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BIOCHEMICAL CHARACTERIZATION OF A SINGULAR MUTANT OF NITRATE REDUCTASE FROM *CHLAMYDOMONAS REINHARDII*

NEW EVIDENCE FOR A HETEROPOLYMERIC ENZYME STRUCTURE

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A singular mutant strain from *Chlamydomohas reinhardii* defective in nitrate reductase has been characterized. Mutant 301 possesses an ammonia-repressible NAD(P)H-cytochrome *c* reductase with the same charge and size properties as the low molecular weight ammonia-repressible diaphorase present in the wild-type strain 6145*c* and is also able to reconstitute NAD(P)H-nitrate reductase activity by in vitro complementation with reduced benzyl viologen-nitrate reductase from mutant 305. Furthermore, a heat-labile costitutive molybdenum cofactor which is fuctionally active is also present in mutant 301. Mutant 301 has the two requirements exhibited by the active nitrate reductase complex from fungi, namely, NAD(P)H-cytochrome *c* reductase activity and molybdenum cofactor, but lacks NAD(P)H-nitrate reductase activity. This fact together with biochemical data presented from other *C. reinhardii* mutants strongly suggest a heteropolymeric model for the nitrate reductase complex of the alga.

Introduction

Mutants deficient in assimilatory NAD(P)H-nitrate reductase have been obtained from fungi [1,2] , algae [3–6] and higher plants [7–9]. Studies on fungal mutants have greatly contributed to configuring a homopolymeric model for nitrate reductase consisting of similar (if not identical) subunits bearing the NADPH-cytochrome *c* reductase activity, assembled by a peptide molybdenum-containing cofactor [1,2]. A similar model has been recently proposed for NADH-nitrate reductase of *Nicotiana tabacum* [10,11].

However, analyses of prosthetic groups and values of molecular weights for subunits of purified nitrate reductases from algae and higher plants suggest a possible heteropolymeric structure for the complex [12,13]. Furthermore, studies of in vitro complementation between mutant strains of *Chlamydomonas reinhardii* strongly support a heteromultimeric model for the NAD(P)H-nitrate reductase of this alga [14].

Mutant strains of *C. reinhardii* nitrate reductase characterized thus far resemble those from *Aspergillus nidulans* or *Neurospora crassa* [1,3] in that they either lack molybdenum cofactor and possess an ammonia-repressible NAD(P)H-cytochrome *c* reductase activity or lack ammonia-repressible NAD(P)H-cytochrome *c* reductase and possess molybdenum cofactor and reduced benzyl viologen-nitrate reductase activity [3,4,14–17].

In this paper, we present new evidence supporting a heteromultimeric structure for *C. reinhardii*

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nitrate reductase. The biochemical characterization of the mutant 301 strain, devoid of NAD(P)H-nitrate reductase and having solely ammonia-repressible NAD(P)H-cytochrome *c* reductase and molybdenum-containing cofactor, both being functionally active, advocates for, at least, two types of subunits in the native nitrate reductase assembled by the molybdenum cofactor and responsible for the two activities of the NAD(P)H-nitrate reductase complex, namely, NAD(P)H-cytochrome *c* reductase and reduced benzyl viologen-nitrate reductase.

Experimental Procedure

Materials. NADPH and horse heart cytochrome *c* were obtained from Boehringer. Hypoxanthine, FAD and Coomassie brilliant blue G-250 were purchased from Sigma. Benzyl viologen, methyl viologen, *p*-nitroblue tetrazolium and phenazine methosulphate were from Serva. All other chemicals used were of the highest quality available.

Growth conditions and preparation of extracts. C. reinhardii mutants 301, 102, 203, 104, 305 and 307 and wild type strain 6145*c* were cultured on ammonia and derepressed with nitrate as previously reported [3,14]. *N. crassa nit*-1 mutant (from the Fungal Genetics Stock Center, Arcata, CA) was grown on ammonia and derepressed with nitrate as described elsewere [14,18]. Cell-free extracts of *C. reinhardii* and *N. crassa nit*-1 mutant were obtained as reported in Ref. 14.

Enzyme assays. NAD(P)H-nitrate reductase, NAD(P)H-cytochrome *c* reductase, reduced benzyl viologen-nitrate reductase and reduced methyl viologen-nitrite reductase activities were determined according to methods described earlier [19]. Spectrophotometric and kinetic data were obtained with a Pye-Unicam SP-8-100 recording spectrophotometer. 1 unit of activity is defined as the amount of enzyme which catalyzes the transformation of 1 μmol substrate per min. Specific activity is expressed in units/mg protein.

Analytical methods. Nitrite was determined colorimetrically according to the method of Snell and Snell [20].

Protein was measured by the method of Lowry et al. [21] as modified by Bailey [22] using bovine serum albumin as standard.

Electrophoresis. Analytical disc gel electrophoresis was performed on 7.5% polycrylamide gels in Tris (3.6 g/l)-glycine (15.3 g/l) buffer (pH 8.3) [23]. Samples with glycerol and bromophenol blue were subjected to electrophoresis at 4 mA/gel until the tracking dye reached the bottom of the cylinder.

Diaphorase activity was visualized on the gels with a staining solution consisting of 0.6 mM NADPH and 0.6 mM *p*-nitroblue tetrazolium in 0.5 M potassium phosphate buffer (pH 7.5) [24].

Xanthine dehydrogenase activity was shown directly on the gels with a solution containing 2 mM hypoxanthine, 2 mM *p*-nitroblue tetrazolium, and 0.6 mM phenazine methosulphate in 0.1 M pyrophosphate buffer (pH 8.0) [10].

Proteins were located by staining with 1% (w/v) Coomassie brilliant blue G-250 in 7% (v/v) acetic acid for 1 h.

Complementation procedures. Reconstitution of NADPH-nitrate reductase activity by using extracts from *C. reinhardii* mutants or from *N. crassa* mutant *nit*-1 was carried out under conditions previously described [14,15].

Molecular weight determination. Molecular weights were determined according to the method of Hedrick and Smith [25]. Extracts from mutant 301 and 104 and wild type 6145*c* nitrate-derepressed cells and protein standards were run in parallel in gels with different total concentrations of acrylamide and, after staining, their corresponding R_F values were calculated. Slopes were obtained by plotting log ($100 \times R_F$) vs. the different acrylamide concentrations. Standards of known molecular weight were: bovine lactalbumin (14400), soybean trypsin inhibitor (20100), bovine carbonic anhydrase (30000), hen ovalbumin (43000), and bovine serum albumin (67000).

Results

Enzymatic activities in mutant 301 of C. reinhardii

Cells of *C. reinhardii* mutant 301 lack NAD(P)H-nitrate reductase and reduced benzyl viologen-nitrate reductase activities but retain ammonia-repressible NAD(P)H-cytochrome *c* reductase (Table I). This ammonia-repressible diaphorase is detected by polyacrylamide gel electrophoresis as a fast new band that appears after

TABLE I

ENZYMATIC ACTIVITIES IN WILD-TYPE AND MUTANT 301 STRAINS OF *CHLAMYDOMONAS REINHARDII*

Wild-type and mutant 301 cells, grown on ammonia, were transferred to media with ammonia (A) or nitrate (N) for 5.5 h. Cell-free extracts were obtained and their activities measured as described in Experimental Procedure

Strain	Specific activity (mU/mg)			
	NADPH-nitrate reductase	NADPH-cytochrome *c* reductase	Reduced benzyl viologen nitrate reductase	Reduced methyl viologen nitrite reductase
6145*c* (A)	0	42	0	0
6145*c* (N)	13	93	20	56
301 (A)	0	44	0	0
301 (N)	0	156	0	51

induction on nitrate of wild-type or mutant strains of *C. reinhardii* [16]. In order to establish possible differences in charge or size between the nitrate-inducible diaphorase from mutant 301 and from the wild-type strain, the corresponding molecular weights were determined. According to the method of Hedrick and Smith [25], proteins with identical size but different charge yield parallel lines in the plot of log $(100 \times R_F)$ vs. acrylamide concentration, whereas proteins with different size and identical or different charge produce lines with different slopes in the same type of plot. When crude extracts of wild-type and mutant 301 and 104 strains from *C. reinhardii* and protein markers were subjected to electrophoresis on polyacrylamide gels containing 6.25, 7.5, 8.75 and 10% (w/v) acrylamide, the same molecular weight value of 45000 was obtained for the nitrate-inducible diaphorase of strains 301, 104 and 6145*c*, without any difference in charge, since identical R_F values were obtained for all of them (results not shown).

As in the wild-type strain, reduced methyl viologen-nitrite reductase is an anzyme repressible by ammonia and, therefore, is not affected by the mutation (Table I).

Molybdenum-containing cofactor occurrence in mutant 301

Mutant 301 possesses a constitutive molybdenum-containing cofactor able to reconstitute the NADPH-nitrate reductase activity of *N. crassa* when mixed with extracts of mutant *nit*-1 derepressed with nitrate. When extracts from ammonia-grown mutant 301 cells were mixed and incubated at 20°C for 1 h with equal volumes of extracts of mutant *nit*-1 derepressed with nitrate, reconstitution of NADPH-nitrate reductase took place (66 nmol NO_2^-/h per mg). On nitrate media the molybdenum cofactor production is enhanced (126 nmol NO_2^-/h per mg). By heating at 45°C for 5 min, the ability of mutant 301 extracts to complement with *nit*-1 is completely abolished (results not shown).

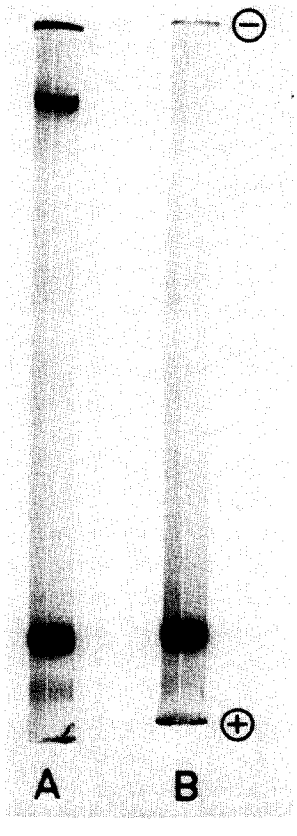

Fig. 1. Visualization of xanthine dehydrogenase activity of mutant 301 of *C. reinhardii* on polyacrylamide gel. Cell-free extracts of nitrate -derepressed mutant 301 were subjected to electrophoresis on polyacrylamide gels and stained for xanthine dehydrogenase activity (A). Gel B was the same as A except that hypoxanthine was omitted in the assay.

The molybdenum-containing cofactor has been reported to be present in several wild-type and mutant strains of *C. reinhardii* which have both xanthine dehydrogenase and terminal nitrate reductase activities [15]. In mutant 301, which lacks nitrate reductase but still has molybdenum cofactor, xanthine dehydrogenase could be detected electrophoretically (Fig. 1).

In vitro complementation between wild-type and mutant strains of C. reinhardii

Table II summarizes all the possible complementation binary mixtures between different mutant strains from *C. reinhardii*. In vitro reconstitution of NADPH-nitrate reductase activity only took place when extracts from mutant 305 derepressed with nitrate were mixed with extracts from nitrate-derepressed mutants 301, 102, 104 and 307. Under such conditions, mutant 305 has reduced benzyl viologen-nitrate reductase activity, mutants 301, 102, 104 and 307 show a new NAD(P)H-cytochrome *c* reductase, and NAD(P)H-nitrate reductase is absent in all the mutant strains (results not shown). Wild-type strain 6145*c*, grown on ammonia, was unable to reconstitute in vitro NAD(P)H-nitrate reductase by complementation with mutant strains which when grown on ammonia, did not complement with mutant 305 derepressed with nitrate (Table II).

Complementation between mutants 301 and 305 dropped drastically when the latter was derepressed on nitrate media containing 0.1 or 1 mM tungstate (Fig. 2). NAD(P)H-cytochrome *c* reductase of mutant 301 derepressed with nitrate in the presence of tungstate up to 0.1 mM was not affected whereas reduced benzyl viologen-nitrate reductase of mutant 305 derepressed with nitrate in the presence of 0.01, 0.1 and 1 mM tungstate followed the same pattern of inactivation as that observed in the complementation mixtures (results not shown), which indicates that complementation takes place between the active form of the terminal nitrate reductase from mutant 305 and an ammonia-repressible diaphorase protein supplied by mutant 301 without participation of the molybdenum cofactor of the latter.

Other possible auxotrophic mutations for precursors of nitrate reductase prosthetic groups, e.g.,

TABLE II

IN VITRO COMPLEMENTATION BETWEEN DIFFERENT MUTANT STRAINS FROM *CHLAMYDOMONAS REINHARDII*

Extracts from strains of *C. reinhardii*, grown on ammonia (A) or derepressed with nitrate (N), were obtained as described in Experimental Procedure. 0.1 ml of each extract, containing 1.5–2.5 mg/ml, was mixed as indicated, incubated at 20°C for 1 h, and then the NADPH-nitrate reductase activity was determined. Activity is expressed as nmol NO_2^- formed/h per mg. *, less than 1 nmol NO_2^-/h per mg.

Strain		Strain											
		301		102		203		104		307		305	
Strain		A	N	A	N	A	N	A	N	A	N	A	N
6145*c*	(A)	*	*	*	*		*	*	*	*	*	*	*
305	(A)	*	*	*	*	*	*	*	*	*	*		
	(N)	*	56	*	175	*	*	*	107	*	153		
307	(A)	*	*	*	*	*	*	*	*				
	(N)	*	*	*	*	*	*	*	*				
104	(A)	*	*	*	*	*	*						
	(N)	*	*	*	*	*	*						
203	(A)	*	*	*	*								
	(N)	*	*	*	*								
102	(A)	*	*										
	(N)	*	*										

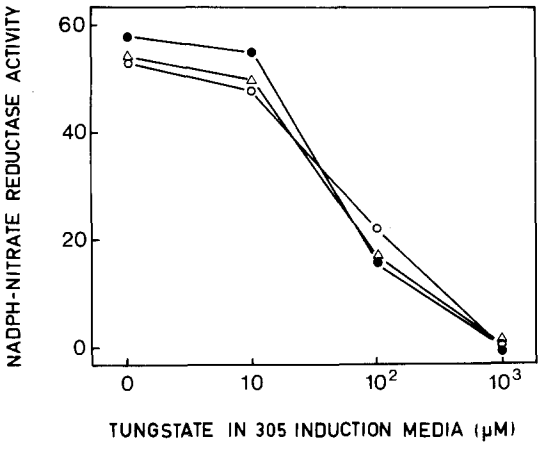

Fig. 2. Effect of tungstate concentration in the derepression media on the in vitro complementation between mutants 301 and 305 of *C. reinhardii*. Cells grown with ammonia in the presence of 100 mM tungstate were derepressed on media with nitrate containing different tungstate concentrations plus 10 nM molybdate. After 5.5 h, the cells were harvested and extracts prepared separately. Equal volumes of extracts of mutant 301 derepressed with 0 (●———●), 10 (○———○) or 100 μM (△———△) tungstate, and of mutant 305 derepressed at the indicated tungstate concentrations, were incubated together at 20°C for 1 h, and the enzymatic activity determined thereafter. Activity is expressed as nmol NO_2^- formed/h per mg.

riboflavin or haemin, were tested by means of growth experiments and gave negative results. Ferricyanide treatment to reactivate possible reduced inactive forms of NAD(P)H-nitrate reductase of mutant 301 was also without effect (results not shown).

Discussion

The above results show that *C. reinhardii* mutant 301 like mutants 102, 104 and 307 [3], lacks NAD(P)H- or reduced benzyl viologen-nitrate reductase activities but has a repressible NAD(P)H-cytochrome *c* reductase, able to reconstitute the NAD(P)H-nitrate reductase activity of *C. reinhardii* by in vitro complementation with active reduced benzyl viologen-nitrate reductase from mutant 305 (Table II). As previously reported [14], full integrity and activity of both proteins were required for complementation of *C. reinhardii* to take place. When one of the mutants remained repressed or mutant 305 was derepressed in the presence of tungstate (Table II and Fig. 2), complementation did not occur. Moreover, the ammonia-repressible diaphorase of mutant 301, detectable as a faster migrating band on polyacrylamide gel electrophoresis [16], is indistinguishable in charge and size from the low molecular weight ammonia-repressible diaphorases of strains 104 and 6145*c* (Fig. 1), very recently reported to be true subunits of the nitrate reductase complex [26].

On the other hand, mutant 301 possesses a molybdenum-containing cofactor capable of complementing in vitro with induced mutant *nit*-1 of *N. crassa*. This cofactor is constitutive, although its synthesis was enhanced in the presence of nitrate, it is heat-labile and resembles in its properties the molybdenum cofactor of mutant 305 [14]. An additional proof of the existence of this cofactor is that xanthine dehydrogenase activity is present in mutant 301 (Fig. 1). Xanthine dehydrogenase and nitrate reductase of *A. nidulans* [1] and *N. crassa* [2] share this common cofactor as do *N. tabacum* [10] and *C. reinhardii* [15]. Unlike mutant 305 of *C. reinhardii*, which has molybdenum-containing cofactor, reduced benzyl viologen-nitrate reductase and xanthine dehydrogenase [15], the mutant 301 strain has molybdenum cofactor and xanthine dehydrogenase but lacks reduced benzyl viologen-nitrate reductase. Mutant 301 is also different from the mutant 203 strain of *C. reinhardii* which has molybdenum cofactor and xanthine dehydrogenase but lacks NAD(P)H-cytochrome *c* reductase and reduced benzyl viologen-nitrate reductase [15]. The mutant 301 molybdenum cofactor does not participate in the vitro complementation with mutant 305 extracts, since tungstate substitution for molybdate in mutant 301 had no effect on the reconstituted nitrate reductase level.

The characteristics of several *C. reinhardii* mutants, from data presented herein and reported previously [14–16], are summarized in Table III. As shown, the ability of *C. reinhardii* mutants to complement with the mutant *nit*-1 extract is concomitant with the existence of xanthine dehydrogenase activity, and the capability of complementing with mutant 305, the sole mutant strain from *C. reinhardii* which possesses terminal nitrate reductase, is accompanied by the presence of an ammonia-repressible diaphorase.

TABLE III

CHARACTERISTICS OF WILD-TYPE AND MUTANT STRAINS FROM *CHLAMYDOMONAS REINHARDII*

All the strains were derepressed with nitrate. (a) Ref. 15 and present work. (b) Ref. 16 and present work. (c) Ref. 14 and present work.

Strain	Xanthine dehydrogenase(a)	Complementation with mutant *nit*-1(a)	Reduced benzyl viologen-nitrate reductase(b)	Ammonia repressible NAD(P)-cytochrome *c* reductase(b)	Complementation with 305 from *C. reinhardii*(c)
301	+	+	−	+	+
305	+	+	+	−	
102, 104 or 307	−	−	−	+	−
203	+	+	−	−	+
6145*c*	+	+	+	+	+

In Table IV, the biochemical characteristics of mutants of *C. reinhardii* and other eukaryotic organisms affected in nitrate assimilation are compared. As observed, mutant 305 is analogous to mutants *nit*-3 of *N. crassa*, *nia* D 10 of *A. nidulans*, *nia*-95 of *N. tabacum*, *Az* 32 of barley and *nit*-A of *C. reinhardii*, whereas mutant 104 is similar to *nit*-1 of *N. crassa* and *cnx* of *A. nidulans* or *N.*

TABLE IV

BIOCHEMICAL CHARACTERISTICS OF STRUCTURAL GENE MUTANTS FOR NITRATE REDUCTASE IN EUKARYOTIC ORGANISMS

n.s., not studied

Mutant	Xanthine dehydrogenase and molybdenum cofactor	Reduced benzyl viologen-nitrate reductase	Ammonia-repressible NAD(P)H-diaphorase	Ref.
N. crassa				
nit-1	−	−	+	2, 27
nit-3	+	+	−	2, 27
A. nidulans				
cnx	−	−	+	1, 28
nia D 10	+	+	−	1, 28
nia D 17	+	−	+	1, 28
nia D 8	+	−	+	1, 28
N. tabacum				
cnx 68/2	−	−	+	11
nia-95	+	+	−	11
nia	+	−	−	11
Barley				
Az 28	n.s	−	+	29
Az 32	n.s.	+	−	29
C. reinhardii				
nit A	n.s.	+	−	17
104	−	−	+	15, 26
305	+	+	−	15, 26
301	+	−	+	

tabacum. Mutant 301 is unique with regard to its properties and has no equal in other organisms. Mutants from *nidulans* possessing molybdenum cofactor and diaphorase activity related to nitrate reductase such, as *nia* D 17, are unable to complement with other *nia* D strains, since they belong to the same group of complementation [1,28,30]. However, in vitro complementation between mutants 301 and 305 from *C. reinhardii* occurs, which indicates that either both strains belong to different complementation groups (mutants 301 and 305 would be affected in different structural genes) or they can give rise to intragenic complementation (mutants 301 and 305 would be affected in different zones of the same structural gene). The latter possibility does not explain why mutant 301, with both functionally active NAD(P)H-cytochrome *c* reductase activity and molybdenum cofactor, lacks nitrate reductase activity, nor why in vitro complementation between the molybdenum cofactor from mutant 301 and ammonia-repressible diaphorase does not take place.

Recently, we have demonstrated that nitrate reductase reconstitution in *C. reinhardii* mutants occurred between integral and fully active NAD(P)H-cytochrome *c* reductase and reduced benzyl viologen-nitrate reductase, proteins dissimilar in size and cofactors, thus concluding that nitrate reductase in this organism consists of, at least, two types of subunits separately responsible for these activities [14]. Molybdenum cofactor and diaphorase activities from suitable mutant or wild-type strains of *C. reinhardii* cannot restore the nitrate reductase complex, thus reinforcing the conclusion that complementation between two different classes of subunits is taking place.

The results of the present work support the foregoing heteromultimeric model proposed for NAD(P)H-nitrate reductase of *C. reinhardii*: its nitrate reductase complex consists of the products of two structural genes, one responsible for the subunits with diaphorase activity and the other for the subunits of the apoprotein of the terminal activity. Other gene(s) code for the molybdenum cofactor needed for the activity of the terminal subunits and, possibly, for the assembly of both kinds of subunits, as has been proposed for fungal nitrate reductase [2,27].

The existence of a low molecular weight ammonia-repressible diaphorase in mutant 301, identical in charge and size with the present in wild-type strain 6145*c* and able to reconstitute the NAD(P)H-nitrate reductase complex by assembly with reduced benzyl viologen-nitrate reductase, as well as the presence in mutant 301 of a functional molybdenum cofactor, associated with xanthine dehydrogenase activity and detected by complementation with mutant *nit*-1 of *N. crassa* and, on the other hand, the absence in mutant 301 of auxotrophic requirements for nitrate reductase prosthetic groups precursors, lead us to propose that mutation in strain 301 has affected the gene(s) coding for the apoprotein responsible for the terminal nitrate reductase activity, thus supporting with new evidence the heteromultimeric model previously proposed for the *C. reinhardii* nitrate reductase complex [14].

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