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PURIFICATION AND PROPERTIES OF THE NAD(P)H:NITRATE REDUCTASE OF THE YEAST *RHODOTORULA GLUTINIS*

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Summary

The assimilatory nitrate reductase from the yeast *Rhodotorula glutinus* has been purified 740-fold, its different catalytic activities have been characterized, and some inhibitors studied. The purified enzyme (150 units per mg protein) contains a cytochrome of the *b*-557 type. An $s_{20,w}$ of 7.9 S was found by the use of sucrose density gradient centrifugation, and a Stokes radius of 7.05 nm was determined by gel filtration. From these values, a molecular weight of 230 000 was estimated for the native enzyme. The purified preparation consisted of two electrophoretically distinguishable proteins, both of which exhibited nitrate reductase activity. The species with the higher electrophoretic mobility which represented the great majority of the total nitrate reductase gave a positive stain for heme and was shown to be composed of subunits with a molecular weight of about 118 000. Thus the molecule contains two subunits of the same size.

Introduction

The molybdoprotein nitrate reductase catalyzes the reduction of nitrate to nitrite, the first step in the assimilatory reduction of nitrate to ammonia. The enzyme is present in many organisms such as bacteria, fungi, algae and higher plants, that have the ability to utilize nitrate as a nitrogen source [1,2].

In spite of many attempts, there has been relatively little success in the purification of assimilatory nitrate reductases. Preparations with reasonably good

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Abbreviations: APAD, acetylpyridine-adenine dinucleotide; APADP, acetylpyridine-adenine dinucleotide phosphate.

values of specific activity (about 15 units per mg protein) have been obtained from the molds *Neurospora crassa* [3] and *Aspergillus nidulans* [4,5], but only the enzyme from *Chlorella vulgaris* has been purified to homogeneity [6,7]. The development by Solomonson [7] of a procedure for purifying *Chlorella* nitrate reductase on a Blue Dextran-Sepharose column has provided a new approach which can be applied to the enzyme from other sources.

Little information has been available about the nitrate reductase of nitrate-utilizing yeasts. A soluble enzyme requiring pyridine nucleotides has been reported in *Hansenula anomala* [8], in *Candida utilis* [9], and in *Torulopsis nitratophila* [10,11]. Such enzymes were studied without much purification. Sims et al. [12] have reported that a particulate fraction, termed "nitrosomes", prepared from *Candida utilis*, can reduce nitrate, nitrite and hydroxylamine with reduced pyridine nucleotides as reductant, but this report has not been substantiated by further publications.

The present paper reports on the purification of nitrate reductase from the yeast *Rhodotorula glutinis*, with a partial characterization of the enzyme and of the reactions it catalyzes.

Materials and Methods

Materials. Bovine serum albumin, yeast alcohol dehydrogenase, bovine liver catalase, *Escherichia coli* RNA polymerase, horse heart cytochrome *c*, NADH, NADPH, and APAD were purchased from Boehringer; protamine sulfate from Nordisk Insulin Laboratories (Copenhagen); *E. coli* β-galactosidase, rabbit muscle phosphorylase *a*, horse heart myoglobin, FAD, FMN, and APADP from Sigma; dithioerythritol from Serva (Heidelberg); Bio-Gel A-1.5 m from Bio Rad Laboratories; Sephadex G-25, Sepharose 4B, and Blue Dextran from Pharmacia. ^{3}H-labelled myoglobin and bovine serum albumin were a gift from Dr. Ruiz-Carrillo and were prepared according to Rice and Means [13]. *C. vulgaris* nitrate reductase was purified by the procedure of Solomonson [7]. Blue Dextran-Sepharose was prepared according to Ryan and Vestling [14].

Growth of cells. R. glutinis strain 0303 from the Institut für Gärungsgewerbe (Berlin) was grown aerobically at 27° C on a medium consisting of 1.17% Difco yeast carbon base and 8 mM KNO_3. After 18 h of growth the cells were harvested by centrifugation and washed with 50 mM sodium phosphate buffer (pH 7.5). The resulting cell paste was stored at −15° C until use, usually within 2 weeks, without any significant loss of nitrate reductase activity.

Analytical methods. Protein was determined by a modification of the method of Lowry et al. [15] with bovine serum albumin as a standard. Nitrite was measured as described by Snell and Snell [16]. Radioactivity measurements were made with a Nuclear Chicago ISOCAP/300 scintillation counter. Up to 0.2 ml of aqueous samples was added to 15 ml of scintillation fluid AQUASOL (New England Nuclear).

Standard nitrate reductase assays. NADPH: and NADH:nitrate reductase activity was estimated spectrophotometrically at 30° C by following the nitrate-dependent NAD(P)H oxidation at 340 nm. The standard assay system contained: 300 μmol sodium phosphate buffer (pH 7.5), 0.3 μmol NAD(P)H, 0.06 μmol FAD, and 20 μmol KNO_3, in a final volume of 3.0 ml. Activity of turbid

extracts was measured by following nitrite formation. Prior to determination of nitrite, NAD(P)H was removed by addition of 0.3 ml of 25% (w/v) $ZnSO_4$ and 0.3 ml of 1 M NaOH. After standing at 0°C for 5 min, the mixture was centrifuged and nitrite was measured on an aliquot of the clear supernatant solution.

NADPH:cytochrome *c* reductase activity was estimated at 30°C by measuring the initial increase in absorbance at 550 nm, due to the reduction of cytochrome *c*. The reaction mixture contained, in a final volume of 3.0 ml: 300 μmol Tris · HCl buffer (pH 7.5), 0.3 μmol NADPH, 0.06 μmol FAD, and 0.15 mg cytochrome *c*.

Reduced methyl viologen:nitrate reductase activity was estimated at 30°C by measuring the formation of nitrite. The 1.0 ml assay mixture contained: 100 μmol sodium phosphate buffer (pH 7.5), 20 μmol KNO_3, 4.6 μmol $Na_2S_2O_4$, 9 μmol $NaHCO_3$, and 0.2 μmol methyl viologen. The reaction was started by the addition of the enzyme and stopped after 5 min by rapid oxidation of the electron donor system in a Vortex mixer.

One unit of NADPH: or NADH:nitrate reductase is that amount which catalyzes the oxidation of 1 μmol of NAD(P)H by nitrate per min. One unit of NADPH:cytochrome *c* reductase is that amount which catalyzes the reduction of 1 μmol of cytochrome *c* by NADPH per min. One unit of reduced methyl viologen:nitrate reductase is that amount which catalyzes the reduction of 1 μmol of nitrate by reduced methyl viologen per min.

Assay of proteins used as standards. Alcohol dehydrogenase activity was estimated as described in Boehringer Biochemica Katalog. Catalase activity was measured as described by Lück [17]. ^{3}H-labelled myoglobin and bovine serum albumin were located by radioactivity measurements. The activity of the NADH:nitrate reductase from *C. vulgaris* was estimated as previously described [18].

Electrophoresis. Analytical disc gel electrophoresis of the native enzyme was performed at 4°C in 7.5% acrylamide gels using a Tris-barbital system [19]. Conditions of the electrophoretic procedure, staining of protein bands and detection of nitrate reductase and dehydrogenase activity have been described by Solomonson et al [6].

Sodium dodecyl sulfate electrophoresis was carried out in 5% acrylamide gel slabs (20 cm length, 1.5 mm thick) with the buffer system of Laemmli [20]. Samples were pretreated as described by Weber and Osborn [21] and aliquots (5—50 μl) in 10% glycerol were applied on the 3% acrylamide stacking gel. Bromphenol blue (0.01%) was used as the tracking dye. Gel slabs were run at 20 mA for about 1 h and then set at 60 mA until the tracking dye front was close to bottom of the slab. After electrophoresis, protein bands were stained by immersing the slab in a 0.1% solution of Coomassie Brilliant Blue G-250 in 50% (w/v) trichloroacetic acid. After 1 h, gels were transferred to 7% acetic acid for destaining. The subunit-molecular weights of standards were as given by Weber and Osborn [21], except that the value for *E. coli* RNA-polymerase was taken from Boehringer Biochemica Information.

Molecular weight determination. The Stokes radius (*a*) of nitrate reductase was determined with a Bio-Gel A-1.5 m (200—400 mesh) column (80 × 2.5 cm) equilibrated with 0.1 M sodium phosphate buffer (pH 7.5) containing 0.1 mM

dithioerythritol, 0.1 mM EDTA and 20 μM FAD. Samples (1.0 ml in volume and containing 10% sucrose to increase their density) were layered onto the surface of the gel under the buffer, and eluted at a flow rate of 10 ml/h. Fractions of 2.0 ml were collected. The void volume (V_o) was determined from the elution volume of Dextran Blue 2000 run under identical conditions. The total volume (V_t) was determined from a calibration of the column with water. Results were plotted according to Laurent and Killander [22] as $(-\log K_{av})^{1/2}$ versus Stokes radius, where $K_{av} = (V_e - V_o)/(V_t - V_o)$. The Stokes radius for the standard proteins were those given by Siegel and Monty [23]. A Stokes radius of 8.9 nm was used for nitrate reductase of *C. vulgaris* [6].

The sedimentation coefficient was determined by sucrose density gradient centrifugation according to the method of Martin and Ames [24]. The linear gradients (12.0 ml volume) used were 5—20% (w/v) sucrose in 0.1 M sodium phosphate buffer (pH 7.5), 0.1 mM dithioerythritol, 0.1 mM EDTA, 20 μM FAD. Samples of 0.2 ml containing the enzyme and/or the standards were applied by using Beckman band-forming caps. Centrifugation was at 3° C for 21 h at 40 000 rev./min in the SW 40 rotor of a Beckman Spinco L2-65 B centrifuge. After centrifugation, the gradient was eluted by pumping in a 40% (w/v) sucrose solution from the bottom of the tube and 3-drop fractions were collected. The sedimentation coefficient of the standard proteins were those given by Siegel and Monty [23].

Results and Discussion

Presence of an inactive form of nitrate reductase in extracts of Rhodotorula

Nitrate reductase in cell-free extracts of cells grown on nitrate was present partially in an inactive form which could be activated by ferricyanide. Maximal activation was obtained with 2.5 mM ferricyanide and the reaction was complete after 1 or 2 min. Higher concentrations of ferricyanide gave lower values of enzyme activity. The proportion of inactive enzyme to total enzyme in different preparations varied from 50 to 90%. The disruption procedure did not have any influence on the activation state of the enzyme in the extracts, the same proportion of inactive enzyme being obtained whether the cells were broken by sonication, or by grinding with alumina in a mortar, or by French Press treatment. Similar inactive forms of nitrate reductase which could be activated by ferricyanide have been found in other cells also [25—28].

Purification of nitrate reductase

All steps of the purification procedure were carried out at 0—4° C. Unless otherwise indicated, all buffers were adjusted to pH 7.5, and contained 0.1 mM dithioerythritol, 0.1 mM EDTA and 20 μM FAD.

Step 1. Crude extract. Cells (100 g wet weight) were thawed and suspended in 500 ml of 10 mM sodium phosphate buffer. The cell suspension was disrupted with an Aminco French Press at a pressure of 10 000 lb/inch^2. The resulting homogenate was centrifuged for 30 min at 40 000 × *g*, and the supernatant filtered through cheese-cloth to eliminate the lipid fraction that remained in the upper part of the centrifuge tubes.

Step 2. Protamine sulfate fractionation. 0.1 volume of 2% (w/v) protamine

sulfate solution (pH 7.0) was added, with stirring, to the crude extract from step 1. After 5 min, the precipitate was collected by centrifugation for 10 min at 27 000 × *g*. The sediment containing nitrate reductase was washed with 10 mM sodium phosphate buffer and later dispersed in 50 ml of 0.2 M sodium phosphate buffer. After standing at 0°C for 1 h, most of the enzyme was solubilized and could be separated from insoluble material by centrifugation.

Step 3. Ammonium sulfate fractionation. The ammonium sulfate precipitations were carried out by addition of a solution of $(NH_4)_2SO_4$ saturated at 0°C and brought to a pH of 7.2 with NH_4OH. The supernatant from step 2 was first brought to 35% $(NH_4)_2SO_4$ saturation and centrifuged. Nitrate reductase remained in the supernatant and was activated by the addition of $K_3Fe(CN)_6$ to a final concentration of 2.5 mM. After 5 min at 0°C, the enzyme was precipitated by adding $(NH_4)_2SO_4$ to 50% saturation, and collected by centrifugation.

Step 4. Bio-Gel A-1.5 m column chromatography. The 35—50% ammonium sulfate fraction was dissolved in 3 ml of 10 mM sodium phosphate buffer and centrifuged to remove insoluble material. The resulting supernatant was applied to a Bio-Gel A-1.5 m column (40 × 2.5 cm) equilibrated with 10 mM sodium phosphate buffer, and eluted with the same buffer. Fractions of 5 ml were collected.

Step 5. Blue Dextran-Sepharose affinity column chromatography. The peak fractions from step 4 were combined and applied to a Blue Dextran-Sepharose column (10 × 2 cm) equilibrated with 10 mM sodium phosphate buffer. The column was washed with 50 ml of the same buffer, and nitrate reductase was eluted with 0.1 mM NADPH in 10 mM sodium phosphate buffer, at a flow rate of about 15 ml/h.

The results of a typical purification are given in Table I. A purification of 740-fold was achieved with about 35% recovery of enzymatic activity. The specific activity of the resulting preparation (148 units per mg protein) was higher than any previously reported for this type of enzyme. Comparable specific activity has been observed only for the nitrate reductase purified from the green alga *C. vulgaris* [6,7]. For the subsequent studies, the active fractions from step 5 were concentrated in an Amicon Model 12 ultrafiltration cell equipped with an UM 2 membrane, and then filtered through a Sephadex G-25

TABLE I

PURIFICATION OF NITRATE REDUCTASE

Step	Volume (ml)	Total protein (mg)	Specific * activity (units per mg)	Purification (fold)	Total activity (units)	Recovery (%)
1. Crude extract	410	2562	0.2 **	1	512.4	100
2. Protamine sulfate	39	515	0.7 **	3.5	360.5	70.4
3. Ammonium sulfate	3.8	155	2.1	10.5	325.5	63.5
4. Bio-Gel A-1.5m	20	27	8.5	42.5	229.5	44.8
5. Blue Dextran-Sepharose	22.5	1.2	148.2	741	177.8	34.7

* Nitrate reductase activity was measured with NADPH as reductant.

** Activity was determined after full activation with $K_3Fe(CN)_6$.

bed equilibrated with 0.1 M sodium phosphate buffer. The enzyme was unstable even after concentration. Low ionic strength caused more loss in activity. The purified enzyme was stored at −20° C in 0.1 M sodium phosphate buffer containing 40% glycerol to prevent freezing. Under these most favorable conditions the half-life of the enzyme was about 2 weeks. Because of their tested protective effect, dithioerythritol, EDTA and FAD were always included in the buffers during the purification procedure and storage.

Purity of the enzyme. Analytical polyacrylamide gel electrophoresis of the enzyme preparations obtained as described above showed the presence of two different protein bands (Fig. 1). The thickest one, which exhibited the higher mobility, always contained more than 90% of the entire protein as determined densitometrically, though the relative amount of the fainter band varied in different preparations, being higher in aged preparations than in the ones recently prepared. Both protein bands were coincident with NADPH dehydrogenase and reduced methyl viologen:nitrate reductase activities. These two proteins might represent isozymes of nitrate reductase, as has been reported for spinach [29]. We are inclined, however, to view the minor component as a degradation product of the native enzyme, because of the fact that there was an increase in the relative amount of the minor component with time of storage of the preparation. The thickest and darkest staining zone also gave a positive stain for heme whereas the other band did not. This could be due to the absence of heme in the protein molecule or, more likely, to the fact that the heme content of the small amount of material was below the sensitivity limit of the staining method.

Fig. 1. Polyacrylamide gel electrophoresis of purified nitrate reductase. Protein, 30 μg.

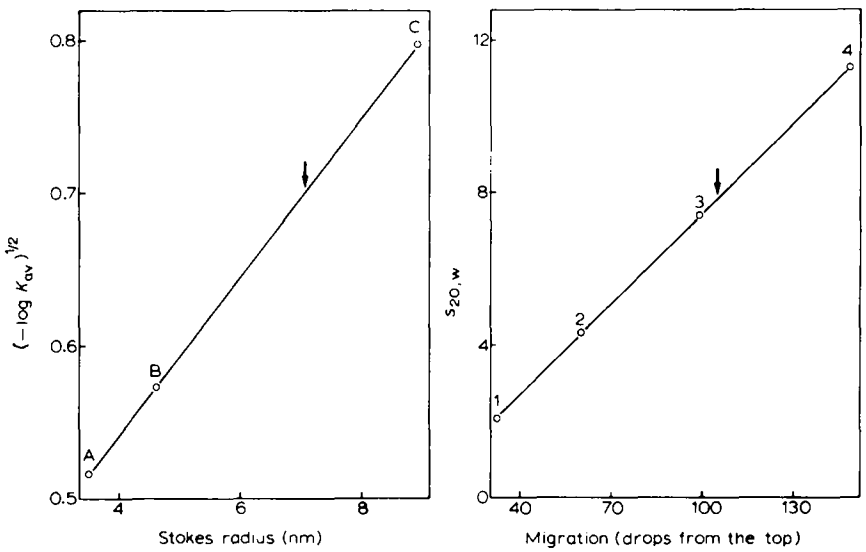

Fig. 2. Determination of the Stokes radius (left) and sedimentation coefficient (right) of nitrate reductase. Stokes radius for the standards used were: A, bovine serum albumin, 3.5 nm; B, yeast alcohol dehydrogenase, 4.6 nm; C, *C. vulgaris* nitrate reductase, 8.9 nm. Standards of known sedimentation coefficient were: 1, myoglobin, 2.04 S; 2, bovine serum albumin, 4.3 S; 3, yeast alcohol dehydrogenase, 7.4 S; 4, catalase, 11.3 S. The arrows locate the nitrate reductase of *Rhodotorula*.

Physicochemical properties of the enzyme

Molecular weight. The molecular weight of the native nitrate reductase from *Rhodotorula* was determined by combining the techniques of density gradient centrifugation and gel filtration, as suggested by Siegel and Monty [23]. The sedimentation coefficient of the enzyme was found to be 7.9 S and the Stokes radius was 7.05 nm (Fig. 2). The molecular weight was calculated by the relationship $M_r = (6\ \pi\eta\ Nas)/(1-\bar{v}\rho)$, where η = viscosity of medium, N = Avogadro's number, a = Stokes radius, s = sedimentation coefficient, $\bar{v}$ = partial specific volume (assumed to be 0.725 ml/g), and ρ = density of medium. A value of 230 000 was obtained for the molecular weight which is similar to those reported for the enzymes of *Aspergillus* [4,5] and *Neurospora* [3].

Subunits. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis the purified enzyme always gave two bands in close proximity. The thickest band, which should correspond to the faster migrating species of nitrate reductase observed in the normal polyacrylamide gel electrophoresis (Fig. 1), had a mobility corresponding to a molecular weight of 118 000 (Fig. 3), a value approximately half of that determined for the size of the native enzyme. A molecular weight of 115 000 was calculated for the second, fainter protein band due to the other enzyme species (Fig. 3).

Absorption spectrum. Spectroscopic examination of purified preparations of nitrate reductase showed the presence of a cytochrome in the oxidized form which could be rapidly reduced on addition of NADPH. The absorption spectra, before and after the addition of reductant are shown in Fig. 4. Wavelength maxima were observed at 557 nm (α), 527 nm (β) and 423 nm (Soret) for the reduced enzyme, and at 412 nm for the oxidized. These band maxima are iden-

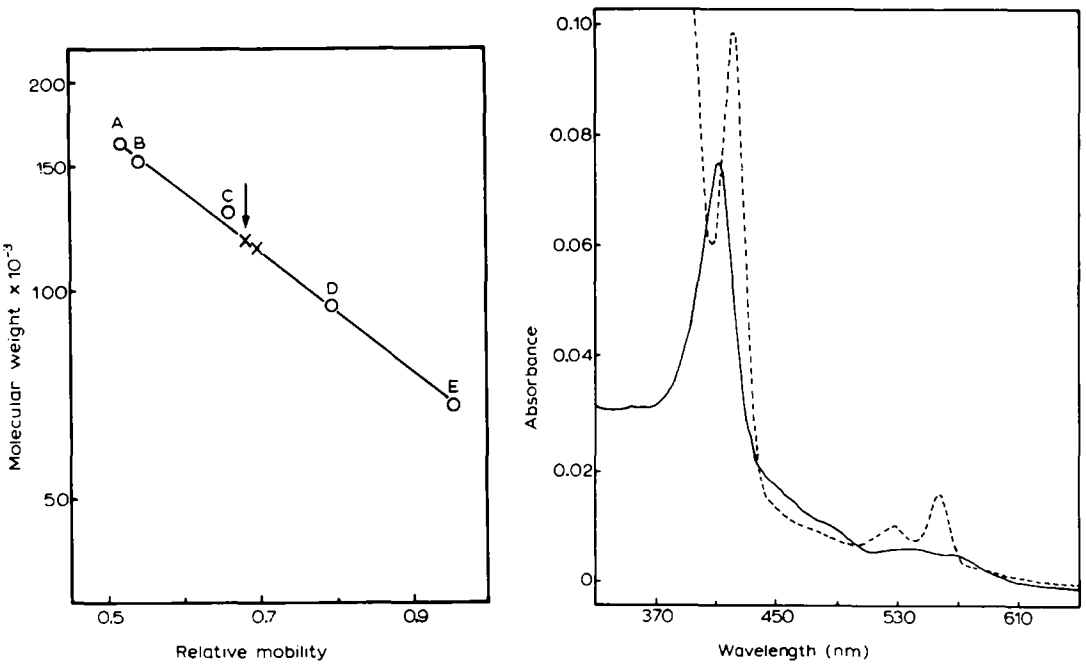

Fig. 3. Estimation of the molecular weight of the subunits of nitrate reductase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Molecular weight standards were: A, *E. coli* RNA polymerase β'-subunit, 165 000; B, *E. coli* RNA polymerase β-subunit, 155 000; C, *E. coli* β-galactosidase, 130 000; D, phosphorylase *a*, 94 000; E, bovine serum albumin, 68 000; X, nitrate reductase. Mobility was measured relative to bromophenol blue. The arrow shows the position of the major protein band due to nitrate reductase.

Fig. 4. Absorption spectra of purified nitrate reductase. The solution contained 0.14 mg protein per ml 0.1 M sodium phosphate buffer (pH 7.5) supplemented with 2 μM FAD. ———, no additions; - - - - - -, a small amount of solid NADPH was added.

tical to those reported for the cytochrome *b*-557 present in the nitrate reductases of *Neurospora* [3,30] and of *Chlorella* [6,31].

The addition of dithionite to the oxidized enzyme gave essentially the same absorption spectrum as that obtained by reduction with NADPH. Addition of nitrate resulted in reappearance of the oxidized form. It is worth stressing that complete reduction of the cytochrome by NADPH only took place when added FAD was present in the enzyme preparation before addition of NADPH. When FAD was removed by filtration of the enzyme through Sephadex G-25, the addition of NADPH did not bring about the reduction of the heme group. Addition of FAD after the NADPH resulted in a partial reduction with appearance of the peaks at 423 and 557 nm, but with lower absorbance values than the corresponding control where FAD was present prior to NADPH addition. These results support the participation of the heme in the transport of electrons from reduced pyridine nucleotides to nitrate, according to the tentative sequence flavin → heme → Mo → NO_3^- [1,2]. The Mo content of the *Rhodotorula* enzyme remains to be determined as does the possible presence of non-heme iron.

Nitrate reductase from *C. vulgaris* has a molecular weight of 356 000 and

seems to be composed of three subunits of about equal size [6]. This enzyme has been shown to contain two heme moieties per molecule of enzyme, with a minimal molecular weight of 152 000 per molecule of heme [6]. The absorbance values at the different wavelength maxima were measured in different preparations of *Rhodotorula* nitrate reductase and compared with those obtained with pure nitrate reductase of *Chlorella*. With equivalents amounts of protein, the values for *Rhodotorula* were 0.7 of those for *Chlorella*. If one assumes that the extinction coefficients for the cytochrome *b*-557 are the same in both enzymes, there should be 1 mol of heme per 220 000 g protein in the *Rhodotorula* enzyme. This agrees quite well with the estimated molecular weight of 230 000. The indirect estimate made here suggest that only one of the two subunits of the *Rhodotorula* enzyme contains heme, whereas there is sufficient heme in the *Chlorella* enzyme to deduce the presence of heme in two subunits (or 2 mol of heme in 1 subunit). Thus, in both cases, there appears to be at least one heme-free subunit in the molecule, or some of the heme is readily lost. In the nitrate reductase of *Aspergillus*, the presence of heme has been disputed [4,5]. The nitrate reductase of spinach contains heme, however [32].

Enzymatic activities of Rhodotorula nitrate reductase

Electron donors. The ability of different reductants to act as electron donors for the reduction of nitrate in the presence of purified nitrate reductase, is shown in Table II. Both NADH and NADPH were good reductants, the latter being more effective than the former when tested at the same concentration. A similar preference for NADPH over NADH as reductant has been reported for the enzyme of *Neurospora* [3]. In contrast, the nitrate reductase of *C. vulgaris* has a strong preference for NADH [31]. Dithionite alone was not an electron donor, but the combination of dithionite with flavin nucleotides or viologens gave positive results. Dithionite-reduced methyl viologen was especially effective, the values for nitrite formation obtained with this reductant being far higher than those observed with NADPH.

TABLE II

ELECTRON DONORS FOR NITRATE REDUCTASE FROM *RHODOTORULA*

Activities with NADH or NADPH were determined as described under Materials and Methods. Conditions for the estimation of activity with dithionite-reduced methyl viologen were those of the corresponding activity assay. Other assays with dithionite as the reductant were carried out under the same conditions, but methyl viologen was either absent or replaced by the same amount of benzyl viologen or flavin nucleotides.

Electron donor	Specific activity (units per mg protein)
NADH	37.5
NADPH	112.5
$S_2O_4^{2-}$, methyl viologen	255.0
$S_2O_4^{2-}$, benzyl viologen	192.5
$S_2O_4^{2-}$, FMN	48.0
$S_2O_4^{2-}$, FAD	33.0
$S_2O_4^{2-}$	0.7
None	0

TABLE III

ELECTRON ACCEPTORS FOR NITRATE REDUCTASE FROM *RHODOTORULA*

Conditions were those of the standard assay for NADPH:nitrate reductase activity except that, where indicated, nitrate was either absent or alternate oxidants were substituted for it. The reaction rates were measured by determining the initial rate of decrease of absorbance at 340 nm, except in the cases where APAD(P), ferricyanide, cytochrome *c* and 2,3-dichlorophenolindophenol were used. The reduction of these compounds was followed by measurements of the initial changes in absorbance at 365, 420, 550 and 600 nm, respectively.

Electron acceptor	Concentration (mM)	NADPH oxidized (μmol/min per mg protein)
KNO_3	6.7	137.5
$NaNO_2$	6.7	0
$KClO_3$	6.7	78.5
Methyl or benzyl viologen	0.1	0
FMN or FAD	0.1	0
APAD or APADP	0.1	0
$K_3Fe(CN)_6$	1.0	478
Cytochrome *c*	0.1	192.5
2,6-Dichlorophenolindophenol	0.1	97.5
None	—	0

Electron acceptors. Several oxidants other than nitrate were enzymatically reduced when either NADPH or NADH were used as reductants. Table III shows the results obtained with NADPH. The enzyme did not exhibit either nitrite reductase or NADPH oxidase activity, but was able to utilize chlorate, in addition to nitrate, as a substrate. No transhydrogenase activity could be detected by using the acetylpyridine derivatives of NAD(P) as possible electron acceptors. Flavin nucleotides and viologens were not reduced at the concentrations tested. On the other hand, cytochrome *c*, 2,6-dichlorophenolindophenol and ferricyanide were effective electron acceptors. NADH could substitute for NADPH in all cases, but the values obtained were always lower (not shown).

Physical association of the different activities

A comparison was made of some of the enzyme activities at different stages of purification. Starting from step 3 (ammonium sulfate fractionation), the ratio of reduced methyl viologen:nitrate reductase to NADPH:nitrate reductase remained constant at about 2.3 : 1, and the ratio of NADPH:cytochrome *c* reductase to NADPH:nitrate reductase remained constant at about 3 : 1. All of these three different activities showed coincident elution profiles from the Bio-Gel and Blue Dextran-Sepharose columns. This coincidence was also maintained during sucrose density gradient centrifugation of the purified enzyme, as shown in Fig. 5. These results demonstrate that the different activities are all catalyzed by the same protein molecule.

Enzyme reaction characteristics

Effect of pH and type of buffer. The same pH optimum of 7.5 was observed for NADPH:nitrate reductase, reduced methyl viologen:nitrate reductase and NADPH:cytochrome *c* reductase, no matter which buffer, Tris · HCl or phosphate, was used. NADPH:nitrate reductase and reduced methyl viologen:nitrate

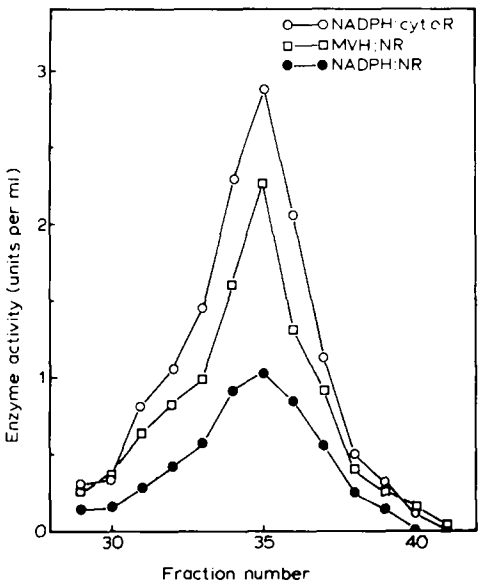

Fig. 5. Distribution of the different enzyme activities after sucrose density gradient centrifugation of nitrate reductase. 0.2 ml of purified enzyme preparation were layered upon 12 ml of a 5—20% sucrose gradient in 0.1 M sodium phosphate buffer (pH 7.5), 0.1 mM dithioerythritol, 0.1 mM EDTA, 20 μM FAD. Centrifugation was at 3°C for 21 h at 40 000 rev./min in the SW 40 rotor of a Beckman Spinco L2 65 B centrifuge. Gradients were fractionated and fractions assayed for activity as described under Materials and Methods. O———O, NADPH:cytochrome *c* reductase activity (NADPH:cyt *c* R); □———□, reduced methyl viologen:nitrate reductase activity (MVH:NR); ●———●, NADPH:nitrate reductase activity (NADPH:NR). Fraction 1 corresponds to the top of the gradient.

reductase activities were both stimulated by phosphate, as the rates of the reactions in Tris · HCl buffers were about 75% of those obtained in phosphate buffers at the same pH. In contrast, NADPH:cytochrome *c* reductase activity was definitely enhanced by Tris · HCl buffers, the reaction rate in Tris · HCl being 1.5-fold higher than that obtained in phosphate.

FAD requirement. Even in crude extracts, added FAD was required in order to obtain maximal activities of NADPH:cytochrome *c* reductase and NADPH: nitrate reductase. An activation of 7—10-fold was obtained with 10 μM FAD, which was saturating. This requirement for FAD, also reported for the nitrate reductase of *Neurospora* [3] and other sources [1,2], suggests that the flavin is rather loosely bound to the enzyme. In contrast, the nitrate reductase of *C. vulgaris* contains FAD which is not readily removed [6]. FAD was not required for the methyl viologen:nitrate reductase activity. On the contrary, this activity was somewhat inhibited by FAD, presumably because of competition between FAD and methyl viologen, since FAD behaved also as an effective reductant for nitrate reductase activity (cf. Table II).

Effect of inhibitors. Table IV shows the effect of various inhibitors on the different enzyme activities. Only *p*-chloromercuribenzoate behaved as an inhibitor of the NADPH:cytochrome *c* reductase activity, whereas it did not affect the reduced methyl viologen:nitrate reductase activity. On the other hand, azide, cyanate and cyanide inhibited the reduced methyl viologen:nitrate reductase, but were without effect on the NADPH:cytochrome *c* reductase

TABLE IV

EFFECT OF INHIBITORS OF NITRATE REDUCTASE ACTIVITIES

Experimental conditions were those of the respective standard assays except that inhibitors were added to the assay system at the indicated final concentrations

Inhibitor	Concentration (M)	Inhibition (%)		
		NADPH:nitrate reductase	NADPH:cytochrome *c* reductase	Reduced methyl viologen:nitrate reductase
p-Chloromercuribenzoate	10^{-4}	100	100	0
	10^{-5}	100	100	0
	10^{-6}	100	100	0
Potassium cyanate	10^{-2}	100	0	94
	10^{-3}	93	0	80
	10^{-4}	60	0	66
Potassium cyanide	10^{-4}	80	0	81
	10^{-5}	12	0	22
Sodium azide	10^{-3}	100	0	*
	10^{-4}	87	0	*
	10^{-5}	39	0	*

* Reduced methyl viologen:nitrate reductase activity was not measured because azide interfered with the nitrite assay.

activity. In its response to these inhibitors, the enzyme of *Rhodotorula* behaves similarly to other pyridine nucleotide nitrate reductases [1,2].

Kinetic properties of NAD(P)H:nitrate reductase

Comparison of NADH and NADPH. The ratio of NADPH:nitrate reductase activity to NADH:nitrate reductase activity, at saturating concentration of nitrate and with reduced pyridine nucleotide at 0.1 mM, was close to 2.5 in both crude extracts and in purified preparations. Both activities were activated to the same extent on addition of ferricyanide to the extracts, both had the same pH optimum, and both were favored by phosphate as compared with Tris. The reduction of nitrate with either NADPH or NADH was linear with the time provided that the concentrations of the substrates were not limiting, and was also proportional to the amount of protein added to the assay mixture. 1 mol of NAD(P)H was oxidized per mol of nitrite formed, and the reaction was inactivated by heating the enzyme (5 min at 100°C).

K_m *values for nitrate and reduced pyridine nucleotides.* The effect of nitrate concentration on nitrate reductase activity assayed either with NADH or NADPH was determined. From Lineweaver-Burk plots of the obtained data, the apparent K_m values for nitrate were calculated to be 125 μM for the case of NADPH and 45 μM with NADH. The effect of the concentration of the reduced pyridine nucleotides on nitrate reductase activity was also studied. The apparent K_m for NADPH was 20 μM, whereas a value of 160 μM was calculated in the case of NADH. At saturating concentrations of both nucleotides, the maximal velocity for the reaction with NADPH as reductant was 1.5-fold

higher than that with NADH. These facts may have significance in the in vivo utilization of nitrate. At high nitrate concentrations, the enzyme would use NADPH preferentially as reductant (higher V and lower K_m). At low nitrate concentrations, or with a large excess of NADH over NADPH, the NADH would be used preferentially.

Inhibitory effect of nitrite. Nitrite, the product of the reaction, inhibited the NAD(P)H:nitrate reductase. This inhibition was competitive with respect to nitrate. The calculated K_i value for nitrite with NADPH as reductant was 180 μM. This K_i value is similar to the K_m value for nitrate, which suggest that nitrite might inhibit nitrate reduction under physiological conditions in *Rhodotorula*. This is not the case for the *C. vulgaris* enzyme, which has a relative high K_i value for nitrite [31]; but the nitrate reductase of *Aspergillus* has also been found to have a K_i value for nitrite which is about the same as the K_m value for nitrate [5].

Effect of temperature. The dependence of NAD(P)H:nitrate reductase reaction rate on the temperature was studied. Between 10 and 25°C, there was a linear relationship between log v and $1/T$. The slope decreased between 25 and 30°C, became zero between 30 and 33°C and changed to negative values when the temperature increased over 33°C. From the linear part of the curve an activation energy of 5300 cal was calculated. Between 10 and 20°C the value for Q_{10} was 1.4. From the data previously reported for the effect of temperature on reaction velocity for the enzyme from *C. vulgaris* [28], one might conclude that the *Chlorella* enzyme is much more unstable at 30°C than the *Rhodotorula* enzyme. The measurements with the *Chlorella* enzyme were made with relatively crude preparations, prior to extensive purification. We therefore repeated the measurements of the effect of temperature on reaction rate with highly purified *C. vulgaris* enzyme. The results were quite similar to those reported here for *Rhodotorula* nitrate reductase.

Cytochrome c. The reduction of cytochrome *c* with NADPH as electron donor showed Michaelis-Menten kinetics with respect to cytochrome *c*. From the data plotted in the double reciprocal form, a K_m value of 8 μM was calculated.

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