Biochimica et Biophysica Acta, 657 (1981) 1—12

BBA 69164

IN VITRO COMPLEMENTATION OF ASSIMILATORY NAD(P)H-NITRATE REDUCTASE FROM MUTANTS OF *CHLAMYDOMONAS REINHARDII*

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(Received July 24th, 1980)

Key words: Complementation; Nitrate reductase; Molybdenum-containing cofactor; (Chlamydomonas reinhardii)

Summary

In vitro complementation of the soluble assimilatory NAD(P)H-nitrate reductase (NAD(P)H:nitrate oxidoreductase, EC 1.6.6.2) was attained by mixing cell-free preparations of *Chlamydomonas reinhardii* mutant 104, uniquely possessing nitrate-inducible NAD(P)H-cytochrome *c* reductase, and mutant 305 which possesses solely the nitrate-inducible $FMNH_2$- and reduced benzyl viologen-nitrate reductase activities.

Full activity and integrity of NAD(P)H-cytochrome *c* reductase from mutant 104 and reduced benzyl viologen-nitrate reductase from mutant 305 are needed for the complementation to take place.

A constitutive and heat-labile molybdenum-containing cofactor, that reconstitutes the NAD(P)H-nitrate reductase activity of *nit*-1 *Neurospora crassa* but is incapable of complementing with 104 from *C. reinhardii*, is present in the wild type and 305 algal strains.

The complemented NAD(P)H-nitrate reductase has been purified 100-fold and was found to be similar to the wild enzyme in sucrose density sedimentation, molecular size, pH optimum, kinetic parameters, substrate affinity and sensitivity to inhibitors and temperature.

From previous data and data presented in this article on 104 and 305 mutant activities, it is concluded that *C. reinhardii* NAD(P)H-nitrate reductase is a heteromultimeric complex consisting of, at least, two types of subunits separately responsible for the NAD(P)H-cytochrome *c* reductase and the reduced benzyl viologen-nitrate reductase activities.

Introduction

Nitrate reductase (NAD(P)H:nitrate oxidoreductase, EC 1.6.6.2), the key enzyme in biological nitrate assimilation, is an enzyme complex extensively studied in microorganisms and higher plants. It is subjected to regulation and has several catalytic activities, including NAD(P)H-, $FMNH_2$- and reduced viologens-nitrate reductase, and NAD(P)H-cytochrome *c* reductase [1,2].

Nitrate reductase-deficient mutants from fungi have greatly contributed to our present knowledge of the structure and mechanism of the nitrate reductase complex [2,3]. In *Neurospora crassa*, NADPH-nitrate reductase is an aggregate of two nitrate-inducible 4.5 S polypeptide subunits bearing NADPH-cytochrome *c* reductase activity, coded by the *nit*-3 gene, and a labile and dialyzable molybdenum-containing cofactor, coded by the *nit*-1 gene, required for both assembly and function of the 7.9 S nitrate reductase complex and shared with other well-known molybdo-enzymes [4,5]. In *Aspergillus nidulans*, the 7.6 S NADPH-nitrate reductase molecule is made up of four polypeptide subunits of the *nia* D gene product, together with a small molecular weight cofactor specified by, at least, two *cnx* genes [3].

Very few biochemically-defined mutants without nitrate reductase have been selected in higher plants and green algae. Nitrate-reductase-deficient mutants have been isolated from barley [6], *Arabidopsis thaliana* [7] and *Nicotiana tabacum* [8]. Reconstitution in vitro of NADH-nitrate reductase from two mutant cell lines (*nia* and *cnx*) in the latter, strongly supports a structure for the nitrate reductase complex very similar to the fungal model: an aggregate composed of several protomers with the NADH-cytochrome *c* reductase activity, coded by the *nia* gene, and a molybdenum-containing cofactor formed by the action of *cnx* genes product(s) [9].

In green algae, nitrate reductase-deficient mutants have also been isolated [10—13]. Mutants from *Chlamydomonas reinhardii*, resembling in enzymic properties the *nit*-3 mutant of *N. crassa* (305), the *nit*-1 of *N. crassa* and *cnx* of *A. nidulans* (104), have been biochemically characterized [13—16]. In the present paper, we report the in vitro complementation attained with these mutants from *C. reinhardii*, the 104 mutant uniquely having nitrate-inducible cytochrome *c* reductase activity, and the 305 mutant possessing only nitrate-inducible reduced benzyl viologen-nitrate reductase activity. This complementation occurs in a different way from that described for other mutant organisms [9,17—19]. The results are interpreted in terms of a new structural model for the NAD(P)H-nitrate reductase complex of green algae and higher plants, which deviates from the fungal model.

Experimental procedure

Materials. NADH, NADPH and horse heart cytochrome *c* were obtained from Boehringer. Ovalbumin, bovine serum albumin, bovine catalase (EC 1.11.1.6), horse ferritin, bovine fibrinogen, Blue Dextran, and benzyl viologen were from Serva. Yeast alcohol dehydrogenase (EC 1.1.1.1), Blue Sepharose CL-6B, FMN and FAD were purchased from Sigma. Bio-Gel A-1.5 m was ob-

tained from Bio-Rad Laboratories, and Sephadex G-25 and G-75 from Pharmacia.

Culture methods. C. reinhardii 6145c, a generous gift from Dr. R. Sager (Hunter College, New York) and mutant strains obtained from this wild type, were grown in liquid media containing 10 mM NH_4Cl, under conditions previously reported [20]. When required, the cells were derepressed in media with 4 mM KNO_3 during 5.5 h, harvested, frozen in liquid nitrogen and stored until use.

N. crassa nit-1 mutant, obtained from the Fungal Genetics Stock Center (Arcata, CA), was grown in liquid media containing 7.5 mM NH_4Cl, harvested at mid-exponential phase of growth and derepressed in media with 20 mM KNO_3 during 3 h, under conditions previously described [21].

Preparation of extracts. C. reinhardii cell pellets, suspended in 0.1 M potassium phosphate buffer (pH 7.0)/0.1 mM dithioerythritol/0.1 mM EDTA/20 μM FAD (7.5 ml/g wet weight) (buffer 1), were broken with a Branson sonicator at 90 W for 15 s. The resulting homogenate was centrifuged at 30 000 $\times g$ for 15 min, and the supernatant was used as source of enzyme.

Crude extracts of *nit*-1 *N. crassa* were prepared by breaking the mycelia in a cold mortar with alumina (3 g of alumina/g wet weight), and then homogenizing in 2 vol. cold buffer 1. The homogenate was centrifuged at 30 000 $\times g$ for 15 min and the supernatant was used in the enzyme assays.

Assays. NADPH-nitrate reductase (EC 1.6.6.2), NADPH-cytochrome *c* reductase, $FMNH_2$-nitrate reductase and reduced benzyl viologen-nitrate reductase activities were determined according to earlier described methods [22].

Alcohol dehydrogenase activity was estimated following the increase in absorbance at 340 nm of the NADH formed by ethanol oxidation [23]. Catalase activity was measured following the absorbance decrease at 240 nm resulting from H_2O_2 decomposition [24]. Fibrinogen, ferritin, bovine serum albumin and ovalbumin were estimated measuring the absorbance at 280 nm. Spectrophotometric and kinetic data were obtained with a Pye-Unicam SP-8-100 recording spectrophotometer.

Activity units are expressed as μmol of substrate transformed per min, and specific activity as units/mg protein.

Analytical methods. Protein was estimated according to the method of Lowry et al. [25] as modified by Bailey [26], using bovine serum albumin as standard.

Nitrite was determined by the diazo-coupling colorimetric assay of Snell and Snell [27].

Molybdenum and tungsten content was measured as described by Cárdenas and Mortenson [28].

Stokes radii determination. Stokes radii of enzymes were determined with a Bio-Gel A-1.5 m (100—200 mesh) column (2.5 $\times$ 46 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 7.5)/0.1 M NaCl/0.1 mM dithioerythritol/0.1 mM EDTA/20 μM FAD. 0.5-ml samples containing 6% sucrose were layered on the top of the gel bed, and eluted at a flow rate of 26 ml/h for the elution volume, V_e, determination. Enzymes and markers were run separately or in combination (1.2-ml fractions). The void volume, V_0, was obtained from the elution volume of Blue Dextran 2000. The total volume, V_t, was

determined by calibration of the empty column with water. Stokes radii used in calculations were those given by Siegel and Monty [29]. Results were plotted as $(-\log K_{av})^{1/2}$ vs. Stokes radius (α), where $K_{av} = V_e - V_0/V_t - V_0$ [30].

Complementation procedure and purification of the complemented enzyme. Equal volumes of crude extracts of mutants 104 and 305 of *C. reinhardii* were mixed and incubated at 20°C for 1 h to achieve complementation. The bulk of reconstituted nitrate reductase was subsequently purified by precipitation with $(NH_4)_2SO_4$ between 25 and 45% saturation. The enzyme precipitate was collected by centrifugation at 20 000 × *g* for 20 min, dissolved in 0.08 M potassium phosphate buffer (pH 7.3)/0.1 mM dithioerythritol/0.1 mM EDTA/20 μM FAD, desalted by passage through a Sephadex G-25 column, and applied to a Blue Sepharose CL-6B bed (1.8 × 5 cm) equilibrated with the same buffer. The affinity chromatography of the enzyme was performed according to Solomonson [31].

Results

In vitro complementation between mutant strains of C. reinhardii

Although NADH and NADPH were equally effective as electron donors in all reactions related with nitrate assimilation of wild and mutant strains of *C. reinhardii* [15,32], here are presented only the results obtained with NADPH.

Table I shows that crude extracts of mutants 305 and 104 of *C. reinhardii* prepared by combined homogenization are capable of complementing in an in vitro system, to yield active NADPH-nitrate reductase. The same results were obtained when crude extracts from 305 and 104 were prepared separately, mixed and their activity measured after 30 min incubation. In all instances, the mutants must be induced in media-containing nitrate where none of them showed NADPH-nitrate reductase activity. NADPH-nitrate reductase was also

TABLE I

IN VITRO FORMATION OF NADPH-NITRATE REDUCTASE ACTIVITY BY COMBINED HOMOGENIZATION OF WILD AND MUTANT STRAINS OF *CHLAMYDOMONAS REINHARDII*

Cells grown on NH_4^+ media were harvested at mix-exponential phase of growth and then transferred into media with NH_4^+ (A) or NO_3^- (N) for 5.5 h. Where indicated, equivalent weights of cells were homogenized and enzymatic activities determined in the cell-free extracts. 100% activity were 6, 125 and 7 mU/mg protein, for NADPH-nitrate reductase, NADPH-cytochrome *c* reductase and benzyl viologen-nitrate reductase, respectively. The figures of cohomogenized mixtures are corrected for the effect of protein dilution.

Strains	Enzyme activity		
	NADPH-nitrate reductase	NADPH-cytochrome *c* reductase	Benzyl viologen-nitrate reductase
6145c (N)	100	100	100
6145c (A)	0	54	0
305 (N)	0	87	176
104 (N)	0	510	0
6145c (A) + 104 (N)	0	338	0
6145c (A) + 305 (N)	0	80	138
305 (N) + 104 (N)	32	373	78

TABLE II

EFFECT OF DIALYSIS ON THE ACTIVITIES OF COMPLEMENTED NITRATE REDUCTASE FROM *CHLAMYDOMONAS REINHARDII*

Complementation was achieved by mixing 0.25 ml of each cell-free extract from 104 and 305, and subsequent incubation of the mixtures at 0°C for 45 min. The complemented enzyme was filtered through a Sephadex G-25 column (1.6 × 6 cm) equilibrated with the elution buffer but without FAD. Enzymatic activities were determined in dialyzed and undialyzed mixtures.

Treatment	Enzyme activity (mU/mg)			
	NADPH-nitrate reductase	NADPH-cytochrome *c* reductase	$FMNH_2$-nitrate reductase	Benzyl viologen-nitrate reductase
None	1.3	67	1.9	2.9
Sephadex G-25	1.2	52	2.1	2.6

undetectable in extracts of uninduced 6145*c* cells. Other mutants from *C. reinhardii* affected in their terminal nitrate reductase, 301 and 102 [13], behaved similarly to 104 in their ability to complement with induced 305. Constitutive NADPH-cytochrome *c* reductases unrelated to NADPH-nitrate reductase, appear in cell-free extracts of wild and mutant strains of *C. reinhardii* grown on ammonia. These diaphorase activities are not nitrate-inducible and separated from the nitrate-induced NADPH-cytochrome *c* reductase of the 104 mutant when subjected to purification.

Characterization of the complementation process

When the buffer used in extractions lacked FAD, no complementation between 305 and 104 was observed. In these conditions, the nitrate-inducible NADPH-cytochrome *c* reductase activity of 104 was very low and did not

TABLE III

EFFECT OF MOLYBDENUM AND TUNGSTEN ADDED TO THE INDUCTION MEDIA ON THE COMPLEMENTED NADPH-NITRATE REDUCTASE ACTIVITY OF *CHLAMYDOMONAS REINHARDII*

Cells grown with NH_4^+ were induced on media with nitrate containing molybdate or tungstate 100 μM, during 5.5 h, then harvested and cell-free extracts prepared separately. Where indicated equal volumes of both 104 and 305 extracts were incubated together at 20°C for 1 h, and then enzymatic and analytical determinations made. N.D. = not determined.

Strain(s)	Metal in the induction medium	Enzyme activity (mU/mg)			Metal contents of crude homogenate (ng/mg protein)	
		NADPH-nitrate reductase	NADPH-cytochrome *c* reductase	Benzyl viologen-nitrate reductase	Mo	W
6145*c*	Mo	11	142	15	14	0
104	Mo	0	127	0	14	0
305	Mo	0	69	60	24	0
305 + 104	Mo	4	186	36	N.D.	N.D.
6145*c*	W	5	194	3	4	26
104	W	0	128	0	4	85
305	W	0	70	4	5	152
305 + 104	W	0	104	2	N.D.	N.D.

increase when exogenous FAD was added. To rule out a side flow of electrons from NADPH to nitrate through FAD, which is an electron acceptor in the 104 diaphorase reaction and, in its reduced form, an electron donor for the 305 terminal nitrate reductase [15,16], the complementation mixture was filtered through a Sephadex G-25 column, equilibrated with buffer 1 without FAD. Table II shows that, once complementation was achieved, the removal of FAD from the mixture had no effect on the reconstituted NADPH-nitrate reductase.

Optimal conditions for full activity of 305 terminal nitrate reductase were also required for complementation to take place. When tungsten substituted for molybdenum in the induction media, all the nitrate reductase activities of 6145*c* and mutant 305 were markedly reduced, whereas NADPH-cytochrome *c* reductase was practically unaffected (Table III). Induction of 305, in 4 mM KNO_3 on the above conditions, did not render a measurable level of NADPH-nitrate reductase with 104. Addition of sodium molybdate up to 10^{-2} M to this complementation mixture had no effect. The same inefficiency to restore the tungsten-inhibited nitrate reductase of 6145*c* and 305 was observed when molybdenum was added to their corresponding homogenates. Tungsten diminished the concentration of molybdenum in the crude homogenates and accumulates preferentially in those of 305 cells.

The time course of complementation at different temperatures between extracts from 104 and 305 is presented in Fig. 1. As observed after 75 min at 20°C the complementation reached a high level. The maximum was attained sooner at higher temperatures although in these conditions a faster thermal degradation of enzymes started taking place.

Occurrence of a cofactor in C. reinhardii able to complement with nit-1 of N. crassa

6145*c* and 305 cells grown with ammonia have a constitutive cofactor that can reconstitute the NADPH-nitrate reductase activity of *N. crassa nit*-1. Both

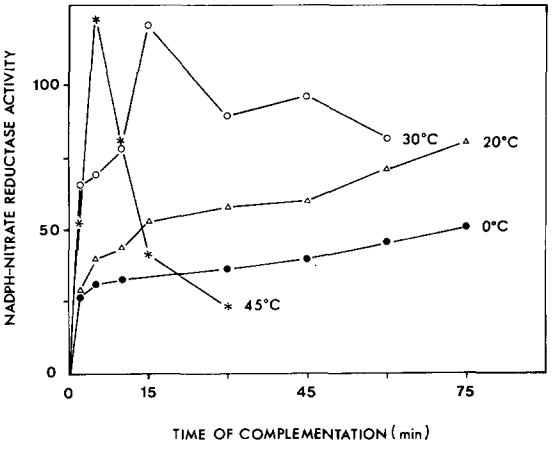

Fig. 1. Time course of complementation at different temperatures. Equal volumes of crude extracts of 305 and 104 were mixed together and incubated. At the indicated times, 0.2 ml of the mixture was removed and assayed for the NADPH-nitrate reductase activity. Activity is expressed as nmol of nitrite formed per ml and per h.

TABLE IV

IN VITRO COMPLEMENTATION OF *NEUROSPORA CRASSA NIT*-1 MUTANT WITH WILD AND MUTANT STRAINS OF *CHLAMYDOMONAS REINHARDII*

nit-1 mycelia, grown on ammonia, were induced with nitrate for 3 h, and *C. reinhardii* cells, grown on ammonia, were induced with ammonia or nitrate, then equal volumes of *nit*-1 and *C. reinhardii* extracts were mixed and incubated at 20°C for 2 h and subsequently, enzymatic activities were determined. All the algal and fungal mutants induced either with nitrate or with ammonia, as well as *C. reinhardii* 6145*c* induced with ammonia, lacked NADPH-nitrate reductase activity. NADPH-nitrate reductase of *C. reinhardii* 6145*c* induced with nitrate was 626 nmol NO_2^-/h per mg protein. Other experimental conditions are as in the caption of Table I.

Complementing strain	Induction medium	NADPH-nitrate reductase (nmol NO_2^-/h per mg)
104	NH_4^+	0
305	NH_4^+	132
6145*c*	NH_4^+	36
104	NO_3^-	0
305	NO_3^-	330
6145*c*	NO_3^-	938

6145*c* and 305 extracts showed an enhanced capability of complementation when induced with nitrate. Contrarily, 104 extracts from either induced or uninduced cells were incapable of restoring the nitrate reductase activity with *nit*-1 (Table IV). Extracts from 305, grown with ammonia and 2 mM Na_2WO_4, did not complement with *nit*-1 extracts. Negligible complementation was also found between *nit*-1 and nitrate and tungstate-induced 305, although normal complementation levels were attained when 10^{-2} M Na_2MoO_4 was present in the complementation mixtures. When nitrate-induced 305 extracts were filtered through a Sephadex G-75 column, two complementing fractions were obtained. The void volume fraction, containing molecules heavier than 70 000, complemented solely with induced 104 extracts, whereas the total volume fraction, corresponding to molecules lighter than 3000, was only able to complement with induced *nit*-1 extracts (results not shown).

Characteristics of N. crassa nit-1 and C. reinhardii complementation systems

The ability exhibited by extracts of 305 cells, either induced or uninduced, to complement with *nit*-1 of *N. crassa* was lost when 305 extracts were subjected previously to heat treatment at 45°C for 5 min. The same treatment, however, had no effect on the complementation between 305 and 104 both induced with nitrate (Table V). When the mixture of induced 305 and 104 was kept at 45°C for 5 min, a better complementation was achieved than that observed after 2 h at 20°C. The same treatment, when applied to induced *nit*-1- and 305 mixtures, prevented nitrate reductase complementation (Table V). In addition, frozen extracts of nitrate-induced 305, kept at —20°C and thawed after 10 h, were not able to reconstitute the NADPH-nitrate reductase with induced *nit*-1, but complemented exceedingly well with extracts from 104 whether or not they were previously frozen (results not shown). Extracts from 305, grown on ammonia and induced with nitrate in the presence of cycloheximide, were still capable of complementing with extracts of induced *nit*-1,

TABLE V

EFFECT OF THERMIC TREATMENT ON THE IN VITRO COMPLEMENTATION OF *NEUROSPORA CRASSA NIT*-1 AND MUTANT STRAINS OF *CHLAMYDOMONAS REINHARDII*

Crude extracts of mutants induced with ammonia (A) or nitrate (N) were obtained separately and then subjected to the indicated treatments. No treatment: 0.2 ml of each crude extract were mixed as indicated, the mixtures incubated at 20°C for 2 h, and their enzymatic activities were determined. Treatment I: 0.2 ml of 305 (A) or 305 (N) crude extracts was heated at 45°C during 5 min, then mixed with 0.2 ml of the indicated extracts, the mixtures were incubated at 20°C for 2 h, and their enzymatic activities determined. Treatment II: 0.2 ml of each crude extract was mixed as indicated, the mixtures incubated at 45°C for 5 min, and then their enzymatic activities determined. Other conditions were as stated in the caption of Table I.

Complementation mixtures	NADPH-nitrate reductase activity (nmol NO_2^-/h per mg)		
	No treatment	Treatment I	Treatment II
305 (A) + *nit*-1 (N)	65	0	3
305 (N) + *nit*-1 (N)	166	0	8
305 (N) + *nit*-1 (A)	0	0	0
305 (A) + 104 (N)	0	0	0
305 (N) + 104 (N)	100	79	118
305 (N) + 104 (A)	0	0	0

although the activity observed was much lower (56 nmol NO_2^-/h per mg) than that observed in the absence of the protein synthesis inhibitor (167 nmol NO_2^-/h per mg). Proteins from both 104 and 305 must be de novo synthesized for the complementation to take place (127 nmol NO_2^-/h per mg), since the presence of cycloheximide in the induction media of either 104 or 305 completely impeded the complementation.

Purification and characterization of complemented NADPH-nitrate reductase of C. reinhardii

The complemented nitrate reductase of *C. reinhardii* was subjected to a purification procedure shown in Table VI. A purification factor of 100 was achieved.

The purified enzyme filtered through a Bio-Gel A-1.5 m column calibrated with standards, was eluted in the same volume as NADPH-nitrate reductase of 6145c cells, corresponding to a Stokes radius of 6.9 nm (Fig. 2). An equivalent

TABLE VI

PARTIAL PURIFICATION OF COMPLEMENTED NADPH-NITRATE REDUCTASE FROM MUTANTS OF *CHLAMYDOMONAS REINHARDII*

Step	Volume (ml)	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)
1. Crude extract	60	180	180	1	100
2. $(NH_4)_2SO_4$ saturation between 25—45%	6	50	486	10	270
3. Affinity chromatography	2	3.4	338	99	187

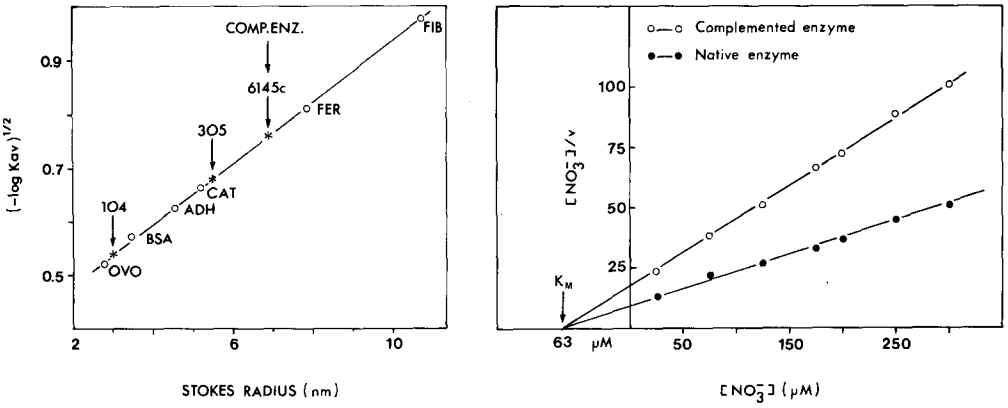

Fig. 2. Determination of Stokes radius of nitrate reductase associated activities of *Chlamydomonas reinhardii* wild and mutant strains. Standards of known Stokes radii were: 2.75 nm, hen ovalbumin (OVO); 3.5 nm, bovine serum albumin (BSA); 4.6 nm, yeast alcohol dehydrogenase (ADH); 5.2 nm, bovine catalase (CAT); 7.8 nm, horse ferritin (FER) and 10.7 nm, bovine fibrinogen (FIB). The calculated Stokes radii for 104 and 305 nitrate induced activities were 3.0 and 5.6 nm, respectively.

Fig. 3. Woolf's plot of nitrate concentration effect on NADPH-nitrate reductases from *C. reinhardi* native and complemented. The assays were carried out with 0.1—0.2 mg of purified proteins (3rd step of purification) and the indicated concentrations of nitrate. Velocity is expressed in arbitrary units.

sedimentation coefficient value of 8.8 S, was also found. The optimum pH for the in vitro assay of both complemented and native NADPH-nitrate reductase was also identical, about 7.0. The apparent K_m values for nitrate of both nitrate reductases were also the same, 63 μM (Fig. 3).

Discussion

The results presented herein show that reconstitution of *C. reinhardii* NADPH-nitrate reductase takes place when combined homogenization of 104 and 305 mutant cells from *C. reinhardii*, both lacking NADPH-nitrate reductase activity, or incubation of cell-free extracts from each line obtained independently, is carried out. For the reconstitution to occur the enzymatic activities of 104, NADPH-cytochrome *c* reductase, and of 305, reduced benzyl viologen-nitrate reductase, must be induced on nitrate media.

Several reconstituted NAD(P)H-nitrate reductase systems from fungal and green plants mutant strains lacking nitrate reductase have been reported. The common trait of these complementation systems is that an NADPH-cytochrome *c* reductase from a mutant line aggregates in the presence of a peptide molybdenum-containing cofactor, producing an NAD(P)H-nitrate reductase identical with the complex from the parent wild strain. It appears that mutations affecting the NAD(P)H-cytochrome *c* reductase activity and the molybdenum-containing cofactor occur at different loci, in all fungi, algae and higher plants [2,8,12]. The mutation in the gene(s) coding for the molybdenum-containing cofactor leads to the loss of terminal nitrate reductase (mutants *nit*-1 of *N. crassa*, *cnx* of *A. nidulans* and *N. tabacum* and 104 of *C. reinhardii*). Mutations in other loci lead to the absence of the NAD(P)H-cytochrome *c* reductase

activity (mutants *nit*-3 of *N. crassa* and 305 of *C. reinhardii*) and, in some cases, of terminal nitrate reductase too (some mutants *nia* of *A. nidulans* and *N. tabacum*.

In *N. crassa* assembly of assimilatory NADPH-nitrate reductase has been obtained by in vitro inter-cistronic complementation of nitrate-induced *nit*-1 mutant extracts, containing NADPH-cytochrome *c* reductase, with either *nit* mutant extracts, induced or uninduced, or uninduced wild type, containing the molybdenum-containing cofactor [17]. The process produced the conversion of the slow sedimenting (4.5 S) inducible NADPH-cytochrome *c* reductase to a larger form (7.9 S) associated with NADPH-nitrate reductase, suggestive of subunit assembly. The source of cofactor could be substituted by acid-treated xanthine oxidase or aldehyde oxidase systems of higher animals [33], or by extracts of different bacteria [34—36]. Nitrate reductase from *A. nidulans* has been reconstituted in vitro by extracting together mycelium from an induced or uninduced *nia* D mutant, cofactor donor, and mycelium from the induced *cnx* strains, core protein donors [18].

The fungal model has also been used to interpret the structure of higher plants nitrate reductases. Mutants *cnx* and *nia*, analogous to those from *A. nidulans* have been isolated and characterized from *Nicotiana tabacum* [8,9, 37]. When nitrate-induced *cnx* cells were cohomogenized with, induced or uninduced, *nia* cells, NADH-nitrate reductase was reconstituted. It is thought that active nitrate reductase from *N. tabacum* is composed of at least the NADH-cytochrome *c* reductase moiety, probable coded by the *nia* gene, and a molybdenum-containing cofactor, product of the *cnx* gene(s) [9].

The integrity and full activity of proteins of the 104 and 305 mutants from *C. reinhardii*, involved in the NADPH-nitrate reductase reconstitution, is an absolute requirement for the complementation to take place. This establishes a clear difference between the earlier types of complementation and that reported herein. The 104 NADPH-cytochrome *c* reductase and 305 terminal nitrate reductase proteins must be induced by nitrate to achieve the complementation. If any of these proteins became inactive, no measurable complemented nitrate reductase was obtained.

Moreover, *C. reinhardii* cells of 6145*c* and 305 have a constitutive cofactor capable of complementing with induced *nit*-1 *N. crassa* mycelia. Such a cofactor is absent in 104 extracts and cannot reconstitute the *C. reinhardii* NADPH-nitrate reductase when extracts of uninduced 6145*c* or 305 are mixed with extracts of induced 104. It is destroyed by heat treatment in conditions that, however, do not affect the nitrate reductase activity, and seems to be peptide in nature since the presence of cycloheximide halts its production. Likewise, this cofactor is dialyzable and presumably contains molybdenum, resembling the cofactor isolated from bacteria [36], fungi [18,38,39], higher plants [9,37] and higher animals [33]. The central role of the molybdenum-containing cofactor in the fungal complementation system is superseded in *C. reinhardii* by an active protein containing molybdenum and bearing the terminal nitrate reductase activity.

The reconstituted enzyme from the mutant strains is physically and chemically indistinguishable from that of the wild parent strains, as concluded from the equality of size, shape, kinetic and enzymatic behaviour of both enzymes.

Molecular weights of 44 500, 165 000 and 248 000 have been calculated for the 104 NADPH-cytochrome *c* reductase, 305 reduced benzyl viologen-nitrate reductase and 6145*c* NADPH-nitrate reductase of *C. reinhardii*, respectively (unpublished data). 104 and 305 enzymatic activities related with nitrate reduction have been well-characterized and shown to be identical with those exhibited by the intact nitrate reductase complex of the wild parent strain [15, 16,22,32,40].

The molybdenum, probably as part of a peptide cofactor, has to be incorporated into a high molecular weight protein bearing the terminal nitrate reductase activity, to complement with the diaphorase, rendering an enzyme identical with the wild parent enzyme. Thus, we propose that NADPH-nitrate reductase of *C. reinhardii* is a heteromultimeric complex consisting of two types of subunit, separately responsible for NADPH-cytochrome *c* reductase and reduced benzyl viologen-nitrate reductase activities, which, assembled by a molybdenum cofactor, produce the native complex. According to available evidence two identical and functional polypeptide chains possibly bear the NADPH-cytochrome *c* reductase, whereas the terminal nitrate reductase is probably built by an unspecified number of protomers of the same or different kind.

This heteropolymeric model contrasts with the homopolymeric one proposed for fungi and green algae. *N. crassa* nitrate reductase (M_r 228 000) consists of two similar (if not identical) subunits of 115 000 [5], corresponding to the 4.5 S diaphorase from *nit*-1 mutants, assembled by a peptide molybdenum cofactor. However, such a proposal has not been universally accepted [41]. *A. nidulans* nitrate reductase (M_r 190 000) was first reported to consist of four protomers of approx. 49 000 each [42], although further work suggested the native enzyme is of heteromultimeric nature [43]. Nitrate reductase from *Chlorella vulgaris* (M_r 280 000) is a globular structure consisting of three very similar subunits of 90 000 each [44]. Very recently eight similar subunits of 58 000 each, possibly of two types — with or without heme — have been reported for nitrate reductase (M_r 460 000) from *Ankistrodesmus braunii* [45].

At present there is no single model which fits the rather incomplete data at hand for different eukaryotic nitrate reductases. In *C. reinhardii*, existing data on cofactors localization, activities characterization, molecular size of 104 NADPH-cytochrome *c* reductase and 305 terminal nitrate reductase (Refs. 15, 16, 32 and unpublished results) and the results presented herein on the successful assembly of these two proteins to yield the whole nitrate reductase complex of the wild parent strain, force us to suggest that NADPH-nitrate reductase of *C. reinhardii* consists of, at least, two types of subunits responsible for the cytochrome *c* reductase and nitrate reductase activities, respectively. Further examination of the possible similarities between these two kinds of subunits, and on the exact number of the protomers, must await the purification of the enzymes and subsequent analysis of their structures.

Acknowledgements

The authors thank Professor M. Losada for continuous help and encouragement. This research was supported by grants from Centro de Estudios de la

Energía (Spain) and Philips Research Laboratories (The Netherlands). The skillful scecretarial assistance of Mrs. Antonia Friend is gratefully acknowledged.

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