The EMBO Journal vol.3 no.6 pp.1403–1407, 1984

Heteromultimeric structure of the nitrate reductase complex of *Chlamydomonas reinhardii*

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Communicated by E. Cerdá-Olmedo

The NAD(P)H-nitrate reductase complex (overall-NR) of *Chlamydomonas reinhardii* exhibits two partial activities: NAD(P)H-cytochrome *c* reductase (diaphorase) and reduced benzyl viologen-NR (terminal-NR). Mild tryptic digestion of the enzyme complex resulted in the loss of both overall and terminal-NR activities, whereas diaphorase activity remained unaltered. The diaphorase activity of mutant 104 and the terminal-NR activity of mutant 305 of *C. reinhardii*, which are the sole activities related to NR present in these mutants, responded to tryptic treatment to the same extent as the corresponding activities of the wild enzyme complex. Trypsin disassembled the 220-kd NR native complex by destroying the aggregation capability of the diaphorase subunits without affecting their activity nor molecular size (45 kd). A 67-kd thermostable protein, containing molybdenum co-factor, was also released from trypsin-treated NR. This protein lacked diaphorase and NR activities but was able to reconstitute the overall-NR complex by complementation with untreated diaphorase subunit of mutant 104. Our results support a tetrameric structure for the *C. reinhardii* NR complex, containing two kinds of subunits.

Key words: Chlamydomonas reinhardii mutants/nitrate reductase/proteolysis

Introduction

The reduction of nitrate with NAD(P)H in eukaryotes is catalyzed by nitrate reductase (NR), a large complex (160–475 kd) which contains FAD, cytochrome b_{557} and molybdenum co-factor as prosthetic groups (Losada, 1976; Garrett and Amy, 1978; Hewitt and Notton, 1980). Depending on the source, NR contains two to eight subunits of 45–145 kd (Hewitt and Notton, 1980; Guerrero *et al.*, 1981; Horner, 1983), which may be identical (Garrett and Amy, 1978; Tomsett and Garrett, 1980; Howard and Solomonson, 1982; Horner, 1983) or different (Hewitt and Notton, 1980; Fernández and Cárdenas, 1982a; De la Rosa, 1983) and are assembled by a molybdenum co-factor (Johnson, 1980).

Two partial activities, namely NAD(P)H-cytochrome *c* reductase (diaphorase) and reduced benzyl viologen-NR (terminal-NR), may be separately assayed in NR (Losada, 1976). Mutants of *C. reinhardii* having lost the NAD(P)H-nitrate reductase complex (overall-NR) activity may retain one of the partial activities and may be able to complement with one another *in vitro* to reconstitute the overall-NR activity (Fernández and Cárdenas, 1981). The biochemical characterization of the mutant enzymes and the *in vitro* complementation suggest that NR of *C. reinhardii* is a heteromultimer with two kinds of subunits, responsible, respectively, for each of the partial activities (Fernández and Cárdenas 1981,1982a,1983c). The diaphorase subunit is a protein of 45 kd, pI 5.05, carrying FAD and cytochrome b_{557} (Fernández and Cárdenas, 1983a,1983b). However, the other kind of subunit has not yet been well defined in molecular terms.

We have found that limited trypsin proteolysis of the NR complex of *C. reinhardii* dissociates the complex without affecting the diaphorase activity, and releases a 67-kd protein, which is thermostable and contains the molybdenum co-factor. We propose that this protein is an inactive terminal subunit, and is required, together with the diaphorase subunit, for the assembly of a functional complex.

Results

Mild tryptic digestion at 4°C of partially purified wild-type NR destroyed both overall and terminal-NR activities of the complex, but did not affect the diaphorase activity (Figure

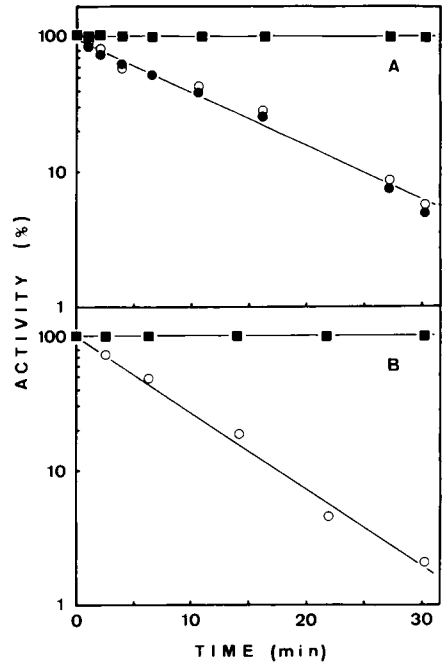

Fig. 1. Effect of trypsin on diaphorase, overall and terminal-NR of wild strain 6145*c* and mutants 104 and 305 of *C. reinhardii*. Partially purified enzyme preparations were incubated with trypsin and assayed as indicated in Materials and methods. **(A)** (■), diaphorase; (○), terminal-NR; (●), overall-NR of wild-type. **(B)** (■), diaphorase of mutant 104; (○), terminal-NR of mutant 305. 100% activity corresponded to 1, 20 and 6 mU/mg, respectively for overall-NR, diaphorase and terminal-NR of wild-type; 116 mU/mg for diaphorase of 104, and 14 mU/mg for terminal-NR of 305.

Table I. Effect of different proteases and lipase on the enzymatic activities of the wild-type NR complex of *C. reinhardii*

Addition	Activity (%)		
	Overall-NR	Terminal-NR	Diaphorase
None	100	100	100
Trypsin	3	4	102
α-Chymotrypsin	5	5	103
Papain	10	8	95
Lipase	100	99	102

Activities were measured in aliquots from partially purified enzyme preparations incubated with 6 μg trypsin, 12.5 μg α-chymotrypsin or 25 μg papain/mg protein at 4°C, for 40 min, or with 5000 U lipase/mg protein at 25°C, for 3 h. 100% activity corresponded to 2, 6 and 17 mU/mg for overall-NR, terminal-NR and diaphorase, respectively.

Table II. Effect of trypsin treatment on complementation between wild 6145*c* and mutant strains 104 and 305 of *C. reinhardii*

Expt. number	Complementation mixtures	Activities (nmol NO_2^-/ml.h)	
		Overall-NR	Terminal-NR
1	104	0	0
2	305	0	390
3	305 + 104	32	420
4	104 (T)	0	0
5	305 + 104 (T)	0	395
6	305 (T)	0	0
7	305 (T) + 104	29	82
8	305 (T) + 104 (T)	0	0
9	6145*c*	153	430
10	6145*c* (T)	0	0
11	6145*c* (T) + 104	24	84
12	6145*c* (T) + 104 (T)	0	0
13	6145*c* (T) + 305	0	390
14	6145*c* (T) + 305 (T)	0	0
15	305 (T), heated + 104	33	96
16	6145*c* (T), heated + 104	25	64

The mixtures of partially purified preparations of strains 104 (390 mU diaphorase/mg protein), 305 (15 mU terminal-NR/mg) and 6145*c* (10 mU overall-NR/mg, 28 mU terminal-NR/mg, and 95 mU diaphorase/mg) were incubated at 30°C for 30 min. (T): before mixing, the extract was treated with trypsin as described in Materials and methods; heated: before mixing, the extract was heated at 45°C for 5 min. Diaphorase activity was unaffected by trypsin treatment.

1A). At 30°C, the loss of the overall and the terminal-NR activities was even faster, but 80% of the diaphorase activity survived after 1-h treatment (results not shown). Mutant 104 of *C. reinhardii*, which lacks molybdenum co-factor and has only the activity of the diaphorase subunit (Fernández and Cárdenas, 1983b), and mutant 305, which lacks diaphorase activity and has only terminal-NR activity (Fernández and Cárdenas, 1983c), have been used throughout this work. The partial activities related to NR of mutants 104 and 305 (Figure 1B) responded to tryptic treatment in the same way as the corresponding partial activities of the wild-type. The effects of papain and α-chymotrypsin (Table I) were very similar to

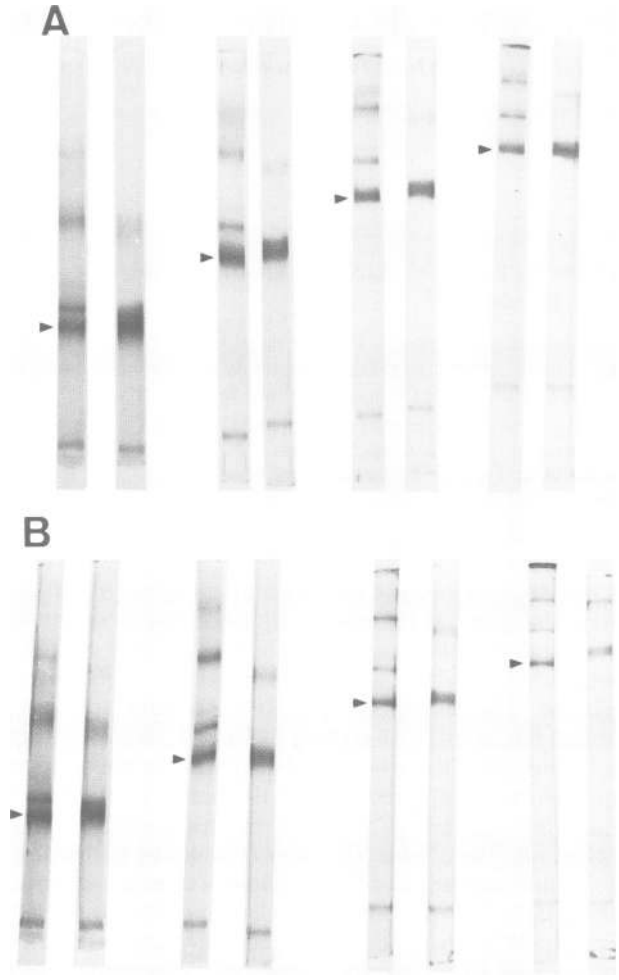

Fig. 2. Polyacrylamide-gel electrophoresis of diaphorases treated with trypsin. Diaphorases from the wild-type (90 mU/mg) (**A**) and mutant 104 (82 mU/mg) (**B**) were incubated at 4°C with trypsin, subjected to electrophoresis on gels of different acrylamide concentrations (6, 8, 10 and 12%, from left to right), and stained for the diaphorase reaction. The diaphorase related to nitrate reduction is indicated by an arrow in the untreated controls shown at the left of each pair. The front band in each gel is the bromophenol blue tracking dye.

those of trypsin. Under the conditions used in these proteolytic digestions, the decay of the two affected activities followed first-order kinetics with half-lives of <10 min. Highly purified lipase had no effect on any of the activities.

Table II summarizes *in vitro* complementation tests between extracts of different strains, both treated and untreated with trypsin. These results may be viewed in the light of our proposal that the NR complex contains two different subunits. One of the subunits carries the diaphorase activity. The other subunit provides the terminal-NR activity, but only when assembled with the first subunit.

Fully-active diaphorase from mutant 104 and terminal-NR from mutant 305 reconstitute *in vitro* an active NR complex which is indistinguishable from the wild-type complex (Fernández and Cárdenas, 1981,1982a and expt. 3 in Table II). Tryptic treatment of mutant 104 extracts rendered them unable to complement *in vitro* with extracts from mutant 305 (expt. 5). Tryptic digestion does not destroy the active site of the diaphorase since this activity remains unaltered, but hinders its aggregation with other subunits to form an active complex. On the contrary, tryptic digestion of mutant 305 extracts, which destroyed their terminal-NR activity (expt. 6),

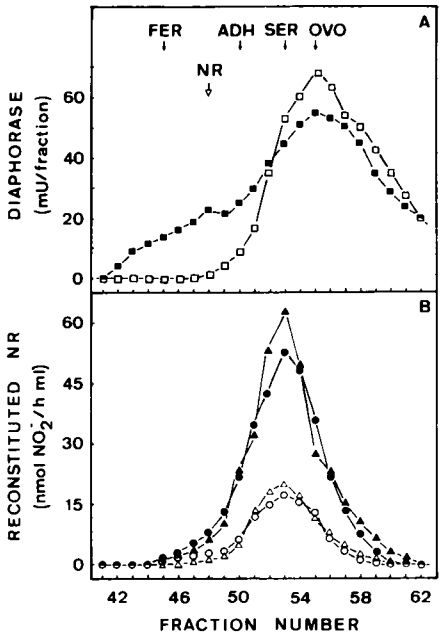

Fig. 3. Disassembly of NR complex by trypsin in the wild-type and mutant strain 305 of *C. reinhardii*. **(A)** Diaphorase activity. Partially purified preparations of wild-type (200 mU diaphorase/mg), either treated (□) or untreated (■) with trypsin, were filtered through a Bio-Gel A-1.5 m column (2.6 x 36 cm). The open arrow indicates the fraction containing maximal NR activity. **(B)** Terminal subunits. Partially purified preparations of wild-type (10 mU overall-NR/mg) and 305 (89 mU terminal-NR/mg) were treated with trypsin and gel-filtered as in **A**. The resulting fractions were complemented at 45°C, for 5 min, with untreated extracts of mutant 104 (117 mU diaphorase/mg). The graphs show reconstituted overall-NR (open symbols) and terminal-NR (filled symbols) for the wild-type (circles) and mutant 305 (triangles). The small arrows indicate the elution volumes of ferritin (FER), alcohol dehydrogenase (ADH), bovine serum albumin (SER) and ovalbumin (OVO).

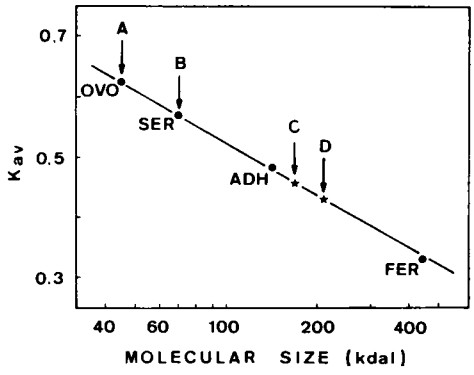

Fig. 4. Mol. wt. determination by gel filtration of the nitrate-reduction related proteins of *C. reinhardii*. **A**: diaphorase subunit (data from Figure 3A); **B**: terminal subunit of either wild-type or mutant 305 (data from Figure 3B); **C**: terminal-NR of mutant 305; **D**: either native overall-NR of the wild-type or overall-NR reconstituted as in Figure 3B.

did not abolish their ability to complement *in vitro* with extracts of mutant 104 (expt. 7). Trypsin-treated wild-type extracts and untreated extracts of mutant 104, both exhibiting only the diaphorase activity, complemented *in vitro* to yield all the activities (expt. 11). The new activities are attributed to the combination of the diaphorase subunits from strain 104 with the terminal subunits of the wild-type or mutant 305. Trypsin treatment of mutant 104 extracts abolishes the aggregation capacity of these diaphorase subunits (expt. 12). Strain 305 is not a source of diaphorase subunits (expts. 13 – 14), as expected.

Extracts of wild-type or mutant 305, treated with trypsin and heated at 45°C for 5 min, complemented *in vitro* with extracts of mutant 104 (expts. 15 – 16). In these experiments the molybdenum co-factor must be provided by the trypsinized and heated extracts, since strain 104 lacks the co-factor altogether (Fernández and Cárdenas, 1981,1982a).

The diaphorase subunit of the NR complex is found in crude extracts of *C. reinhardii* as a fast moving band of R_f = 0.58 on 7.5% polyacrylamide gels (Fernández and Cárdenas, 1982b,1983a,1983b). Diaphorase subunit bands, on polyacrylamide gels made with different acrylamide concentrations, were broader after trypsin treatment without any modification of their electrophoretic mobility (Figure 2). According to Hedrick and Smith (1968), this indicates that trypsin does not cause any significant change in the charge or size of the diaphorase subunits of wild-type or mutant 104. In both cases, they showed identical size (45 kd), as calculated from comparison of their electrophoretic mobilities with those of known standards. Bands of constitutive diaphorases, unrelated to NR, were, on the contrary, greatly modified by trypsin digestion.

When wild-type extracts were filtered through a gel column, two peaks of diaphorase activity were found. The first (Figure 3A, fraction 48) was associated with the overall-NR activity. The second (fraction 55) was shown by electrophoresis to contain the diaphorase subunit of NR and constitutive diaphorases unrelated to NR. Trypsin caused the loss of the first peak and an increase of the second, which suggests that tryptic digestion disassembles the NR complex into its subunits.

Terminal subunits cannot be directly assayed. Figure 3B shows that the terminal subunits from NR of wild-type or mutant 305, detected by *in vitro* complementation with diaphorase-containing extracts of mutant 104, have 67 kd. New gel filtration runs (Figure 4) showed that the overall-NR, reconstituted as in Figure 3B, has the same size (220 kd) as the native enzyme. For comparison, the diaphorase subunit has a size of 45 kd, the terminal subunit, 67 kd, and the enzyme from mutant 305 (showing terminal-NR activity only) has 167 kd.

Affinity chromatography allows the separation of diaphorase and terminal subunits as different and independent entities. When trypsin-treated preparations of wild-type were filtered through a Blue-Agarose column, the terminal subunit was eluted in the void volume of the column (Figure 5, fraction 4). The diaphorase subunit was retained in the agarose bed and had to be eluted with 0.25 M KCl. The diaphorase-containing fractions were incapable of reconstituting native NR with extracts of either mutant 305 (containing terminal-NR) or mutant 104 (containing diaphorase).

Discussion

Our results can be explained by assuming that the NR complex of *C. reinhardii* consists of two kinds of subunits, assembled as shown in the model of Figure 6. The 220-kd NR complex of wild-type is attributed to two 45-kd diaphorase subunits and to two 67-kd terminal subunits containing the molybdenum co-factor, and the 167-kd NR of mutant 305 to two terminal subunits and two fragments of diaphorase subunits. The diaphorase subunit is active by itself. The terminal subunit has no enzyme activity, but, joined to the

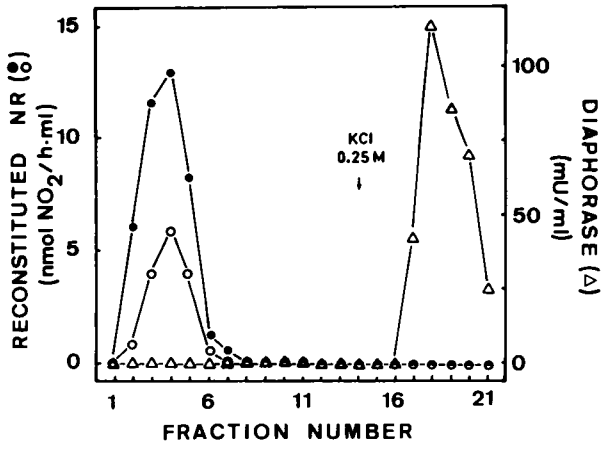

Fig. 5. Separation by affinity chromatography of the diaphorase and terminal subunits of NR from *C. reinhardii*. Partially purified preparations of wild-type in phosphate buffer 20 mM, pH 6.5, 0.1 mM dithioerythritol, 0.1 mM EDTA, 10 μM FAD, were filtered through a Blue-Agarose column (0.8 x 6 cm) at a flow rate of 4 ml/h. Fractions (1.5 ml) were collected and assayed for diaphorase (△). Each fraction was mixed with an extract of mutant 104 at 30°C. After 30 min, reconstituted overall (○) and terminal-NR (●) were determined.

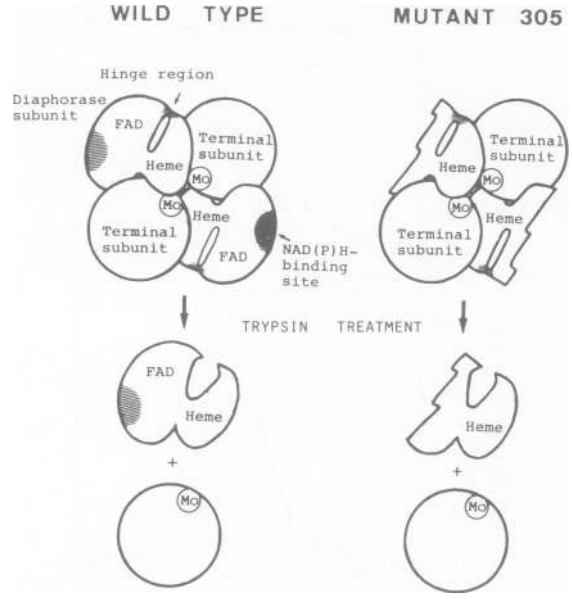

Fig. 6. Tentative model for NR of wild-type and mutant 305 of *C. reinhardii*. The wild-type complex contains two diaphorase and two terminal subunits. Trypsin treatment is assumed to disaggregate the complex by cleaving a hinge region in the diaphorase subunit. Mutant 305 contains the terminal subunits and the fragments of the diaphorase subunits necessary for the terminal activity, including the cytochrome b_{557} and the trypsin-sensitive site.

diaphorase subunit, is responsible for the terminal- and overall-NR activities. Other enzyme complexes such as bacterial tryptophan synthetase (Yanofsky and Crawford, 1972) and anthranilate synthetase (Zabin and Villarejo, 1975) have two kinds of subunits, able to catalyse separate reactions, which require to be assembled to exhibit the whole activity. The present results may be viewed in the light of the above model.

Protease-induced loss of the terminal and overall activities of wild NR (Figure 1 and Table I) was not due to a modification of the terminal subunit but of the diaphorase subunit, as deduced from the complementation data (Table II). The modified diaphorase subunit retained its activity and did not change its size (Figures 1 and 2), but was unable to aggregate with the terminal subunit (Figure 3A). This effect can be explained by assuming that the enzymatic activity and the aggregation ability of the diaphorase subunit reside in separate regions of the macromolecule. Trypsin might cleave a small section of the diaphorase polypeptide essential for aggregation.

NR of *C. reinhardii* seems to contain regions hypersensitive to proteolysis, whose integrity is required for the overall and terminal activities, but not for the diaphorase activity, as deduced from the short half-lives of NR in the presence of proteases. Protease-sensitive hinge zones, joining distinct polypeptide domains, have been described in proteins as diverse as bacterial DNA polymerase I (Kornberg and Kornberg, 1974), rat liver sulfite oxidase (Johnson and Rajagopalan, 1977), chicken liver xanthine dehydrogenase (Guiard and Lederer, 1977) and yeast flavocytochrome b_2 (Ghrir and Lederer, 1981). Consequently, an alternative explanation of the trypsin effect on the subunits of *C. reinhardii* NR is that, after tryptic digestion of a peptide hinge, certain domains of the diaphorase subunit, possibly flavin and heme domains, might remain clamped to the rest, as reported for chicken liver xanthine dehydrogenase (Coughlan *et al.*, 1979). Recently, a heme-binding domain, homologous to cytochrome b_5, has been found in NR of *Neurospora crassa* (Lê and Lederer, 1983).

Proteolysis of the NR complex from maize scutellum or barley leaves maintains its diaphorase activity, but causes a large reduction in size, attributed to partial digestion of the two identical subunits which make up the complex (Brown *et al.*, 1981; Batt and Wallace, 1983). In contrast, trypsin inactivates the overall-NR and the diaphorase of *Chlorella vulgaris* without affecting terminal-NR (Yamaya *et al.*, 1980). This suggests structural differences between the NR of *C. vulgaris*, on the one hand, and *C. reinhardii* and higher plants, on the other.

We have identified the terminal subunit of the NR complex of *C. reinhardii* as the 67-kd protein which was able to complement with diaphorase donors to reconstitute the whole complex (Figure 3B). Proteases destroyed the activity of the enzyme from mutant 305 and released the terminal subunit of 67 kd (Figures 1 and 3). Thus, mutant 305 NR, which has lost a portion of the diaphorase subunit (Fernández and Cárdenas, 1983c), must retain, besides the heme domain, the trypsin-sensitive site.

The 67-kd terminal subunit has no enzyme activity outside of the complex, either because it requires the participation of some co-factors(s) present in the diaphorase subunit, or because the assembly of the diaphorase and terminal subunits is essential for the correct building of the active site for nitrate reduction. It is thus not surprising that the attempts to separate physically the two partial activities of NR have been unsuccessful.

The molybdenum co-factor, indispensable for both terminal- and overall-NR activities (Johnson, 1980), must be integrated within the 67-kd terminal subunit since this subunit complemented extracts from mutant 104 devoid of the co-factor (Figure 3B). Once integrated into the subunit, the molybdenum became resistant to heat exposures (Table II) which destroy the free co-factor (Fernández and Cárdenas, 1981).

Materials and methods

Chemicals and enzymes

NADPH and horse heart cytochrome *c* were purchased from Boehringer, Mannheim, FRG; FAD, Coomassie Brilliant Blue G-250, Blue-Agarose, alcohol dehydrogenase (yeast), trypsin (Type III from bovine pancreas), trypsin inhibitor (type II-0 from chicken egg), lipase (type III from *Rhizopus arrhizus*), α-chymotrypsin (type I-S from bovine pancreas) and papain (type III from *Papaya latex*) were from Sigma, St. Louis, MO, USA; hen ovalbumin, bovine serum albumin (BSA), ferritin, benzyl viologen and *p*-nitrobluetetrazolium chloride from Serva, Heidelberg, FRG; DEAE-Sephacel from Pharmacia, Uppsala, Sweden, and Bio-Gel A-1.5 m from Bio-Rad, Richmond, CA, USA. All other reagents were of analytical grade.

Cells, growth conditions and preparation of extracts

Strain 6145c is a wild-type obtained from Dr. Ruth Sager (Hunter College, New York). The mutant strains 104 and 305 were obtained by Sosa *et al.* (1978).

Cell-free extracts from *C. reinhardii* cells, cultured with ammonia and derepressed with nitrate, were obtained under conditions previously reported (Fernández and Cárdenas, 1981).

Enzyme preparation

Crude extracts from wild-type and mutant 305 were partially purified in a DEAE-Sephacel column (1.5 x 11 cm), equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl, 0.1 mM dithioerythritol, 0.1 mM EDTA, 10 μM FAD. NR was eluted with a linear gradient (0.05 – 0.3 M) in the same buffer at ~0.25 M NaCl, and the eluate was routinely used as source of enzyme.

Enzyme assays and analytical methods

NADPH-nitrate reductase (EC 1.6.6.2), NADPH-cytochrome *c* reductase and reduced benzyl viologen-nitrate reductase activities were determined as described elsewhere (Fernández and Cárdenas, 1982b). Alcohol dehydrogenase was assayed spectrophotometrically by following changes in absorbance at 340 nm (Bergmeyer *et al.*, 1974). Other standard proteins were estimated by measuring the absorbance at 280 nm. Spectrophotometric and kinetic determinations were performed with a Pye-Unicam SP-8-100 recording spectrophotometer. A unit of activity (U) is defined as the amount of enzyme which transforms 1 μmol of substrate/min. Specific activity is expressed in units per mg protein.

Nitrite was determined colorimetrically by the diazotization method of Snell and Snell (1949). Protein was measured spectrophotometrically according to Bradford (1976), using BSA as standard.

Tryptic treatment

Enzyme samples were incubated with 6 μg of trypsin/mg protein at 4°C in kinetic experiments, or with 100 μg trypsin/mg protein at 30°C in complementation experiments. The treatment was stopped by adding 5 μg trypsin inhibitor/μg trypsin.

Complementation procedures

Reconstitution experiments of overall-NR activity were carried out with crude extracts as previously described (Fernández and Cárdenas, 1981).

Molecular weight determination

Mol. wts. were determined by gel electrophoresis on polyacrylamide gels of different concentrations after Hedrick and Smith (1968) or by filtration through a Bio-Gel A-1.5 m (100 – 200 mesh) column (2.6 x 36 cm) according to Andrews (1964). Diaphorase activity was located on the gels with *p*-nitrobluetetrazolium chloride as described by Wang and Raper (1970), and standard proteins by staining for 1 h with 1% (w/v) Coomassie Blue G-250 in 7% acetic acid and 5% methanol. Densitograms of stained gels were obtained with a Pye-Unicam densitometer mod. 790826.

Standards of known mol. wt. were bovine lactalbumin (14.4 kd), soybean trypsin inhibitor (20.1 kd), bovine carbonic anhydrase (30 kd), hen ovalbumin (43 kd), BSA (67 kd), phosphorylase *b* (94 kd), yeast alcohol dehydrogenase (141 kd) and ferritin (440 kd).

Acknowledgements

The authors thank Prof. M. Losada for provision of facilities. The improvements of the manuscript and the valuable criticisms of Prof. E. Cerdá are also acknowledged.

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Received on 16 January 1984; revised on 15 February 1984