreated one [4]. In addition, the differences observed between the FAD-requirements of NADH-diaphorase activity and NADH-induced inactivation in *p*HMB-treated preparations agree with the suggested independence of the two processes [12].

The present paper reports the effects of different denaturing agents on the 3 enzymic activities of the spinach nitrate reductase complex, in the presence and absence of FAD. It also describes a simple purification procedure for spinach nitrate reductase that gives a preparation with a higher sp. act. than previously reported [13].

RESULTS

Partial purification of spinach nitrate reductase

Nitrate reductase was obtained from spinach leaves (*Spinacia oleracea* L.). The same day in which the plants were harvested, the leaves were washed with tap water and either used in the purification of nitrate reductase or kept at −20° until use. At −20° the spinach leaves maintain the enzymic activities of the nitrate reductase complex without appreciable losses for 3 or 4 months.

All the operations of the following purification procedure which are summarized in Table 1, were conducted between 0 and 5°.

(*i*) *Preparation of the cell-free extract.* One Kg of

Table 1. Purification of spinach nitrate reductase

Fraction	1 Protein (mg)	2 Total activity (nkat)	3 Specific activity (nkat/mg)	4 NADH-NO_3Rase / FNH_2-NO_3Rase
I. Homogenate	20000	2170	0.11	1.2
II. $(NH_4)_2SO_4$ precipitate (25–45%)	11000	1500	0.14	0.80
III. Calcium phosphate gel eluate	166	833	5.02	0.80
IV. $(NH_4)_2SO_4$ precipitate (30–40%)	22	367	16.7	0.73
V. Agarose column eluate	1.5	67	44.7	0.73

spinach leaves were homogenized with 1 l. of 10 mM potassium phosphate (pH 7.5), 1 mM EDTA, 20 μM FAD and 10 mM KNO_3. The homogenate was filtered through three sheets of cheesecloth, centrifuged at 27000 g for 20 min, and the supernatant collected (Fraction I).

(ii) Ammonium sulphate precipitation (25–45%). To the supernatant obtained in step (i), solid $(NH_4)_2SO_4$ was added to a final concentration of 25% saturation, with the pH maintained at 7.5 by addition of 2 M NH_4OH. After stirring for 15 min, the preparation was centrifuged at 27000 g for 10 min. To the supernatant thus obtained, solid $(NH_4)_2SO_4$ was added to a final concentration of 45% saturation. After stirring for 15 min, the precipitate was collected by centrifugation at 27000 g for 10 min, and dissolved in 300 ml of 10 mM Tris–HCl (pH 7.5) (Fraction II).

(iii) Calcium phosphate gel treatment. To the solution obtained in step (ii), calcium phosphate gel (10 mg/ml prepared as described by Wood [16] was added to a final concentration of 1 mg of gel per 25 mg of protein. After 20 min, the suspension was centrifuged at 5000 g for 5 min. The sediment was suspended in 20 ml of 50 mM potassium phosphate (pH 7.5), and centrifuged at 5000 g for 5 min. The supernatant was discarded and the sediment suspended in 40 ml of 0.2 M potassium phosphate (pH 7.5) containing 1 mM EDTA, 20 μM FAD and 10 mM KNO_3. This suspension was centrifuged at 27000 g for 10 min and the supernatant collected (Fraction III).

(iv) Ammonium sulphate precipitation (30–40%). Saturated $(NH_4)_2SO_4$ was added to Fraction III to a final concentration of 30% saturation, maintaining the pH at 7.5 by addition of 2 M NH_4OH. After 20 min, the suspension was centrifuged at 27000 g for 10 min. To the supernatant thus obtained, saturated $(NH_4)_2SO_4$ was added to a final concentration of 40% saturation. After 20 min, the suspension was centrifuged at 27000 g for 10 min and the precipitate dissolved in 5 ml of 0.2M potassium phosphate (pH 7.5) containing 1 mM EDTA, 20 μM FAD and 10 mM KNO_3 (Fraction IV).

(v) Agarose chromatography. Fraction IV was passed through an agarose column (Bio gel A–1.5 m., 100–200 mesh; 2.5 cm i. d. and 60 cm high) equilibrated with the last mentioned solution. Fractions of 5 ml each were collected and those of higher sp. act. were pooled to constitute Fraction V.

Effects of different denaturing agents

To study the effects of denaturing agents on nitrate reductase, Fraction III (Table 1) was used. This preparation can be stored at 0–5° without substantial loss of the nitrate reductase enzymic activities for at least a week. Immediately before use, the purified preparation was equilibrated with the required solution by passage through Sephadex G-25.

Temperature. The effect of temperature on the functional stability of nitrate reductase, in the absence of FAD, is shown in Fig. 1A. Incubation at 25° for 20 min did not affect any of the activities of the complex. Higher temperatures produced inactivation. NADH-diaphorase and NADH-nitrate reductase were more susceptible than

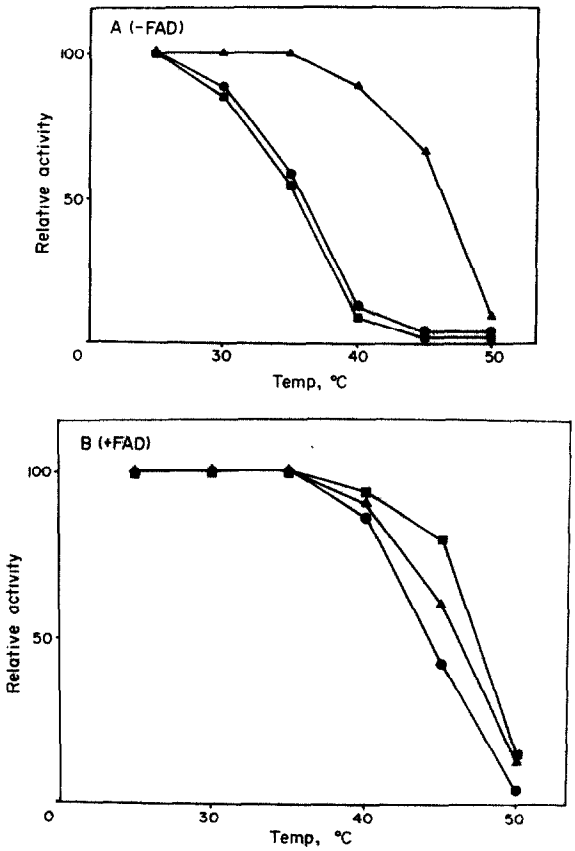

Fig. 1. Stability of nitrate reductase at different temperatures in the presence and absence of FAD. Nitrate reductase (3 mg/ml) was incubated in the absence (A) and the presence (B) of 20 μM FAD, with 0.2 M KPi (pH 7.5), 10 mM KNO_3 and 1 mM EDTA, at the indicated temperatures. After 20 min of incubation, NADH-nitrate reductase (●), NADH-diaphorase (■) and FNH_2-nitrate reductase (▲) activities were determined in aliquots of the incubation mixtures. Activities are expressed as percentages of the corresponding activity at time zero of incubation.

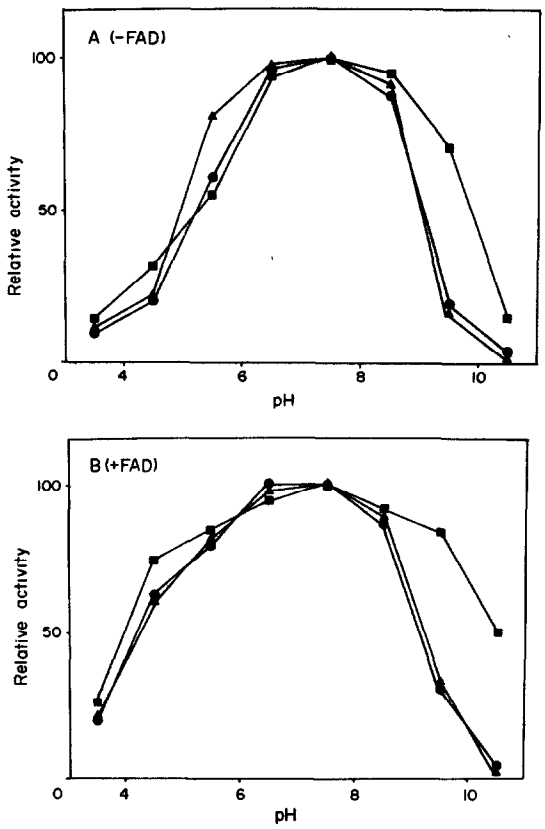

Fig. 2. Effect of pH on the stability of nitrate reductase in the presence and absence of FAD. Nitrate reductase (2 mg/ml) was incubated at 25° in the absence (A) and the presence (B) of 20 μM FAD, with 1 mM EDTA and each of the following buffers: 0.05 M citrate–Pi (pH 3.5, 4.5, 5.5 and 6.5); 0.05 M KPi (pH 7.5); 0.2 M Tris–HCl (pH 8.5); and 0.2 M carbonate–bicarbonate (pH 9.5 and 10.5). After 1 hr of incubation, NADH-nitrate reductase (●), NADH-diaphorase (■) and FNH_2-nitrate reductase (▲) activities were determined in aliquots of the incubation mixtures. Activities are expressed as percentages of the corresponding activity of the incubation mixture at pH 7.5 and time zero of incubation.

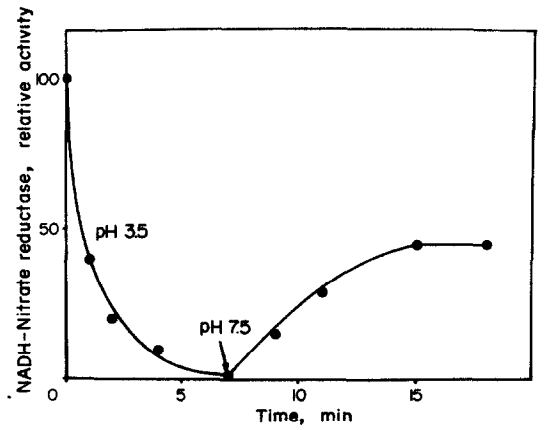

Fig. 3. Reversible inactivation of NADH-nitrate reductase at pH 3.5. Nitrate reductase (3 mg/ml) was incubated at 25° with 0.025M citrate–Pi buffer (pH 3.5), 20 μM FAD, 10 mM KNO_3 and 1 mM EDTA. At the time indicated in the figure, the incubation mixture was diluted with its volume of 0.5 M KPi (pH 7.5), 20 μM FAD, 10 mM KNO_3 and 1 mM EDTA. NADH-nitrate reductase was determined in aliquots of the incubation mixtures at the times indicated.

FNH_2-nitrate reductase, being almost completely inactivated at 40°, while FNH_2-nitrate reductase was 90% inactivated only at 50°. In the presence of FAD (Fig. 1B) the stability of NADH-diaphorase and NADH-nitrate reductase increased and the 3 activities were inactivated with temperature in a similar way.

pH. Fig. 2A shows the enzymic activities of the nitrate reductase complex after incubation for 1 hr under different conditions of pH, in the absence of FAD. The 3 activities were fairly stable between pH 6.5 and pH 8.5. Outside this interval of pH the activities decreases in a parallel way, except for NADH-diaphorase which was a little more stable in the alkaline region. The presence of FAD in the incubation mixtures caused some protection of all 3 activities at acid pH and of NADH-diaphorase at alkaline pH (Fig. 2B).

The inactivation of NADH-nitrate reductase at acid pH was partially reversible upon neutralization. Fig. 3 shows the inactivation of NADH-nitrate reductase at pH 3.5 and the reactivation produced after readjustment of pH to 7.5.

The NADH-induced inactivation of NADH-nitrate reductase was affected by pH, as shown in Fig. 4. At pH 5.5 no inactivation was detected in 1 hr, whereas at pH 8.5 practically complete inactivation was obtained in 30 min.

Urea. The effects of incubation with urea, in the presence of FAD, on the 3 activities of the nitrate reductase complex are shown in Table 2. Incubation with 4M urea produced inactivation of FNH_2-nitrate reductase and NADH-nitrate reductase, but only a slight effect on the NADH-diaphorase activity. In the absence of FAD, all 3 activities were equally inactivated by urea. Incubation of the nitrate reductase complex with 3M urea produced a pseudo-first order inactivation of NADH-diaphorase, with a decrease to 50% of the original activity in 7.5 min.

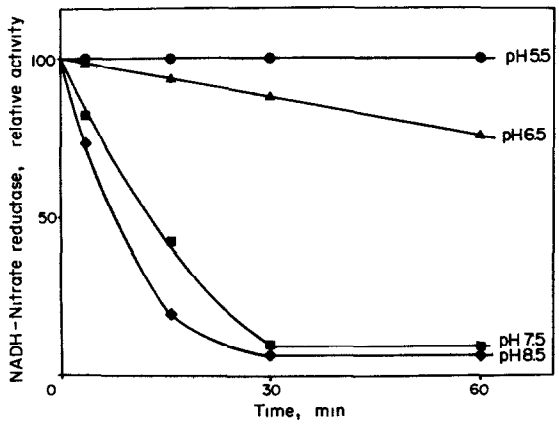

Fig. 4. Effect of pH on the NADH-induced inactivation of nitrate reductase. Nitrate reductase (1.5 mg/ml) was incubated at 25° in the presence of 20 μM FAD, 0.5 mM NADH and the buffers described in the legend of Fig. 2 which correspond to the values of pH indicated in the present figure. NADH-nitrate reductase activity was determined in aliquots of the incubation mixtures at the times indicated, and is expressed as percentage of the corresponding control without NADH.

Table 2. Effects of urea on the activities of the nitrate reductase complex

Urea (M)	Relative activities NADH-nitrate reductase	FNH_2-nitrate reductase	NADH diaphorase
0	100	100	100
2	70	70	95
4	10	15	90
6	<5	<5	10

Table 4. Effects of sodium dodecyl sulphate on the activities of the nitrate reductase complex

SDS (%)	Relative activities NADH-nitrate reductase	FNH_2-nitrate reductase	NADH-diaphorase
0	100	100	100
0.02	75	90	75
0.03	<5	60	5
0.04	<5	10	<2

When 20 μM FAD was also included no appreciable inactivation was observed in the same period of time. The urea-inactivated enzyme was partially reactivated during activity assay, as shown in Fig. 5. This reactivation was very likely due to the decrease in urea concentration (60 times) which accompanied the preparation of the activity assay mixtures.

Guanidine hydrochloride. The effects of incubation with guanidine hydrochloride on the enzymic activities of the nitrate reductase complex were similar to those produced by incubation with urea. Table 3 shows that, in the presence of FAD, the NADH-diaphorase activity was practically unaffected by incubation with M guanidine hydrochloride, while FNH_2-nitrate reductase and NADH-nitrate reductase were 80% inactivated. In the absence of FAD all 3 activities were inactivated to a similar extent. As in the case of urea, incubation of nitrate reductase with M guanidine hydrochloride produced a pseudo-first order inactivation of NADH-diaphorase, with a decrease to 50% of the original activity in 6.7 min. FAD (20 μM) gave almost complete protection against this inactivation.

Sodium dodecyl sulphate. The effects of incubation with SDS on the enzymic activities of the nitrate reductase complex are quite different from those produced by urea and guanidine hydrochloride. In this case the inactivation preferentially affects the NADH-diaphorase moiety of the complex. Table 4 shows that incubation with 0.03% SDS produced almost complete inactivation of NADH-diaphorase and NADH-nitrate reductase, while FNH_2-nitrate reductase retained 60% of the original activity. FAD had no protecting effect on NADH-diaphorase.

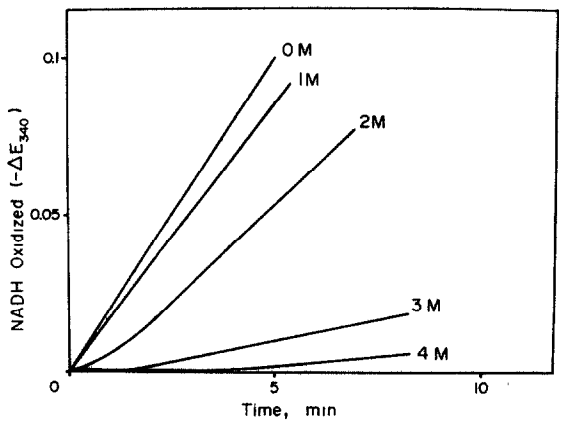

Fig. 5. Reactivation of urea-inactivated nitrate reductase. Nitrate reductase (10 mg/ml) was incubated at 4° in the presence of 0.2 M KPi (pH 7.5), 10 mM KNO_3, 1 mM EDTA, 20 μM FAD and urea at the concentrations indicated. After 35 min of incubation NADH-nitrate reductase activity was followed spectrophotometrically in aliquots of the incubation mixtures, which were diluted ×60 to prepare the assay mixtures.

DISCUSSION

Nitrate reductase from spinach leaves, like those from other sources, appears to contain FAD as a prosthetic group which is required for activity [1]. Passage of a spinach preparation through an agarose column produces a considerable loss of NADH-diaphorase activity which can be partially recovered by incubation with FAD [3]. FAD was also shown to protect NADH-diaphorase against inactivation by heating at 45° for 5 min and by incubation with *p*HMB [3]. The NADH-induced inactivation of native spinach nitrate reductase does not require the addition of FAD. However, *p*HMB-treated preparations require the addition of FAD to obtain inactivation by NADH [12]. This FAD-requirement of NADH-induced inactivation is different from the FAD-requirement for NADH-diaphorase activity, since the NADH-diaphorase activity of *p*HMB-treated preparations is not affected by added FAD [12]. Conversely, the nitrate reductase preparation passed through an agarose column, whose NADH-diaphorase activity is restored by incubation with FAD, does not require added FAD to be inactivated by NADH [12]. These results might indicate the existence of at least two different binding sites for

Table 3. Effects of guanidine hydrochloride on the activities of the nitrate reductase complex

Guanidine hydrochloride (M)	Relative activities NADH-nitrate reductase	FNH_2-nitrate reductase	NADH-diaphorase
0	100	100	100
0.5	90	85	100
1	20	20	95

FAD in the nitrate reductase complex. In addition, the protecting effect of FAD against inactivation by different denaturing agents reported in this paper shows the important role of FAD in the stabilization of the nitrate reductase structure, especially that required for the NADH-diaphorase function.

The NADH-induced inactivation of spinach nitrate reductase has a pH-dependence similar to that reported for the nitrate reductase of *Chlorella* [9,17], showing higher rates of inactivation at alkaline pH values. This dependence on pH suggests that ionization of a chemical group of the enzyme may be the rate-limiting event in the inactivation process.

The two functional moieties of the spinach nitrate reductase complex are not affected to the same extent by the denaturing agents used in the present study. In the presence of FAD, inactivation with 4M urea or M guanidine hydrochloride causes the inactivation of the FNH_2-nitrate reductase moiety, while NADH-diaphorase activity is only slightly affected. By contrast, incubation with 0.03 % SDS or heating at 45° for 5 min in the absence of FAD produces the preferential inactivation of the NADH-diaphorase moiety. These results indicate differences in the structural requirement of the two partial activities NADH-diaphorase and FNH_2-nitrate reductase. We have not been able to reconstruct the NADH-nitrate reductase activity by combining under mild denaturing conditions one nitrate reductase preparation lacking NADH-diaphorase activity with another lacking FNH_2-nitrate reductase activity, subsequently eliminating the denaturing agent (unpublished work). However, this kind of hybridization is still possible, since the conditions required for dissociation and reconstitution of the complex may be very specific and quite different from those we have employed.

EXPERIMENTAL

The enzymic activities were determined as described in ref. [5]: NADH-nitrate reductase and FNH_2-nitrate reductase by measuring nitrite formed and NADH-diaphorase by following spectrophotometrically the reduction of cytochrome *c*. NADH-nitrate reductase was also measured by following at 340 nm the oxidation of NADH (see Fig. 5).

Protein concentration. Estimated by the method of ref. [14]. In those preps containing NH_3 the spectrophotometric method of ref. [15] was used.

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