Isoelectric Focusing of the NAD(P)H-Cytochrome *c* Reductase Subunit of *Chlamydomonas reinhardii* Nitrate Reductase

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Z. Naturforsch. **38c**, 35–38 (1983); received August 2/October 7, 1982

Isoelectric Focusing, Nitrate Reductase, Diaphorase, *Chlamydomonas reinhardii*

Chlamydomonas reinhardii 104 mutant strain cells contain many NAD(P)H-cytochrome *c* reductase activities unrelated to nitrate reduction in addition to an NAD(P)H-cytochrome *c* reductase related to NAD(P)H-nitrate reductase, which have molecular weights from 37000 to 124000. By combining chromatographic procedures and preparative isoelectric focusing, three major fractions of NAD(P)H-cytochrome *c* reductase were isolated with pI at 7.1, 5.5 and 5.05, respectively. Of these fractions, only the last one was identified as the diaphorase subunit of the NAD(P)H-nitrate reductase by its ability to reconstitute *in vitro* the nitrate reductase native complex upon complementation with active terminal nitrate reductase of 305 mutant of *C. reinhardii*.

Introduction

In *Chlamydomonas reinhardii* wild and mutant strains grown on, or derepressed with, nitrate many NAD(P)H-cytochrome *c* reductases have been detected [1–3]. Most of these diaphorase activities have been demonstrated to be unrelated to assimilatory nitrate reduction and only one of them, ammonia repressible, has been proposed to be the diaphorase subunit of the native NAD(P)H-nitrate reductase enzyme complex [2, 3]. These diaphorase activities were very resistant to separation by means of the usual chromatographic methods (ion exchange, molecular exclusion and affinity chromatography) due to their similarity in charge and size [2].

In the present paper, we report the isolation of the NAD(P)H-diaphorase nitrate reductase subunit by combining ion exchange chromatography and isoelectric focusing. Since mutant 104 of *C. reinhardii*, which lacks the terminal nitrate reductase activity of the complex [2, 4], has large amounts of NAD(P)H-diaphorase subunit, we used this mutant as source of enzyme throughout all the work.

Experimental

Chemicals

FAD, EDTA and Tris were obtained from Sigma (St. Louis, MO, USA), NADH, NADPH, and horse heart cytochrome *c* were from Boehringer (Manheim, FRG). 4-Nitrobluetetrazolium chloride and dithioerythritol were from Serva (Heidelberg, FRG). Ampholine pH 3.5–5 and 5–8 were purchased from LKB (Bromma, Sweden), and Sephadex G-100 and DEAE-Sephacel from Pharmacia (Uppsala, Sweden).

Culture methods and preparation of extracts

6145 *c* cells and mutant strains 104 and 305 of *C. reinhardii* were cultured and derepressed with nitrate, harvested, frozen in liquid nitrogen and stored, under conditions previously established [3, 4].

Cell pellets were disrupted by thawing with gentle stirring, 1 h, in 50 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl, 0.1 mM dithioerythritol, 0.1 mM EDTA and 10 μM FAD (buffer 1). The resulting suspension was then centrifuged at 30000 × *g*, 15 min, and the supernatant used as source for enzyme purification.

Purification of NAD(P)H-cytochrome c reductases

A) *Chromatographic procedures:* Enzyme extracts were adsorbed on a DEAE-Sephacel column (6 × 3.2 cm), and eluted with a linear gradient of 0.05 – 0.3 M NaCl in buffer 1. The resulting eluate was precipitated with ammonium sulfate, 30–60% saturation, centrifuged at 30000 × *g*, 15 min, and the sediment resuspended in 5 mM potassium phos-

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phate buffer, pH 7.0, containing 50 μM dithioerythritol, 50 μM EDTA and 10 μM FAD. The solution was filtered through a Sephadex G-100 column (22 × 3 cm).

B) *Isoelectric focusing:* The most active fractions from the above column were pooled and subjected to isoelectric focusing at 4 °C in an LKB UNIPHOR 7900 electrophoresis system with a linear density gradient of 0–45% (w/v) sucrose, containing 0.5% Ampholine pH 3.5–5, and 0.6% (w/v) Ampholine pH 5–8, according to Vesterberg [6]. The electrode solutions were: cathode, 0.2 M NaOH, and anode, 0.45 M H_2SO_4 containing 50% (w/v) sucrose. Starting current (10 mA) had to be occassionally adjusted during the first 12 h until it became stable (2 mA). After 40 h, 2.5 ml fractions were collected.

pH values were measured with a Beckman Expandomatic SS-2 pH-meter endowed with a full-range combination electrode.

Enzyme assays

NAD(P)H-cytochrome *c* reductase and NAD(P)H-nitrate reductase activities were determined spectrophotometrically according to earlier described methods [7].

NAD(P)H-cytochrome *c* reductase ammonia repressible related to nitrate reduction was identified by reconstitution of NAD(P)H-nitrate reductase complex through *in vitro* complementation with terminal nitrate reductase of mutant 305 [3].

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

Analytical methods

Protein was estimated spectrophotometrically by the method of Bailey [8] using bovine serum albumin as standard. Nitrite was measured as described by Snell and Snell [9]. Spectrophotometric determinations were performed in a PYE-UNICAM SP-8-100 recording spectrophotometer.

Electrophoresis

Analytical disc gel electrophoresis was carried out at 4 °C in 7.5% acrylamide gels according to Jovin *et al.* [10] in a Shandon apparatus. NAD(P)H-diaphorase activities were located on the gels after the run with 4-nitrobluetetrazolium as described by Wang and Raper [11].

Molecular weight determination

Molecular weight of diaphorases were determined by running extracts and protein standards on polyacrylamide gels of 3.75, 5, 6.25 and 7.5% (w/v) total acrylamide concentration as described by Hedrick and Smith [12]. Standards used were: carbonic anhydrase (M_r 30000), ovalbumin (M_r 43000), bovine serum albumin (M_r 67000), lactate dehydrogenase (M_r 140000), and catalase (M_r 232000).

Results and Discussion

Wild and 104 mutant strain of *C. reinhardii* grown on ammonia and derepressed with nitrate showed the same diaphorase bands distribution on 7.5% polyacrylamide gels, with a rapidly migrating band of activity repressible by ammonia in the running front which is the sole NAD(P)H-diaphorase activity capable of reconstituting the NAD(P)H-nitrate reductase native complex by complementation with extracts of 305 derepressed with nitrate [1–3].

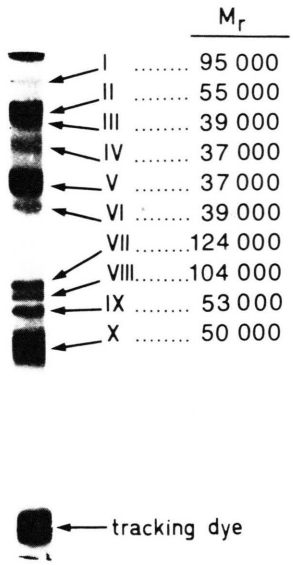

Fig. 1. Molecular weights of NADH-diaphorases of *C. reinhardii* 104 mutant. 0.2 mg samples of crude extracts of nitrate derepressed 104 and protein standards were subjected to disc gel electrophoresis with different concentrations of total acrylamide and stained for NADH-diaphorase activity as described in Experimental. Molecular weights were calculated from plots of log (100 × R_F) vs. acrylamide concentrations and from slopes vs. molecular weights according to Hedrick and Smith [12]. On the left side, the NADH-diaphorases are visualized on 7.5% polyacrylamide gels. On the right side, their corresponding molecular weights are indicated.

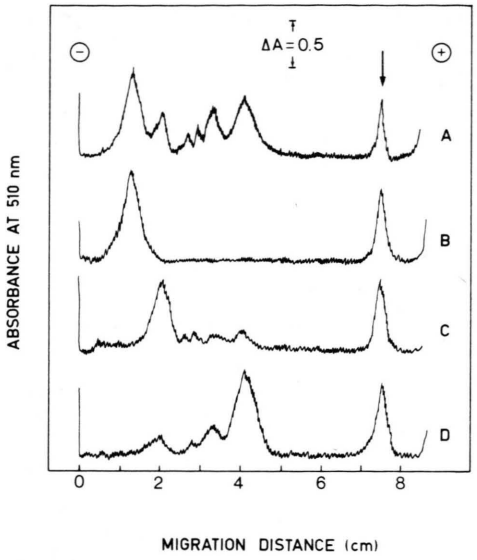

Fig. 2. Densitograms of polyacrylamide gels stained for NADH-diaphorases from *C. reinhardii* 104 mutant. A: partially purified diaphorase preparation before isoelectric focusing; B: fraction 36 from isoelectric focusing separation (Fig. 3); C: fraction 49 from Fig. 3; D: fraction 52 from Fig. 3. The arrow indicates the tracking dye absorption.

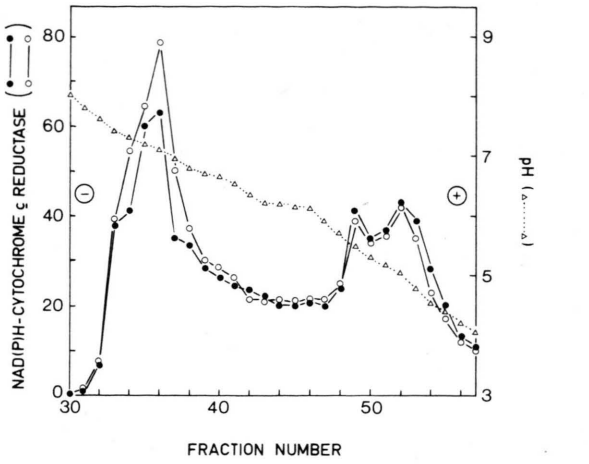

Fig. 3. Isoelectric focusing profile of partially purified diaphorase from *C. reinhardii* 104 mutant. One hundred ml of partially purified 104 diaphorase (19 U, 285 mg) was subjected to isoelectric focusing in a sucrose density gradient as detailed in Experimental. At the end of the process, 2.5 ml fractions were collected and pH (△ – – △) and NADH- (○—○) and NADPH- (●—●) cytochrome *c* reductase activities were measured. Activity is expressed as mU/ml. The recovery was 12 per cent of the starting activity.

In Fig. 1, the bands of NADH-diaphorases of derepressed 104 mutant and their corresponding molecular weight values (37000 – 124000) calculated by the method of Hedrick and Smith [12] are presented. Several diaphorases of molecular weights covering a similar range have been described as isoenzymes of ferredoxin-$NADP^+$ reductase in spinach [13, 14]. A molecular weight of 50000, very close to that previously found by other methods was calculated for the ammonia repressible NAD(P)H-diaphorase, very recently characterized as the diaphorase subunit of the native nitrate reductase enzyme complex [2, 3, 15].

Isolation of this diaphorase activity from all the others unrelated to nitrate reductase, by using conventional chromatographic procedures, was a difficult task since always remained at least three NAD(P)H-cytochrome *c* reductases of similar charge and size [2]. Hence, we proceeded to eliminate most of the diaphorases of low migration velocity and unrelated to nitrate reductase by the purification procedure outlined in Experimental.

The eluate from the last purification step still contained a mixture of ammonia repressible NAD(P)H-cytochrome *c* reductase and several diaphorases unrelated to nitrate reductase (Fig. 2A). When this preparation was subjected to preparative isoelectric focusing, three diaphorase fractions with isoelectric points of 7.1, 5.5 and 5.05 were obtained (Fig. 3).

NADH acted as better electron donor than NADPH for cytochrome *c* reductase of diaphorase of pI 7.1, whereas NADPH was slightly more efficient for those of pI 5.5 and 5.05. The diaphorase of pI 7.1 showed a unique band of activity when subjected to analytical electrophoresis on 7.5% polyacrylamide gels (Fig. 2B). The diaphorase fraction of pI 5.5 exhibited a main diaphorase band together with other minor components (Fig. 2C), whereas that of pI 5.05 contained predominantly the ammonia repressible diaphorase with high migration rate and related to nitrate reductase (Fig. 2D). In this respect, it is worth remembering that NADPH has been described as better donor than NADH for the diaphorase activity of 6145 *c* and for the diaphorase related to nitrate reduction of derepressed 104 mutant [16].

When the above diaphorase fractions were tested for complementation *in vitro* with partially purified preparations of terminal nitrate reductase from *C. reinhardii* 305 mutant, only the NAD(P)H-cytochrome *c* reductase fraction of pI 5.05 was capable

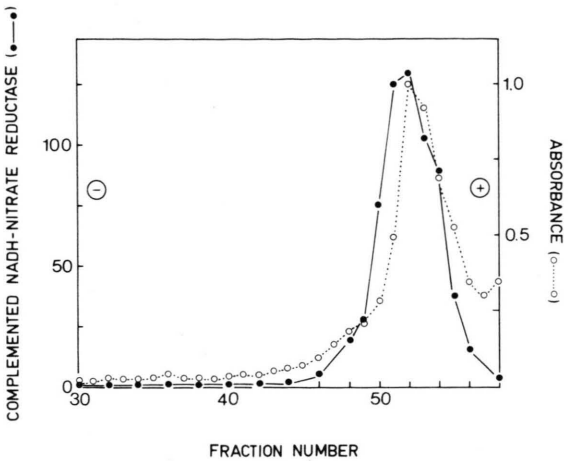

Fig. 4. Identification of the subunit NAD(P)H-cytochrome *c* reductase of *C. reinhardii* nitrate reductase by *in vitro* complementation with partially purified extracts of 305 mutant. 0.2 ml of fractions from isoelectric focusing was mixed with 0.05 ml of an enzymatic preparation of 305 mutant (120 mU of terminal nitrate reductase/ml), incubated at 45 °C, 5 min, and thereafter NADH-nitrate reductase activity, expressed as nmol NO_2^-/h · ml, and absorbance at 280 nm were measured.

of reconstituting the NAD(P)H-nitrate reductase complex (Fig. 4). A similar value of isoelectric point of 5.0 has been reported for the spinach nitrate reductase complex [17].

The isoelectric focusing technique has been also used in the purification of *Neurospora crassa* NADPH-nitrate reductase [18] although no similar utilization has been made with ammonia repressible low molecular weight NADPH-cytochrome *c* reductases which have been described as true subunits of nitrate reductase complex of *N. crassa*, *Aspergillus nidulans*, *C. reinhardii*, and spinach on the basis of *in vitro* specific reconstitution experiments [2, 3, 19–22].

Acknowledgements

The authors thank Prof. M. Losada for the provision of facilities. This work was supported in part by grants from Comisión Asesora de Investigación (Spain) and Philips Research Laboratories (The Netherlands). One of us (E.F.) thanks Ministerio de Educación y Ciencia (Spain) for a fellowship.

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