SHORT COMMUNICATION

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Enzymatic reduction of nitrate with flavin nucleotides reduced by a new chloroplast NADH-specific diaphorase

In previous papers we have reported the reduction of NO_3^- to NO_2^- in spinach leaves, catalyzed by the enzyme nitrate reductase (NAD(P)H:nitrate oxidoreductase, EC 1.6.6.2). The reduction of NO_3^- with NADPH (ref. 1) required FMN and two different proteins, NADPH diaphorase[2-4] (NADPH:ferredoxin oxidoreductase, EC 1.6.99.4) and nitrate reductase[5]. When NADH was used as electron donor[6], the reduction of NO_3^- did not, however, require the addition of any external cofactor or enzyme, for even the most purified nitrate reductase preparation contained a NADH-specific diaphorase which could couple directly with nitrate reductase itself in the transfer of electrons from NADH to NO_3^-.

More recently[7] it was further found that when the nitrate reductase preparation was heated at 45° for 5 min, or when certain specific inhibitors were added to the reaction mixture, the activity of NADH diaphorase disappeared and, in consequence, NADH could no longer act as electron donor for the reduction of NO_3^-. These treatments did not, however, affect the activity of nitrate reductase itself, as assayed with chemically reduced flavin nucleotide as reductant[5].

We report now that chloroplast extract contains a new NADH-specific diaphorase which can reduce either FMN or FAD, and that this system can be coupled to the one involved in the reduction of NO_3^- to NO_2^-, using nitrate reductase lacking the activity of the previously described NADH diaphorase.

The new NADH-specific diaphorase was purified from spinach chloroplast extract, prepared according to WHATLEY, ALLEN AND ARNON[8], through a procedure which included: (1) adsorption on alumina C_γ and elution with 0.085 M sodium phosphate (pH 7), and (2) adsorption on a DEAE-cellulose bed and washing with 0.05 M sodium phosphate (pH 7.0) before finally eluting NADH diaphorase with 0.17 M sodium phosphate (pH 7.0).

Table I shows the specificity of the diaphorase for NADH as measured spectrophotometrically by the oxidation of reduced nicotinamide nucleotides with FMN. In the absence of FMN (or FAD) no change in absorbance occurred. The reaction did not take place either when the enzyme was heated for 5 min at 45° or when 0.1 mM *p*-chloromercuribenzoate was added to the reaction mixture. By contrast with the previously described NADH-specific diaphorase present in the highly purified nitrate reductase preparation, which cannot use flavin nucleotides as electron acceptors, the new enzyme was unable to reduce cytochrome *c* directly, although the reaction could take place in the presence of added flavin nucleotides. On the other hand, the specificity of this enzyme for NADH clearly distinguishes it from those of AVRON AND JAGENDORF[2,3] and LAZZARINI AND SAN PIETRO[9]. Ferricyanide, but neither

menadione nor spinach ferredoxin[10], was also found to be an effective electron acceptor for the new NADH diaphorase.

TABLE I

NADH-SPECIFIC DIAPHORASE PURIFIED FROM CHLOROPLAST EXTRACT

The complete reaction mixture included, in a final volume of 3 ml, 0.5 mg NADH diaphorase, 0.2 μmole FMN, and 200 μmoles sodium phosphate (pH 7.0). Where indicated, 0.3 μmole *p*-chloromercuribenzoate was added. At zero time, 0.3 μmole of either NADH or NADPH was added and their oxidations followed spectrophotometrically at 340 mμ under air.

System	*Electron donor*	
	NADH	*NADPH*
	(Change in absorbance per min)	
Complete	0.060	0.002
Minus FMN	0.001	—
Complete, enzyme heated	0.003	—
Complete *plus* *p*-chloromercuribenzoate	0.003	—

Table II shows the reduction of nitrate by nitrate reductase with FMN reduced by the NADH–NADH diaphorase system. In the absence of the electron carrier or of any of the enzymes, no reduction of NO_3^- to NO_2^- occurred.

TABLE II

REDUCTION OF NITRATE WITH NADH THROUGH THE COMBINED ACTION OF NADH DIAPHORASE, FMN, AND NITRATE REDUCTASE

The complete reaction included, in a final volume of 3 ml, 1 mg NADH diaphorase, 1 mg heated (45°, 5 min) nitrate reductase[5], 200 μmoles sodium phosphate (pH 7.0), 0.2 μmole FMN, 0.6 μmole NADH, and 10 μmoles KNO_3. The reactions were carried out at 22° for 20 min in Warburg manometer flasks under argon. At the end of the experiment, NO_2^- was estimated by the method of SNELL AND SNELL[11] after eliminating NADH by precipitating with barium acetate and ethanol[12].

System	NO_2^- *formed* *(mμmoles)*
Complete	225
Minus NADH diaphorase	18
Minus FMN	3
Minus nitrate reductase	6

The reduction of NO_3^- to NO_2^- with NADH, under the specified conditions, is thus a reaction which proceeds in two independent enzymatic steps, each catalyzed by a different protein: (1) reduction of FMN by the NADH–NADH diaphorase system, and (2) oxidation of FMNH by the nitrate–nitrate reductase system[1]. It is worth noting, however, that, according to present evidence from our laboratory[7], spinach nitrate reductase is not a flavoprotein.

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