Purification and characterization of assimilatory nitrite reductase from *Candida utilis*

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Nitrate assimilation in many plants, algae, yeasts and bacteria is mediated by two enzymes, nitrate reductase (EC 1.6.6.2) and nitrite reductase (EC 1.7.7.1). They catalyse the stepwise reduction of nitrate to nitrite and nitrite to ammonia respectively. The nitrite reductase from an industrially important yeast, *Candida utilis*, has been purified to homogeneity. Purified nitrite reductase is a heterodimer and the molecular masses of the two subunits are 58 and 66 kDa. The native enzyme exhibits a molecular mass of 126 kDa as analysed by gel filtration. The identity of the two subunits of nitrite reductase was confirmed by immunoblotting using antibody for *Cucurbita pepo* leaf nitrite reductase. The presence of two different sized transcripts coding for the two subunits was confirmed by (a) *in vitro* translation of mRNA from nitrate-induced *C. utilis* followed by immunoprecipitation of the *in vitro* translated products with heterologous nitrite reductase antibody and (b) Northern-blot analysis. The 66 kDa subunit is acidic in nature which is probably due to its phosphorylated status. The enzyme is stable over a range of temperatures. Both subunits can catalyse nitrite reduction, and the reconstituted enzyme, at a higher protein concentration, shows an activity similar to that of the purified enzyme. Each of these subunits has been shown to contain a few unique peptides in addition to a large number of common peptides. Reduced Methyl Viologen has been found to be as effective an electron donor as NADPH in the catalytic process, a phenomenon not commonly seen for nitrite reductases from other systems.

INTRODUCTION

Nitrate is a major source of inorganic nitrogen utilized by most plants [1,2], algae [3], yeast [4] and bacteria [5]. The nitrate-assimilatory pathway is mediated by two enzymes, nitrate reductase (EC 1.6.6.2) and nitrite reductase (EC 1.7.7.1), which catalyse the stepwise reduction of nitrate to nitrite and nitrite to ammonia respectively.

Nitrite reductase was first identified by Losada and Peneque [6] as an enzyme that catalyses the six-electron reduction of nitrite to ammonia. The enzyme has since been purified from a variety of plants, including spinach [7–9], calabash [10], *Cucurbita pepo* [11], *Chlorella* [12], barley [13,14] and pea [15]. The enzymes from cyanobacterial species [5, 16] and red algae [17] have also been purified. The enzyme in general is known to be composed of one polypeptide [5, 9, 13, 18], although it has also been reported as a heterodimer composed of two subunits 61–64 kDa and 24–35 kDa [19, 20]. In photosynthetic organisms, the physiological electron donor is ferredoxin. Nitrite reductases from these organisms can use reduced Methyl Viologen as an artificial substitute for ferredoxin. For non-photosynthetic organisms the electron donor used under physiological conditions is NADPH, which is also the donor used in *in vitro* assays of the enzyme [21].

The *Candida* species belongs to the yeast family which is now universally recognized as an important experimental model system. *Candida utilis*, owing to its high protein content (72 %), is considered to be a fodder yeast and a potential microbial source of protein for animal feed as well for human consumption. It has been industrially exploited for the production of compounds such as nucleotides, nucleosides, glutathione, NAD^+ and CoA. Besides its commercial importance, *C. utilis* provides an ideal system for studying nitrogen assimilation by virtue of its rapid growth, non-pathogenicity and adaptability to growth on nitrate as the sole source of nitrogen.

By a genetic screen, a gene cluster which also contains the nitrite reductase gene has been cloned from a fungus, *Aspergillus nidulans* [22]. However, the purification and characterization of this enzyme has not been reported from any of the yeasts, many of which also use nitrate as a source of assimilable nitrogen. Here we report for the first time the purification of nitrite reductase from a yeast, *C. utilis*, and provide evidence for its heterodimeric nature and its ability to use both NADPH and Methyl Viologen as electron donor under *in vitro* conditions. We also report the separation of the individual subunits of the enzyme, and demonstrate their catalytic potential before and after renaturation.

MATERIALS AND METHODS

Chemicals

Rabbit reticulocyte lysate was obtained from Amersham International, Amersham, Bucks, U.K. L-[^{35}S]Methionine (1200 Ci/mmol), [α-^{32}P]dATP (specific radioactivity 3000 Ci/mmol) and [^{32}P]orthophosphate were obtained from Bhaba Atomic Research Centre, Bombay, India. X-Ray film XR-2, developer and fixer were obtained from the Indian Photographic Company Bombay, India. Oligo(dT) cellulose type 7, Sephacryl S-300 HR column and DEAE-Sephacel were purchased from Pharmacia, Uppsala, Sweden. Acrylamide, protein molecular-mass markers, agarose, Mops, Coomassie Brilliant Blue G, diethyl pyrocarbonate, DEAE-cellulose, 2-mercaptoethanol, *N*-(1-naphthyl)ethylenediamine dihydrochloride, *N*,*N*′-methylenebisacrylamide, SDS, zymolase, guanidinium isothiocyanate, Trizma base, Triton X-100 and 2,5-diphenyloxazole were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. Two-dimensional PAGE standards were purchased from Bio-Rad Laboratories, Hercules, CA, U.S.A., and silver nitrate was

Abbreviations used: poly(A)$^+$, polyadenylated; IEF, isoelectric focusing.

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obtained from Merck India. Yeast extract and peptone were obtained from Difco Laboratories, Detroit, MI, U.S.A. All other chemicals of analytical grade were purchased from BDH, Bombay, India or Sarabhai Chemicals, Baroda, India. A reverse-phase C_8 column was purchased from Vydac Separation Group.

Organism and growth conditions

C. utilis CBS 4511 (wild-type), obtained from the Centraalbureau voor Schimmelcultures, Delft, The Netherlands, was used in all experiments. It was maintained by monthly transfers on Sabouraud's dextrose/agar slants containing 1 % neopeptone, 1 % dextrose, 0.2 % yeast extract and 2 % agar. The basal medium used to grow *C. utilis* was essentially as described by Wickerham [23] with a few modifications. The medium was supplemented with 1 % glucose as carbon source and 50 mM potassium nitrate as the sole nitrogen source. The medium (200 ml) was inoculated with a seed culture grown for 12 h at a level of 2.5 % (v/v), and was grown on a gyratory shaker (200 rev./min) at 30 °C for 8 h. The cells were harvested by centrifugation at 3000 ***g*** for 10 min at 4 °C in a Hitachi Himac 20B2 centrifuge, washed twice with chilled distilled water and finally with 1 M sorbitol and centrifuged again.

Preparation of spheroplasts

The cells of *C. utilis* were grown for maximum induction of nitrite reductase under the standardized growth conditions mentioned above. The cells (3 g) from 8 h culture were harvested. Spheroplasting of the cells was carried out essentially by the method of Winterberger et al. [24]. The harvested cells were resuspended in spheroplasting buffer composed in 1 M sorbitol, 50 mM potassium phosphate, pH 7.5, and 15 mM 2-mercaptoethanol. After the addition of 2-mercaptoethanol (4 μl/ml), the suspension was kept at 30 °C for 10 min. Zymolase was added to yield a final concentration of 1 mg/g of cells and incubation was continued at 30 °C for 1–2 h with slow agitation. The progress of the spheroplast formation was monitored spectrophotometrically, whereby, on addition of 0.1 % SDS, the A_{600} of the spheroplast suspension reduces to one-tenth of the original. The spheroplasts were pelleted by centrifugation at 4000 ***g*** for 10 min, washed with 1 M sorbitol and centrifuged again. The resultant pelleted spheroplasts were used for isolation of total RNA.

Preparation of cell-free extracts

The cells (30 g) were suspended in 30 ml of buffer A (10 mM Tris/HCl, pH 8, containing 1 mM EDTA, 0.1 mM PMSF, 10 mM 2-mercaptoethanol and 10 % glycerol) and disrupted in a French pressure cell at 103.5 MPa (15000 lbf/in²) twice. The slurry was centrifuged at 8000 ***g*** for 20 min at 4 °C. The supernatant (cell-free extract) was used in the subsequent steps.

Purification of nitrite reductase

Crude extract was subjected to acetone precipitation (0–35 %) at −10 °C (using an ice/salt mixture). After 15 min of stirring, the suspension was centrifuged at 10000 ***g*** for 10 min. The precipitate was resuspended in buffer B (buffer A containing 200 mM NaCl) and stirred for 15 min, allowed to stand on ice for 30 min and centrifuged at 10000 ***g*** for 10 min. The supernatant was loaded on a DEAE-cellulose column (15 cm × 2 cm) previously equilibrated with buffer B. The washing obtained from the DEAE-cellulose column with the same buffer B was subjected to 0–35 %- and 35–70 %-satd. $(NH_4)_2SO_4$ precipitations at 4 °C, the pH being maintained at 8.0 by the addition of 1 M solution of Tris base. The pellet obtained from the 35–70 %-satd. $(NH_4)_2SO_4$ precipitation by centrifugation at 12000 ***g*** for 30 min was dissolved in a minimal amount of buffer A and dialysed against buffer C (buffer A containing 100 mM NaCl) for 4 h at 4 °C, with a change of dialysis buffer after 2 h.

The dialysed sample was loaded on a second DEAE-cellulose column (20 cm × 1.5 cm) previously equilibrated with buffer C. The column was washed with 3 column vol. of buffer D (buffer A containing 135 mM NaCl). The proteins were then eluted with buffer E (buffer A containing 170 mM NaCl), and 3 ml fractions were collected. The active fractions were pooled, the volume was measured, and the pool was subsequently diluted with buffer A so that the final NaCl concentration was 100 mM. The diluted sample was then applied to a DEAE-Sephacel column (10 cm × 1.5 cm) previously equilibrated with buffer D. Buffer D (2 bed vol.) was used for washing. Elution was carried out with buffer E, and 5 ml fractions were collected. The fractions with enzyme activity were again pooled and subjected to $(NH_4)_2SO_4$ precipitation (0–50 % saturation) at 4 °C, maintaining the pH at 8.0 by the addition of a solution of 1 M Tris base. After being stirred at 4 °C for 30 min, the precipitated protein was pelleted by centrifugation at 12000 ***g*** for 15 min and dissolved in a minimum volume of buffer F (100 nM phosphate buffer, pH 7.5, containing 0.1 mM PMSF, 1 mM EDTA, 10 mM 2-mercaptoethanol and 50 % glycerol) and stored at −20 °C.

The entire purification protocol was also carried out with buffers that lacked 2-mercaptoethanol and the purified enzyme was similarly stored at −20 °C.

Estimation of protein

The protein content was determined by the method of Lowry as modified by Hartree [25], with crystalline BSA as standard.

Assay of nitrite reductase

Methyl Viologen nitrite reductase activity was assayed by the method of Ida [8] with minor modifications. The reaction mixture in a final volume of 1 ml contained: 75 mM Tris/HCl (pH 8.0), 2 mM KNO_2, 1.5 mM Methyl Viologen, 20 mM potassium dithionite (freshly dissolved in 0.29 M $NaHCO_3$) and an appropriate amount of the enzyme. The reaction was carried out for 5 min at 30 °C and stopped by vigorous shaking to obtain complete oxidation of the excess reductant. Nitrite disappearance was determined after a 100-fold dilution of the reaction mixture by the diazo coupling method [26].

NADPH nitrite reductase activity was determined by the method of Garrett [27] with minor modifications. The reaction mixture contained: 25 mM potassium phosphate buffer (pH 7.5), 0.1 mM KNO_2, 0.01 mM FAD^+, 0.1 mM NADPH, enzyme and distilled water to make up the volume to 1 ml. The reaction was initiated by the addition of enzyme and the activity was estimated spectrophotometrically at 30 °C by monitoring the disappearance of NADPH at 340 nm.

The apparent K_m for nitrite was estimated from the Lineweaver–Burk plots for both NADPH and Methyl Viologen nitrite reductase activities. These assays were carried out exactly as above except that the concentration of nitrite was progressively increased. The amount of the enzyme used for these assays was 15 μg (protein) and the enzyme reaction was stopped after 3 min. Blank tubes without the enzyme at each nitrite concentration were included. The range for the nitrite concentrations for NADPH nitrite reductase assay was 0.8–7.5 mM and that for the

Methyl Viologen nitrite reductase assay was 0.2–2 mM. For each experimental point in the Lineweaver–Burk plot, the mean value from three sets of experiments (each in duplicate) is given. Values that differ by more than 10 % have been discarded. The apparent values of V_{max} and K_m have also been confirmed by Hofstee plots. The plots were drawn by using the Grapher software package. For the determination of fold purification, the dependence of reaction velocity on enzyme concentration and the optimum temperature, Methyl Viologen nitrite reductase activity was measured, and for the determination of optimum pH and the time course of the purified nitrite reductase, activities with both Methyl Viologen and NADPH were separately measured.

Electrophoresis

Non-denaturing PAGE (native PAGE) was carried out in vertical slab gels as described by Davis [28]. The protein band(s) were visualized by silver staining [29]. SDS/PAGE was carried out by the method of Laemmli [30]. Nitrite reductase purified using buffers containing 2-mercaptoethanol was used in sample buffer containing 0.1 % SDS and 0.35 mM 2-mercaptoethanol in Tris/HCl, pH 6.8. Sample buffer without 2-mercaptoethanol was used for the enzyme that had been purified with buffers from which 2-mercaptoethanol had been omitted. The samples were heated for 3 min at 100 °C and loaded on to the gels.

Western-blot analysis

Immunoblotting was carried out essentially by the method of Towbin et al. [31]. The enzyme (25 μg of protein) was subjected to SDS/PAGE (10 % gel), and transferred to poly(vinylidene difluoride) membrane in a Pharmacia LKB Novablot apparatus using a transfer buffer containing 25 mM Tris, 192 mM glycine, 0.1 % SDS and 20 % methanol. Polyclonal antibody raised against *C. pepo* nitrite reductase [15] at a dilution of 1:1500 was used as the primary antibody. Anti-rabbit goat IgG (conjugated to horseradish peroxidase) at a dilution of 1:1000 was used as the secondary antibody. The colour reaction was carried out in citrate/phosphate buffer (438 mg of citric acid, 1.3 g of disodium hydrogen phosphate, pH adjusted to 5.0 with HCl) containing 1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride and 3 μl of H_2O_2/ml of solution.

Determination of molecular mass

The subunit molecular mass of the enzyme was determined from their relative mobilities on an SDS/polyacrylamide gel compared with standard proteins. The molecular mass of the native enzyme was determined by FPLC using a Sephacryl S-300 HR gel-filtration column, comparing the relative elution volume of nitrite reductase with those of several standard proteins. Molecular-mass standards used were: Blue Dextran, 2000 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; egg albumin, 45 kDa. The elution buffer used for gel filtration contained 10 mM Tris/HCl, pH 8.0, and 200 mM NaCl.

Isolation of poly(A)$^+$ RNA

The total RNA from spheroplasts was extracted by the method of Chomczynski and Sacchi [32]. From the total RNA, poly(A)$^+$ RNA was separated as described by Sambrook et al. [33]. After the final step of ethanol precipitation the RNA pellet was dissolved in diethyl pyrocarbonate-treated water.

In vitro translation of poly(A)$^+$ RNA

Freshly isolated poly(A)$^+$ RNA (10 μg) was used to prime a rabbit reticulocyte system in the presence of 15 μCi of [^{35}S]methionine. The *in vitro* translation was followed by either the incorporation of [^{35}S]methionine into trichloroacetate-precipitable proteins or by subjecting the *in vitro* translated products to SDS/PAGE followed by fluorography [34] and autoradiography. A control in which no mRNA was added was also carried out.

Northern-blot analysis

For the Northern blots, 20 μg of the poly(A)$^+$ RNA was denatured in a mixture of 12.5 μl of deionized formamide, 2.5 μl of 10 × Mops (0.2 M Mops, 0.05 M sodium acetate, 0.01 M EDTA, pH 7.5) and 4 μl formaldehyde at 65 °C and electrophoretically separated on an agarose/formaldehyde gel [1.2 % (w/v) agarose in 1 × Mops buffer containing 6 % formaldehyde] [33]. The RNA was transferred to nylon membranes in 20 × SSC (3 M NaCl/0.3 M sodium citrate, pH 7.0) and UV-fixed.

The blots were prehybridized for 8 h in solution containing 50 % formamide, 5 × SSC, 10 × Denhardt's solution (100 × Denhardt's solution is 2 % each of BSA, Ficoll and polyvinylpyrrolidine 40), 50 mM sodium phosphate buffer (pH 7.0), 0.2 % SDS, 7.5 % dextran sulphate and 100 μg/ml denatured salmon sperm DNA. Hybridization was carried out for 24 h at 42 °C in the same buffer containing the labelled denatured DNA probe.

The nitrite reductase cDNA from *Aspergillus nidulans* (pSTA 2,3,4 and 8) [22] was labelled with [α-^{32}P]dATP by nick translation [35] to a specific radioactivity of 3×10^8–6×10^8 c.p.m./μg of DNA. The filters were finally washed with 0.1 % SDS in 2 × SSC for 30 min at 42 °C, and subjected to autoradiography. After detection of the signal, the filters were stripped of the probe by submerging them in 0.1 % SDS for 30 min at 100 °C and rehybridized to radiolabelled *Candida albicans* actin gene [36], to serve as an internal control.

Immunoprecipitation

Immunoprecipitations were carried out by the method of Perbal [37] with the products obtained from *in vitro* translation of the poly(A)$^+$ RNA from *C. utilis*. The polyclonal antibody used (at a dilution of 1:1500) in the study was raised against *C. pepo* nitrite reductase. The immunoprecipitated products were subsequently separated by SDS/PAGE. The gels were fluorographed, dried and autoradiographed.

Two-dimensional gel electrophoresis

Purified nitrite reductase (25 μg) was subjected to two-dimensional gel electrophoresis by the method of O'Farrell [38]. The proteins were separated in the first dimension by isoelectric focusing (IEF) in the presence of ampholytes (40 % stock of pH range 3–10). The electrophoretic conditions were: 200 and 400 V for the first and second hour respectively, followed by 900 V for the remaining 10 h.

In the second dimension, the tube gel was layered on a polyacrylamide SDS/7.5 % gel and electrophoresed at 80 V. The gel was subsequently silver stained to detect protein spots. The pI of the subunits was calculated with reference to the two-dimensional PAGE standard proteins which were run under identical conditions and silver stained. The standards used were hen egg conalbumin, BSA, bovine muscle actin, rabbit muscle glyceraldehyde-phosphate dehydrogenase, bovine carbonic anhydrase, soya bean trypsin inhibitor and equine myoglobin.

In vivo labelling of the *C. utilis* cells

For *in vivo* labelling of the *C. utilis* cells with [^{32}P]orthophosphate, the cells were grown in the presence of radioactive phosphate (100 μCi/ml) in phosphate-free Wickerham medium under the growth conditions described above. The labelled cells were harvested, washed and disrupted in a French pressure cell. The lysate was centrifuged at 8000 ***g*** for 20 min at 4 °C, and the supernatant was used for immunoprecipitation.

Separation of active subunits

The two subunits of nitrite reductase were eluted from the SDS/polyacrylamide gels essentially by the method of Hager and Burgess [39]. The two subunits of the purified enzyme were visualized by 0.25 M KCl staining [39], the relevant regions of the gels were cut out, crushed and the protein was eluted at room temperature for 1 h. SDS was removed by subjecting the sample to a 4 vol. acetone precipitation. The precipitate was dissolved in 6 M guanidinium chloride in a dilution buffer [50 mM Tris/HCl, pH 8.0, 20 % glycerol, 150 mM NaCl, 1 mM dithiothreitol and 0.1 mM EDTA] and allowed to stand for 30 min. The solution was diluted 50-fold and the subunits were renatured for 12–24 h at room temperature.

Peptide mapping

The purified subunits (100 μg of protein) were subjected to a 2 % tryptic digestion for 3 h at 37 °C. The digest was separated on a Vydac reverse-phase C_8 column (pore size 0.6 nm) in a Shimadzu LC 6A HPLC machine with an acetonitrile gradient of 0–100 % for 100 min. The data were analysed using chromatopac integrator CR 4A from Shimadzu.

RESULTS

Purification of nitrite reductase

The nitrite reductase from *C. utilis* was purified over 200-fold. A specific activity around 16.0 and a yield of 4 % were achieved by a combination of acetone fractionation, $(NH_4)_2SO_4$ precipitations, DEAE-cellulose chromatography and DEAE-Sephacel chromatography (Table 1).

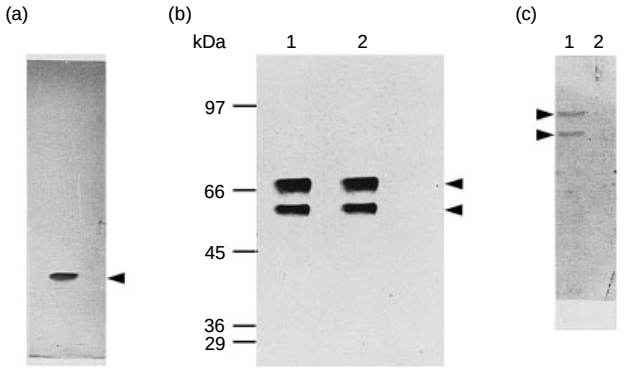

Figure 1 Homogeneity of nitrite reductase from *C. utilis*

(**a**) The purified nitrite reductase (20 μg of protein) was run on a 6 % native polacrylamide gel and stained with silver nitrate. (**b**) The purified sample (20 μg of protein) was analysed by an SDS/PAGE (10 % gel) and subjected to silver staining. Lane 1, enzyme purified without 2-mercaptoethanol in buffer and loaded after mixing with a sample buffer without 2-mercaptoethanol; lane 2, same as in lane 1 but with 2-merceptoethanol. (**c**) The purified enzyme (25 μg of protein) was subjected to SDS/PAGE (10 % gel), transferred to poly(vinylidene difluoride) membrane and probed with nitrite reductase antibody (lane 1) or with normal rabbit serum (lane 2).

Electrophoretic analysis of the purified enzyme

The enzyme obtained after the second $(NH_4)_2SO_4$ step was electrophoresed on 6, 7.5, 10 and 12 % native polyacrylamide gels and silver stained. A single band was always obtained indicating the homogeneity of the purified enzyme. A representative picture of the single band visualized on staining in a 6 % non-denaturing gel is shown in Figure 1(a). When the same preparation was run on an SDS/10 % polyacrylamide gel, two bands of molecular mass 66 and 58 kDa were observed irrespective of whether the sample loading buffer contained 2-mercaptoethanol or not (Figure 1b, lanes 1 and 2).

Immunoblot analysis of the purified enzyme

Two polypeptide bands of molecular mass 66 and 58 kDa were observed on immunoblotting of purified enzyme with a heterologous polyclonal antibody against *Cuc. pepo* leaf nitrite re-

Table 1 Purification of nitrite reductase from *C. utilis*

One unit of nitrite reductase is defined as the amount of enzyme that catalyses the reduction of 1 μmol of nitrite/min under standard assay conditions. For the crude extract, 30 g of cell mass was used.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
Crude extract	150	1650.0	128.7	0.07	100.00	1.00
Acetone precipitation (0–35 %)	48	528.0	96.1	0.18	74.66	2.6
$(NH_4)_2SO_4$ precipitation I (35–70 %)	18	126.3	73.3	0.58	56.95	8.3
DEAE-cellulose II	72	5.37	64.4	11.99	50.00	171.3
DEAE-Sephacel	52.5	3.76	47.8	12.7	37.14	181.4
$(NH_4)_2SO_4$ precipitation II (0–50 %)	6.9	0.33	5.3	16.06	4.11	229.4

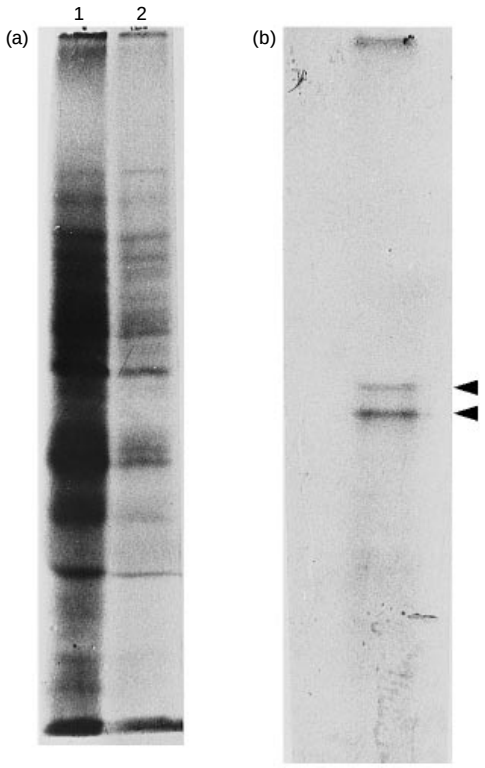

Figure 2 *In vitro* translation of poly(A)$^+$ RNA from *C. utilis* and immunoprecipitation of the *in vitro* translated products

(**a**) Poly(A)$^+$ RNA (10 μg) was used for *in vitro* translation using a rabbit reticulocyte lysate system in presence of 15 μCi of [^{35}S]methionine. The *in vitro* translated products were separated by SDS/PAGE (10% gel): lanes 1 and 2 contained 60000 c.p.m. and 15000 c.p.m. of radiolabelled proteins respectively. (**b**) The *in vitro* translated proteins were immunoprecipitated with *C. pepo* nitrite reductase antibody (details in the Materials and methods section). The immunoprecipitated products were subjected to SDS/PAGE (10% gel).

ductase (Figure 1c, lane 1). When normal rabbit serum was used as the primary antibody no bands were observed (Figure 1c, lane 2), indicating the specificity of the antigen–antibody interaction.

In vitro translation of poly(A)$^+$ RNA and the immunoprecipitation of the translated products

In vitro translation of the poly(A)$^+$ RNA (isolated from the optimally induced *C. utilis* cells) showed a more than 30-fold increase in incorporation of [^{35}S]methionine into the trichloroacetate-precipitable radioactivity (results not shown). SDS/PAGE and fluorography of the *in vitro* translated products indicated that polypeptides with a wide range of molecular mass had been synthesized (Figure 2a, lanes 1 and 2). Immunoprecipitation of the *in vitro* translated products with the *C. pepo* leaf nitrite reductase antibody, on SDS/PAGE and fluorography, showed the presence of two bands corresponding to 66 and 58 kDa (Figure 2b). In a control experiment in which no mRNA was added to rabbit reticulocyte lysate in the presence of [^{35}S]methionine, no incorporation of the radioactivity in the trichloroacetate-precipitable material occurred. On SDS/PAGE of this translation mixture, no newly synthesized proteins were observed (results not shown).

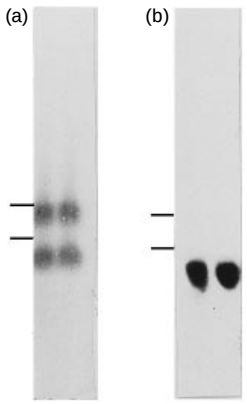

Figure 3 Northern-blot analysis of poly(A)$^+$ RNA from *C. utilis*

Poly(A)$^+$ RNA (20 μg) isolated from *C. utilis* was used for Northern-blot analysis as described in the Materials and methods section. The blot was first probed with (**a**) heterologous nitrite reductase clone from *A. nidulans* and (**b**) subsequently deprobed and reprobed with actin gene from *C. albicans*.

Detection of transcripts coding for nitrite reductase

When the poly(A)$^+$ RNA isolated from *C. utilis* cells was probed with heterologous nitrite reductase gene, two transcripts of approximate size 2–3.5 kb were detected (Figure 3a). The actin-specific transcript was also observed when the same blot was deprobed and reprobed with heterologous actin gene (Figure 3b).

Characteristics of the purified enzyme

Electron donors

The effect of nitrite concentrations on both NADPH nitrite reductase activity and Methyl Viologen nitrite reductase activity is presented in Figures 4(a) and 4(b) respectively. It is apparent that this enzyme is capable of utilizing both NADPH and reduced Methyl Viologen as the electron donor. From the plot of the reciprocal of nitrite concentrations against the reciprocal of reaction velocities, the apparent kinetic constants were calculated. When NADPH was used as the electron donor, the values were: V_{max} (20.50 ± 1.32 nmol/min per μg of protein) and K_m (69.6 ± 0.4 μM). When Methyl Viologen was used, the values were: V_{max} 30.68 ± 0.79 nmol/min per μg of protein) and K_m (80.1 ± 0.9 μM).

Molecular-mass determination

Gel-filtration chromatography under non-denaturing conditions in a Sephacryl S-300 HR column indicated a mass of 126 kDa for the native enzyme.

Biochemical parameters

The initial rate of nitrite reduction was proportional to the enzyme concentration up to 15 μg of protein/ml of reaction mixture. The linearity of nitrite reduction was maintained up to 3 min. Maximum enzyme activity was obtained at pH 8.0 with appreciable amounts of activity in the pH range 7.8–8.3 in the Tris/HCl buffering system. In the phosphate-buffering system

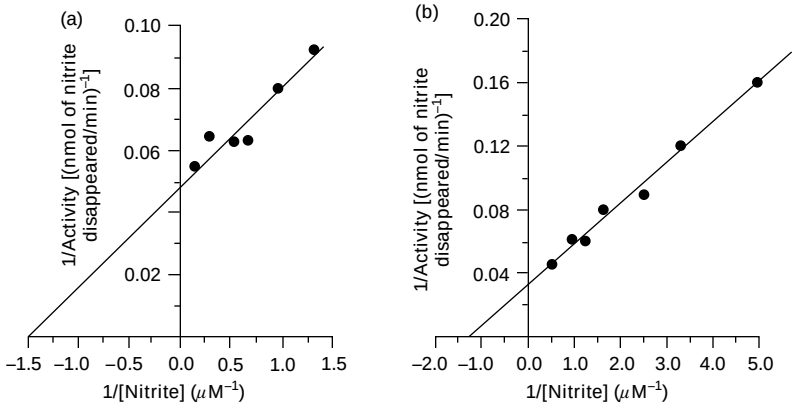

Figure 4 Lineweaver–Burk plot of nitrite reductase activity as a function of nitrite concentration

Assay conditions were essentially as described as in the Materials and methods section. The enzyme (15 μg of protein) was incubated for 3 min for the kinetic analysis. NADPH (**a**) and reduced Methyl Viologen (**b**) were used as electron donors. From the plot, the apparent kinetic constants were calculated.

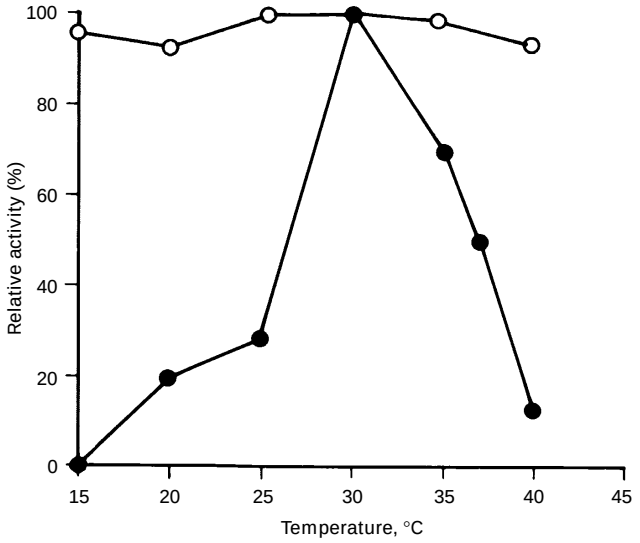

Figure 5 Effect of temperature on the stability of nitrite reductase

The enzyme (15 μg) was incubated for 4 min at the indicated fixed temperatures and the assay for residual nitrite reductase activity was carried out at 30 °C after an incubation of 1 min (○). The same amount of enzyme was assayed for residual activity after incubation with the assay components at the different fixed temperatures for 5 min (●). The term 'relative activity' indicates the relative amount of nitrite reductase activity/min at the different fixed temperatures with respect to the activity at 30 °C.

the enzyme showed an optimum pH of 7.5. Purified nitrite reductase showed no marked decrease in activity when it was assayed at 30 °C after a preincubation over a range of temperatures for 4 min (Figure 5). However, when the assays were carried out at the same fixed temperatures, different amounts of residual nitrite reductase activity were observed, indicating that the optimum temperature of the assay of the enzyme is 30 °C.

Two-dimensional analysis

Two-dimensional electrophoretic analysis (IEF followed by SDS/PAGE) of the purified nitrite reductase showed the presence

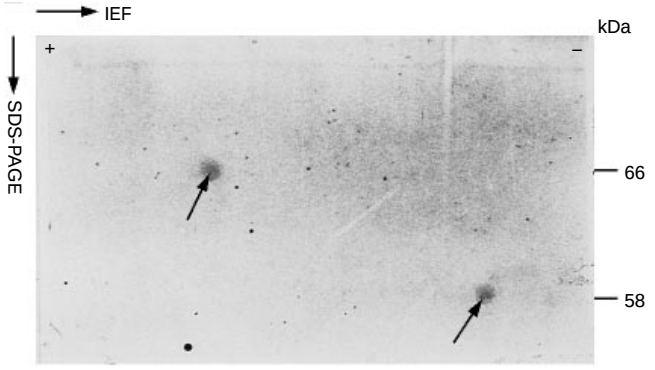

Figure 6 Two-dimensional electrophoresis of *C. utilis* nitrite reductase

Purified nitrite reductase (20 μg of protein) was used for IEF with ampholyte (pH range 3–10) in the first dimension and SDS/PAGE (7.5% gel) in the second dimension. Other conditions were as described in the Materials and methods section. The gel was subsequently silver stained.

of two polypeptides on silver staining (Figure 6). The 66 kDa subunit lies in the acidic range whereas the 58 kDa subunit lies in the basic range. Based on a comparison with two-dimensional PAGE standards, the pI value for the 66 kDa subunit was calculated to be 5.4 and that of the 58 kDa subunit 7.2.

Phosphorylation status

Immunoprecipitation of the [^{32}P]orthophosphate-labelled cell-free extract with the *C. pepo* nitrite reductase antibody was carried out. The immunoprecipitate was electrophoresed in an SDS/10% polyacrylamide gel. Parallel to the labelled immunoprecipitate, protein molecular mass markers were run. After the electrophoresis, the marker lane was cut out and silver stained. On the basis of standard molecular-mass proteins, it was found that the 66 kDa subunit is phosphorylated (Figure 7).

Purification of the subunits

The purified and renatured subunits of nitrite reductase were subjected to SDS/PAGE (10% gel) and subsequently silver stained. Two bands corresponding to the expected molecular

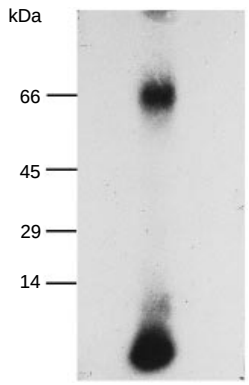

Figure 7 Phosphorylation of nitrite reductase

The [^{32}P]orthophosphate-labelled cell extract from *C. utilis* was immunoprecipitated with *C. pepo* nitrite reductase antibody. The immunoprecipitate was subjected to SDS/PAGE (10% gel), and the gel was subsequently dried and autoradiographed.

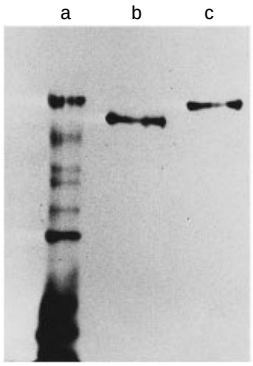

Figure 8 Separation of subunits of nitrite reductase

The two subunits were purified from a polyacrylamide gel under denaturing conditions and renatured (details in the Materials and methods section). Each of the subunits (20 μg of protein) was run on an SDS/10% polyacrylamide gel and silver stained. Lane a, molecular-mass markers; lane b, 58 kDa subunit; lane c, 66 kDa subunit.

masses (58 and 66 kDa) were observed (Figure 8), indicating the homogeneity of the respective subunits.

Enzymic activity of the individual subunits

Individual subunits were used for measurement of Methyl Viologen-linked nitrite reductase activity, in parallel with the reconstituted enzyme (Table 2). The subunits showed markedly different nitrite reductase activities, the 66 kDa subunit having a 3–4-fold higher activity than the 58 kDa subunit. When the two subunits were mixed to facilitate reconstitution and assayed, the activity obtained was almost equal to the sum of the activities of the individual subunits.

In contrast with the native enzyme, there is a proportional increase in the enzyme activity up to 50 μg of the protein for the individual subunits as well as the reconstituted enzyme. The final percentages of nitrite reduced by the native enzyme and the reconstituted enzyme were almost equal, although at different protein concentrations (results not shown).

Table 2 Activity of nitrite reductase and its subunits

The assays for the subunits, reconstituted enzyme and purified enzyme were all carried out as described in the Materials and methods section. Methyl Viologen nitrite reductase activity was measured. The values are means obtained over three independent experiments.

Amount of protein	Nitrite reduced per min (%)
12.5 μg of 58 kDa subunit	2.1
12.5 μg of 66 kDa subunit	7.9
12.5 μg of 58 kDa subunit + 12.55 μg of 66 kDa subunit	9.4
12.5 μg of purified nitrite reductase	32.4

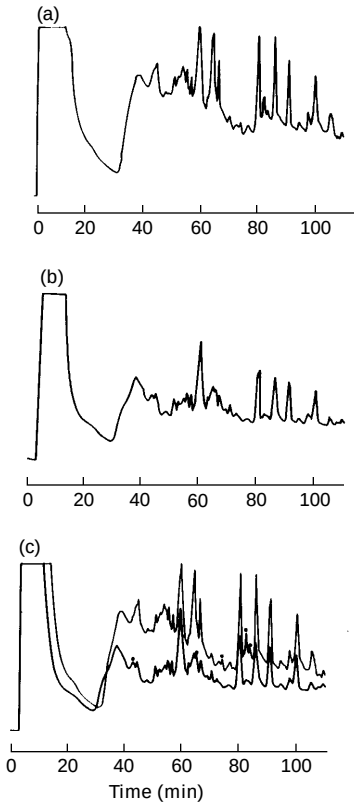

Figure 9 Elution profiles of the peptides obtained by tryptic digestion of the subunits of nitrite reductase

Each of the subunits (100 μg of protein) was subjected to tryptic digestion and separated on a reverse-phase C_8 column by HPLC (details in the Materials and methods section). (**a**) Elution profile of the peptides obtained from the 66 kDa subunit; (**b**) elution profile of the peptides obtained from the 58 kDa subunit; (**c**) superimposition of the peptide profiles obtained from the 66 and 58 kDa subunits.

Peptide mapping of the subunits

The HPLC profiles of the peptides obtained from the tryptic digest of the individual subunits are shown in Figures 9(a) and 9(b). The individual peptide maps have been superimposed to generate Figure 9(c), which gives an idea of the peaks common to both or unique to either of the two subunits. A few peaks, marked by dots, are unique to either one or other of the subunits.

However, most of the peaks are common to both the subunits as indicated by the similar elution profiles.

DISCUSSION

Although assimilatory nitrite reductase has been purified from plants, bacteria and algae, this is the first report of its purification from a yeast. The reported specific activities of the homogeneous preparations vary greatly: 3.4 for cell culture of Paul's Scarlet Rose [40], 6.5 for pea [41], 42 for *Chlorella* [12], 84 for barley [13] and 108 for spinach [18]. The specific activity of the homogeneous enzyme obtained in the present study is 16 (Table 1). However, this specific activity has been achieved only by the use of conventional purification procedures, and ferredoxin–Sepharose affinity column chromatography has not been included in the protocol. In fact the specific activity of the homogeneous enzyme obtained compares favourably with those purification protocols that do not employ the ferredoxin–Sepharose affinity-purification step [12,17,42].

The presence of two bands on the silver-stained SDS/polyacrylamide gel (Figure 1b) using the homogeneous preparation of the native enzyme (Figure 1a) indicates that the enzyme may be a heterodimer. Very few heterodimeric nitrite reductases have been reported [19,20]. In the cases that have, the subunit molecular masses reported are 61–64 kDa for the larger subunit and 24–35 kDa for the smaller subunit. Here we report the presence of two subunits, 66 and 58 kDa, differing very little in their molecular mass. The evidence that these two subunits constitute the native nitrite reductase enzyme is obtained from the gel-filtration data and the fact that a single band is observed when the native enzyme is electrophoresed under non-denaturing conditions in gels of four different concentrations.

In the case of spinach nitrite reductase, 85 kDa protein is separated into two subunits of 61 and 24 kDa in the presence of 2-mercaptoethanol [42]. In the present study, the entire purification procedure was carried out in duplicate, in both the presence and absence of 2-mercaptoethanol and in the continuous presence of PMSF, a proteinase inhibitor. The enzyme obtained from the respective preparations was electrophoresed under non-denaturing conditions in the presence or absence of 2-mercaptoethanol in the sample buffer. In both cases, 58 and 66 kDa polypeptides were obtained, indicating the absence of any intermolecular disulphide linkage (Figure 1b).

It has been reported that nitrite reductase may be synthesized as a larger precursor which is later processed into a smaller polypeptide [43]. So the presence of two polypeptides raises the question of whether there is one or two transcripts. The presence of two transcripts was confirmed by immunoprecipitation (Figure 2b) of polypeptides obtained by *in vitro* translation of poly(A)$^+$ RNA from *C. utilis* (Figure 2a). The presence of two bands corresponding to the molecular masses of the subunits of the purified enzyme is observed. The differences in band intensities may simply be a reflection of the different number of methionine residues in the two polypeptides. However, similar band intensities of the two subunits were seen in Western-blot analysis of the purified protein (Figure 1c, lane 1). Therefore we proposed that two different mRNAs are present which code for the two subunits. This has been confirmed by Northern-blot analysis of the poly(A)$^+$ RNA of *C. utilis* by probing it with nitrite reductase cDNA of *A. nidulans* [22] (Figure 3a). Such transcripts may be encoded by two separate genes or may be obtained from a single gene by a splicing reaction, which is reported to occur in yeast.

The antibody used in the immunoprecipitations was made against the monomeric *C. pepo* leaf nitrite reductase. The very fact that such an antibody recognizes both subunits of the purified nitrite reductase equally indicates the possible presence of some common epitopes in the two subunits. This was proved by the peptide maps (Figures 9a and 9b) of the individual subunits of nitrite reductase which were eluted from the gel and renatured. On superimposition of the peptide maps (Figure 9c) we can see the presence of many peptides that are common to both the subunits. This perhaps accounts for the recognition of both of them with equal intensity by the heterologous antibody. However, there are some unique peptides in both the subunits (indicated with dots in Figure 9c). These unique peptides may explain the differences in molecular mass between the two subunits; their functions are yet to be investigated.

One of the properties shared by the two subunits is the Methyl Viologen nitrite reductase activity (Table 2). Both the subunits have the ability to reduce nitrite, albeit to different levels. The additive activity on reconstitution of the two subunits indicates the functional role of both during the process of nitrite reduction. The differences in the activity of the two subunits may be due to either some differences in the three-dimensional conformation of the active site or the differential binding of the electron donor to the individual subunits.

Some of the biochemical properties of nitrite reductase have also been described in this work; they are mostly similar to those reported for the homogeneous nitrite reductases obtained from other sources. For most enzymes, the optimum temperature is not a useful biochemical parameter to measure because it is the resultant of the effect of temperature on the enzyme's stability. However, nitrite reductase was found to be stable for at least 4 min over a range of temperatures tested. However, the extent of nitrite reduction varied when the assays were carried out at whose fixed temperatures. The optimum temperature of the assay was found to be at 30 °C (Figure 5). In fact at 30 °C, the enzyme was found to retain its activity for up to 30 min (results not shown). The stability of nitrite reductase is unusual and has not been reported for the enzyme from other systems where, in fact, it has been reported to be heat-labile [7,13]. The two-dimensional analysis (Figure 6) of the purified enzyme indicates the presence of an acidic high-molecular-mass negatively charged subunit (66 kDa) which may be due to its phosphorylated nature (Figure 7). The question of whether phosphorylation is involved in the regulation of this enzyme is currently under investigation.

The apparent K_m for nitrite when Methyl Viologen (reduced by dithionite) is the electron donor ranges from 40 μM in *Phormidium laminosum* [5] to 250 μM in barley [13]. The value of 80.1 ± 0.9 μM for the *C. utilis* enzyme falls in this range. Besides reduced Methyl Viologen, the enzyme is able to use its expected electron donor NADPH. The apparent K_m value for nitrite using NADPH as the electron donor is 69.6 ± 0.4 μM (from Figure 4).

In photosynthetic organisms, the physiological electron donor is ferredoxin whereas in non-photosynthetic organisms the donor is NADPH. However, in *C. utilis*, we find a situation where the enzyme is capable of utilizing both NADPH and a ferredoxin analogue, at least under *in vitro* assays conditions.

A comparison of the cDNA sequences of the enzyme from maize [44], spinach [45] and *A. nidulans* [22] reveals that the functional domains of NADPH and ferrodoxin nitrite reductases are laid out in linear arrays (reviewed in ref. [46]). The very fact that two mutually exclusive activities are present in the same enzyme leads us to speculate that this enzyme is a member of a family of enzymes which have multiple redox centres. The first enzyme in the nitrate-assimilation pathway of *C. utilis*, nitrate reductase, was also found to possess two associated activities, NADPH nitrate reductase activity and Methyl Viologen nitrate reductase activity (T. Satyabhama, M. S. Shaila and G. R. Rao, unpublished work). Similar observations on the utilization of

NADPH and reduced Methyl Viologen as electron donors by nitrate reductase have also been reported for the yeast, *Rhodotorula glutinis* [47]. For nitrite reductase, only the enzyme from *Azotobacter chroococcum* is capable of utilizing both NADH and reduced Methyl Viologen as the electron donor. However, Methyl Viologen was utilized by the enzyme with much lower efficiency than the nicotinamide nucleotides [48]. So it is quite possible that some yeasts like *C. utilis* are also capable of assimilating nitrate by two enzymes, nitrate reductase and nitrite reductase, using either NADPH or ferredoxin at each step.

The ability to utilize Methyl Viologen raises the possibility of the presence of ferredoxin or ferredoxin-like substances in yeasts. Apart from photosynthetic bacteria, algae and higher plants, ferredoxin has been known to be present in anaerobic nitrogen-fixing bacteria [49], anaerobic parasite and free-living protozoa [50] and even in vertebrates [51,52] where ferredoxins are known to function in the transfer of reducing equivalents from NADPH oxidoreductase to cytochrome *P*-450 enzymes involved in steroid metabolism. Moreover, a recently identified family of proteins exhibit the so-called 'ferredoxin fold', and these are potentially capable of binding to ferredoxin or ferredoxin-like substances. NAD kinase [53], the RNA-binding domain of U1, the activation domain of ATCase, acyl phosphatase, the allosteric domain of procarboxypeptidase [54] and the flavoprotein ubiquinone oxidoreductase [55] are all members of this family. *Candida lipolytica* aconitase has been found to be complexed with two ferredoxins [56]. Nitrite reductase itself has been shown to form electrostatically stabilized complexes with ferredoxin [57]. These results raise the possibility of nitrite reductase also being a member of the 'ferredoxin fold' family and ferredoxin itself being present in *C. utilis*. There may be present a cryptic or functional region in the nitrate and nitrite reductases of this organism which can bind with ferredoxin or ferredoxin-like substances.

In summary, nitrite reductase has been purified and characterized from the industrially important yeast *C. utilis*; it is the first such report of the enzyme in any yeast. It has been shown to possess certain distinct and unique properties, one of them being the heterodimeric nature of the subunits, with both displaying enzyme activity. The two subunits are also differentially phosphorylated and each of them contains some unique peptides. The enzyme is also appreciably stable over a range of temperatures. Most interestingly the enzyme can utilize both Methyl Viologen and NADPH as electron donor, a capability that is not shown by nitrite reductases from other systems.

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