REACTIVATION BY FLAVIN NUCLEOTIDES OF THE NAD(P)H-INACTIVATED SPINACH NITRATE REDUCTASE

Francisco F. de la ROSA, Francisco CASTILLO, José M. MÉNDEZ and Enrique PALACIÁN
Departamento de Bioquímica, Facultad de Ciencias y C.S.I.C., Universidad de Sevilla, Spain

Received 26 February 1976

1. Introduction

The enzyme complex NADH-nitrate reductase (EC 1.6.6.1) from spinach leaves is inactivated by reduction with NADH, NADPH or thiols, and reactivated by oxidation with ferricyanide [1]. This interconversion between an oxidized active form of the enzyme and a reduced inactive one, observed in assimilatory nitrate reductases from different sources, seems to be of physiological significance [2,3]. Of the two functional moieties of the spinach complex, only the FNH_2-nitrate reductase, activity, which can use $FADH_2$ and $FMNH_2$ as electron donors, is affected by the inactivation [1]. Although NADH-diaphorase activity, the other functional moiety of the complex, seemed to be required for NAD(P)H-inactivation [1], subsequent studies showed that preparations lacking detectable NADH-diaphorase activity can be inactivated by NADH [4]. These results agree with the postulated existence of a regulatory site for pyridine nucleotides different from the NADH-diaphorase catalytic site.

Dichlorophenolindophenol, ammonium persulfate and cytochrome *c*, in addition to ferricyanide, have been shown to reactivate nitrate reductases from different sources which had been previously inactivated by reduced pyridine nucleotides with or without cyanide [5,6]. Of all these activating compounds, ferricyanide turned out to be the most effective.

Although some reports on the reactivating effects of nitrate, NAD^+ and $NADP^+$ have been presented [7–9], little is known about the physiological mechanism of reactivation. The present paper reports the reactivating effects of riboflavin, FMN and FAD on NADH-nitrate reductase from spinach leaves inactivated by NAD(P)H, and studies the possible involvement in reactivation of some compounds of physiological interest.

2. Materials and methods

Nitrate reductase from spinach leaves (*Spinacea oleracea* L.) was partially purified and stored as previously described [4]. Immediately before use, the purified preparation was equilibrated with 0.2 M potassium phosphate (pH 7.5) by passage through a Sephadex G-25 column. Enzyme activity units are defined as micromoles of NADH utilized or nitrite formed per min at 30°C. The NADH-nitrate reductase specific activity of the purified preparation was about 300 milliunits/mg, with ratios of NADH-diaphorase and FNH_2-nitrate reductase to NADH-nitrate reductase activity equal to 5 and 1.25, respectively.

The enzymatic activities were determined as previously described [1]: NADH-nitrate reductase and FNH_2-nitrate reductase by measuring nitrite formed, and NADH-diaphorase by following spectrophotometrically the reduction of cytochrome *c*. Protein concentration was estimated by the method of Lowry et al. [10].

3. Results and discussion

Spinach nitrate reductase inactivated by NADH can be reactivated by FMN and by FAD. Fig.1 shows the effects of these flavin nucleotides on the three enzymatic activities of a nitrate reductase preparation previously inactivated by NADH. NADH-nitrate

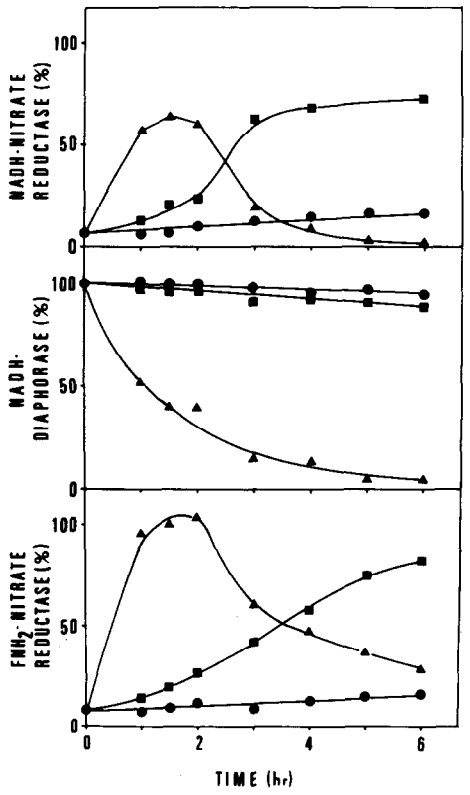

Fig.1. Effects of FMN and FAD on NADH-inactivated nitrate reductase. To obtain the NADH-inactivated preparation, nitrate reductase (5 mg/ml) was incubated at room temperature with 0.2 M potassium phosphate (pH 7.5) and 1 mM NADH. After incubation for 1 h, the preparation was passed through a Sephadex G-25 column to eliminate NADH. The NADH-inactivated nitrate reductase (2.5 mg/ml) was incubated at room temperature: in the presence of 0.1 mM FMN (▲); in the presence of 0.1 mM FAD (■); and in their absence (●). At the times of incubation indicated in the figure, NADH-nitrate reductase, NADH-diaphorase and FNH_2-nitrate reductase activities were determined in aliquots of the incubation mixtures. Enzymatic activities are expressed as percentages of the corresponding specific activity of the original preparation before NADH-inactivation.

reductase and FNH_2-nitrate reductase were reactivated by incubation in the presence of FMN or FAD. FMN produced an initial reactivation which was in both cases followed by inactivation. In the presence of FAD, reactivation took place at a slower rate than with FMN, and no inactivation phase was observed during the 6 h of incubation. NADH-diaphorase, which is not affected by the NADH-treatment, was almost completely inactivated in the presence of FMN. However, with FAD or in the absence of flavin nucleotides, incubation was accompanied by only slight inactivation. The inactivating effect of FMN on the NADH-diaphorase moiety of the complex explains the lower relative values of NADH-nitrate reductase activity as compared with those of FNH_2-nitrate reductase. The inactivating phase of FMN on FNH_2-nitrate reductase might be the result of the structural changes brought about in the complex by the inactivation of NADH-diaphorase. On the contrary, FAD, which has no effect on NADH-diaphorase activity, does not produce any inactivation of FNH_2-nitrate reductase. This result is not surprising, since FAD but not FMN has been shown to protect the NADH-diaphorase activity against different inactivating agents [11].

Of all the compounds tested in the experiment described in table 1, only riboflavin, FMN and FAD produced reactivation of FNH_2-nitrate reductase. Neither NAD^+ nor $NADP^+$ showed any reactivating effect, in contrast with nitrate reductase from *Nitrobacter agilis* [9]. Table 1 also shows a decrease of activity in the presence of riboflavin and FMN after the initial reactivation. Riboflavin produced effects on the three enzymatic activities of the complex entirely similar to those of FMN shown in fig.1.

Table 1
Effects of different compounds on the FNH_2-nitrate reductase activity of an NADH-inactivated nitrate reductase

Addition	FNH_2-nitrate reductase activity (%) Incubation for 2 h	Incubation for 4 h
None	11	18
0.1 mM Riboflavin	62	39
0.1 mM FMN	60	38
0.1 mM FAD	22	92
0.3 mM NAD^+	12	20
0.3 mM $NADP^+$	15	24
0.3 mM AMP	13	19
0.3 mM ADP	14	20
0.3 mM ATP	10	17

The NADH-inactivated nitrate reductase, obtained as described in the legend of fig.1, was incubated at room temperature with the compounds indicated in the table. After 2 and 4 h of incubation, FNH_2-nitrate reductase activity was determined in aliquots of the incubation mixtures. Activities are expressed as percentages of the specific activity of the original preparation before NADH-inactivation.

Table 2
Reactivation by FAD of preparations of nitrate reductase inactivated in different ways

Preparation inactivated with	FNH_2-nitrate reductase activity (%) Incubation without FAD	Incubation with FAD
(a) NADH	15	75
(b) NADPH	18	81
(c) NADH plus cyanide	17	70

The inactivated preparations were obtained by incubation of nitrate reductase (5 mg/ml) at room temperature, in the presence of 0.2 M potassium phosphate (pH 7.5), with: 1 mM NADH (a) or 1 mM NADPH (b), for 90 min; or 0.1 mM NADH plus 5 μM KCN, for 30 min. After incubation, the different preparations were passed through Sephadex G-25 columns to get rid of the pyridine nucleotides and free cyanide. The preparations obtained in this way presented 5% or less of the specific FNH_2-nitrate reductase activity of the original untreated preparation. The three inactivated preparations (2.5 mg/ml) were incubated at room temperature in the presence and absence of 0.1 mM FAD. After incubation for 5 h, FNH_2-nitrate reductase activity was determined in aliquots of the incubation mixtures. Enzymatic activities are expressed as percentages of the specific activity of the original untreated preparation.

The physiological compounds nitrate (10 mM) and NAD^+ (0.3 mM), previously reported as reactivating agents of nitrate reductases from other sources [7–9], did not cooperate with FAD (0.1 mM), either individually or in pairs, to bring about reactivation.

Table 2 shows that the reactivating effect of FAD takes place not only on preparations inactivated by NADH, but also on those inactivated by either NADPH or low concentration of NADH in the presence of cyanide.

The reactivation obtained with riboflavin, FMN and FAD might be due to either direct oxidation of the enzyme by these compounds, or to induction of an enzyme structure susceptible to oxidation. Since the effect is not specific of FAD, the first possibility seems more likely. The higher effectiveness of riboflavin and FMN in producing rapid initial reactivation might be a consequence of the smaller size of these compounds as compared with FAD.

Acknowledgments

We acknowledge the helpful advice and encouragement of Professor M. Losada and the useful criticism of Professor A. Paneque. This work was aided by a grant to Professor Losada from the National Science Foundation, GF-44115 (USA).

References

[1] Palacián, E., de la Rosa, F. F., Castillo, F. and Gómez-Moreno, C. (1974) Arch. Biochem. Biophys. 161, 441–447.
[2] Hewitt, E. J. (1975) Ann. Rev. Plant Physiol. 26, 73–100.
[3] Losada, M. (1974) Third International Symposium on Metabolic Interconversion of Enzymes, Seattle, pp. 257–270, Springer-Verlag, Berlin.
[4] Castillo, F., de la Rosa, F. F. and Palacián, E. (1975) Biochem. Biophys. Res. Commun. 64, 546–552.
[5] Garrett, R. H. and Greenbaum, P. (1973) Biochim. Biophys. Acta 302, 24–32.
[6] Solomonson, L. P., Jetschmann, K. and Vennesland, B. (1973) Biochim. Biophys. Acta 309, 32–43.
[7] Solomonson, L. P. and Vennesland, B. (1972) Biochim. Biophys. Acta, 267, 544–557.
[8] Herrera, J., Paneque, A., Maldonado, J. M., Barea, J. L. and Losada, M. (1972) Biochem. Biophys. Res. Commun. 48, 996–1003.
[9] Herrera, J. and Nicholas, D. J. D. (1974) Biochim. Biophys. Acta 368, 54–60.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Relimpio, A. M., Aparicio, P. J., Paneque, A. and Losada, M. (1971) FEBS Lett. 17, 226–230.