REVISTA ESPAÑOLA DE FISIOLOGIA, 33, 287-290. 1977

Nitrate Reductase From *Spinacea oleracea*. FAD and the Reactivation of the Enzyme Treated with *p*-Hydroxymercuribenzoate *

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(Received on January 27, 1977)

F. CASTILLO, F. F. DE LA ROSA and E. PALACIAN. *Nitrate Reductase From* Spinacea oleracea. *FAD and the Reactivation of the Enzyme Treated with p-hydroxymercuribenzoate.* Rev. esp. Fisiol., 33, 287-290. 1977.

Spinach nitrate reductase complex previously inactivated by treatment with mercurials p-hydroxymercuribenzoate or p-hydroxymercuriphenyl suplhonate can be reactivated by incubation with dithioerythritol.

The reactivation of NADH-diaphorase seems to be FAD-dependent, whereas that of FNH_2-nitrate reductase is not.

The requirement of FAD for NADH-inactivation of nitrate reductase treated with p-hydroxymercuribenzoate disappears after treatment with dithioerythritol.

The spinach nitrate reductase complex (E.C. 1.6.6.1) has two functional moieties that can be assayed separately (5, 8, 10): NADH-diaphorase and FNH_2-nitrate reductase **. When the enzyme lacks either of them, no NADH-nitrate reductase activity is detected. The two partial activities are affected independently by certain treatments and inhibitors (5, 9, 10). NADH-diaphorase is easily inactivated by sulphydryl-group reagents, while FNH_2-nitrate reductase is much less susceptible to inactivation by these compounds (3, 10). FNH_2-nitrate reductase can be inactivated by reduction with NADH, NADPH or thiols and reactivated by oxidation with differents compounds (1, 4, 5, 9). This interconversion between an oxidized active form of the enzyme and a reduced inactive one seems to be of regulatory significance (5, 6).

Spinach nitrate reductase appears to

* This work was aided by a Grant to Professor M. Losada from the National Science Foundation, GF-44115 (U.S.A.) and Philips Research Laboratories (The Netherlands).

** *Abbreviations:* DTE, 2.3-dihydroxy-1, 4-dithiolbutane (dithioerythritol); FNH_2, reduced FAD or FMN; *p*-HMB, p-hydroxymercuribenzoate; *p*-HMBS, p-hydroxymercuriphenylsulphonate.

contain FAD as a prosthetic group required for activity (5, 10). The present paper reports a stimulating effect of FAD on the reactivation by DTE of *p*-HMB-treated nitrate reductase. After DTE-treatment of the *p*-HMB-modified enzyme the requirement of FAD for inactivation of nitrate reductase by NADH was eliminated.

Materials and Methods

Nitrate reductase from spinach leaves (*Spinacea oleracea* L.) was partially purified and stored as previously described (3). Immediately before use, the purified preparation was equilibrated with 0.1 M potassium phosphate (pH 7.5) and 1 mM EDTA by passage through a Sephadex G-25 column. All the incubations took place at 30° C.

The enzymatic activities were determined as previously described (9): NADH-nitrate reductase and FNH_2nitrate reductase by measuring nitrite formed, and NADH-diaphorase by following spectrophotometrically the reduction of cytochrome *c*. The NADH-nitrate reductase, FNH_2-nitrate reductase and NADH-diaphorase specific activities of the purified preparation were about 400, 450 and 900 mU/mg, respectively. Protein concentration was estimated by the method of LOWRY *et al.* (7).

Results and Discussion

Spinach nitrate reductase inactivated by the sulphydryl-group reagents *p*-HMB or *p*-HMBS can be reactivated by incubation with thiols. Table I shows the inactivation of the enzymatic activities of nitrate reductase by *p*-HMB and *p*HMBS, and the reactivation of all three activities by incubation with DTE. To determine NADH-diaphorase activity in preparations treated with DTE, this thiol was eliminated from the preparation, since it interferes with the activity assay by reducing the artificial substrate cytochrome *c* chemically.

When free FAD was absent during the DTE-treatment, the extent of reactivation of NADH-diaphorase was notably lower (table II). If tightly bound FAD required for NADH-diaphorase activity is lost upon incubation with *p*HBM, free FAD will be needed during the DTE treatment to reform the active enzyme. After incubation with DTE, addition of FAD had

Table I. *Inactivation of nitrate reductase by pHMB and pHMBS and reactivation by DTE.* Nitrate reductase (2 mg/ml) was incubated for 10 min with 0.1 M potassium phosphate (pH 7.5), 20 μM FAD, 1 mM EDTA and the additions indicated in the table. Enzymatic activities were determined in aliquots of the incubation mixtures.

Addition (mM)	Relative specific activities					
	NADH-diaphorase		FNH_2-nitrate reductase		NADH-nitrate reductase	
		Treated with DTE *		Treated with DTE *		Treated with DTE *
None	100	120	100	95	100	92
*p*HMB (0.2)	<1	100	67	90	<5	60
*p*HMBS (0.5)	<1	100	40	85	<5	40

* After treatment with the sulphydryl-group reagents, the enzymatic preparations were incubated for 5 min with 1 mM DTE, and passed though Sephadex G-25 columns equilibrated with 0.1 M potassium phosphate (pH 7.5), 20 μM FAD and 1 mM EDTA to eliminate the added reagents. The enzymatic activities were determined in aliquots of the incubation mixtures after elution from the Sephadex columns.

Table II. *Effect of FAD on the DTE-reactivation of NADH-diaphorase previously inactivated by pHMB.*

Nitrate reductase (5 mg/ml) was incubated for 10 min in the presence of 0.1 M potassium phosphate (pH 7.5), 1 mM EDTA and *p*HMB (30 nmoles/mg protein). After this treatment, the enzyme was incubated with 1 mM DTE in the presence and absence of 0.2 mM FAD for 15 min, and the preparations were passed through Sephadex G-25 columns equilibrated with 0.1 M potassium phosphate (pH 7.5) and 1 mM EDTA. NADH-diaphorase activity was determined in aliquots of the incubation mixtures before DTE-addition and after elution from the Sephadex columns. The activities are expressed as percentages of the activity of a control preparation that was not incubated with *p*HMB.

Nitrate reductase preparation	NADH-diaphorase *
Before DTE-treatment	< 1
After DTE-treatment	
In the absence of FAD	30
In the presence of FAD	70

* Relative specific activity (%).

Table III. *Inactivation by NADH after DTE-treatment.*

Nitrate reductase (5 mg/ml) was incubated in the presence of 0.1 M potassium phosphate (pH 7.5), 1 mM EDTA and *p*HMB (30 nmoles/mg protein). After this treatment, the enzyme was incubated for 5 min with 1 mM DTE in the presence and absence of 0.2 mM FAD, and the preparations were passed through Sephadex G-25 columns equilibrated with 0.1 M potassium phosphate (pH 7.5) and 1 mM EDTA. The eluted enzymes were incubated for 1 h in the presence of the compounds indicated in the table at the following concentrations: NADH, 0.5 mM and FAD, 20 μM. FNH_2-nitrate reductase activity was determined in aliquots of the incubation mixtures.

DTE-treatment	Addition	FNH_2-nitrate reductase *
In the absence of FAD	None	100
	NADH	30
	NADH, FAD	25
In the presence of FAD	None	100
	NADH	25
	NADH, FAD	23

* Relative specific activity (%).

no effect on NADH-diaphorase activity, probably because its binding site is no longer accessible. No effect of FAD was observed on the FNH_2-nitrate reductase reactivation by DTE.

It has been reported that treatment of spinach nitrate reductase with low concentration of *p*HMB (25-50 nmoles/mg protein) produced complete inactivation of NADH-diaphorase but no decrease in FNH_2-nitrate reductase activity (3). This preparation, in contrast with the native enzyme, presented a FAD-requirement for inactivation by NADH of FNH_2-nitrate reductase (2). After DTE-treatment, either in the presence of absence of FAD, of nitrate reductase modified by *p*HMB, no effect of FAD on the NADH-induced inactivation was observed (table III). This result indicates that the requirement of FAD for NADH-induced inactivation is a property of the *p*HMB-modified enzyme.

Acknowledgments

We acknowledge the helpful advice and encouragement of Professor M. Losada and the useful criticism of Professor A. Paneque.

Resumen

La nitrato reductasa de espinaca inactivada por los mercuriales *p*-hidroximercuribenzoato y *p*-hidroximercurifenil sulfonato se reactiva mediante un tratamiento con ditioeritritol. La reactivación de la NADH-diaforasa parece ser dependiente de FAD, mientras que la reactivación de la FMH_2-nitrato reductasa no lo es.

El requerimiento de FAD para la inactivación por NADH de la nitrato reductasa previamente tratada con *p*-hidroximercuribenzoato desaparece después del tratamiento con ditioeritritol.

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