BBA 66665

FERREDOXIN:NITRITE OXIDOREDUCTASE FROM *CHLORELLA* PURIFICATION AND PROPERTIES

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(Received March 3rd, 1972)

SUMMARY

Ferredoxin:nitrite oxidoreductase from *Chlorella fusca* was obtained in an electrophoretically homogeneous state with a specific activity of 51.7 μmoles nitrite reduced per min per mg protein. With reduced ferredoxin as electron donor the product of the reaction is exclusively ammonia. The preparation consists of two electrophoretically distinguishable proteins, both independently capable of the reduction of nitrite and also hydroxylamine at a slower rate. The molecular weight of both forms of the enzyme is 63 000. The visible spectrum shows absorption maxima at 384, 573, 630 and 692 nm. Nitrite reductase contains two atoms of iron which are probably bound in the chromophoric group. The enzyme consists of 600 amino acids with ten residues of cysteine and a slight predominance of acidic amino acids.

INTRODUCTION

The assimilatory reduction of nitrate by algae and higher plants proceeds in two steps each catalyzed by a different enzyme. For *Chlorella* evidence has been presented that the first step, the reduction of nitrate to nitrite, is mediated by a high molecular weight enzyme complex containing FAD[1], molybdenum[2,3] and most probably also iron[4].

Further reduction of nitrite to ammonia[5] is accomplished by a second enzyme for which Ramirez *et al.*[6] proposed the systematic name ferredoxin: nitrite oxidoreductase. Nitrite reductase has been isolated from spinach[6–8], marrow[9], corn[10] and the blue-green alga *Anabaena*[11]. In each case only partially purified enzymes have been obtained with, except of marrow[9], rather low specific activities. The purpose of this paper is to describe a purification method which yields an electrophoretically homogeneous nitrite reductase from *Chlorella* and to report on some biochemical characteristics of the enzyme.

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MATERIALS AND METHODS

The organism and preparation of a cell-free extract

Chlorella fusca Shihira et Krauss, Strain 211-15 from the culture collection at Göttingen was grown in 6-l flasks with an inorganic medium containing nitrate as only nitrogen source[12]. After 4 days approx. 3 g wet weight of algae per l medium were obtained. 200 g of cells were mixed with 160 ml of 50 mM Tris buffer (pH 8.0) and disrupted by glass beads in a vibration homogenizer. The cell debris was stirred in the cold with an excess of 10 mM Tris buffer (pH 7.5) freed from glass beads and centrifuged for 90 min at 53 000 × *g*. Nitrite reductase, NADP reductase (EC 1.6.99.4), ferredoxin and flavodoxin were purified from the green supernatant. All steps were performed in the cold. Tris buffers were adjusted with HCl.

Enzyme assay

The standard assay for nitrite reductase was run in open test tubes with chemically reduced methyl viologen as electron donor[6,7]. The pH of the reaction mixture was adjusted to 7.5. One unit of nitrite reductase corresponds to an amount of enzyme that reduces 1 μmole nitrite in 1 min under the conditions of the standard assay. The enzymatic oxidation of reduced methyl viologen under anaerobic conditions by nitrite, hydroxylamine or sulfite was studied by the method of Asada[13].

Purification of NADP reductase

NADP reductase was purified by the use of similar fractionation steps as those for nitrite reductase[14].

Polyacrylamide gel electrophoresis

Analytical separations were performed in tubes of 5 mm diameter with the discontinuous gel system of Jovin *et al.*[15]. Nitrite reductase was localized in the gel slabs by soaking them in a standard mixture from which nitrite has been omitted. Subsequent incubation in a buffered nitrite solution reveals the position of nitrite reductase by the formation of a colorless band in the blue gel (J. Cardenas, personal communication). On a preparative scale separation of nitrite reductase was achieved with the new ultraphor electrophoresis apparatus having a flat gel chamber of 150 mm × 6 mm (Colora, Lorch)[16,17].

Analytical methods

Nitrite was determined according to Snell and Snell[18]. NADPH was eliminated from the reaction mixture by precipitation with ethanolic barium acetate[19]. Ammonia was measured by Nessler's reagent after microdiffusion in 0.005 M H_2SO_4[20]. Protein was determined by the method of Lowry *et al.*[21] with bovine serum albumin as a standard. Metals were determined with a Beckman atomic absorption spectrophotometer Model 440. In addition, iron content of nitrite reductase was determined colorimetrically[22]. The molecular weight of nitrite reductase was determined by polyacrylamide gel electrophoresis in the presence of dodecyl sulfate; 0.5 g of cross linker was used[23]. The presence of heme groups in the enzyme was examined with the method of pyridine hemochrome formation[24] or acid cleavage from the protein

with subsequent butanone extraction[25]. Amino acid analysis was performed with a Biocal analyzer[27,26]. Spectral measurements were done with a Zeiss PMQ II and a Beckman DB-GT spectrophotometer.

Chemicals

Chemicals were obtained from the following commercial sources: NADH, NADPH, FAD and FMN from Boehringer (Mannheim); myoglobin, ovalbumin, bovine serum albumin, chymotrypsinogen, hemoglobin, *p*-chloromercuribenzoate, methyl viologen, benzyl viologen, protamine sulfate and all chemicals for polyacrylamide gel electrophoresis from Serva (Heidelberg); DNAase I from Worthington (Freehold); Sephadex G-100 from Pharmacia (Uppsala); DEAE-cellulose (DE-52, 1.0 mequiv) from Whatman (London) and metal standards for atomic absorption spectroscopy from E. Merck (Darmstadt). Other chemicals were of analytical grade. RNAase T_1 was purified from *Aspergillus oryzae*[28].

RESULTS

Purification of nitrite reductase

(1) First DEAE-cellulose chromatography. The crude extract obtained from 200 g of cells was stirred for 45 min with approx 100 g preswollen and equilibrated cellulose to adsorb the enzyme. A column of 20–30 cm × 2.5 cm was packed with the sedimented cellulose and the proteins were eluted at 30 ml/h by a linear sodium chloride gradient (0.045 to 0.3 M in 10 mM Tris, pH 7.5). The gradient volume was 800 ml. Nitrite reductase was eluted from the column at about 0.11 N Cl^- followed by NADP reductase, flavodoxin and ferredoxin.

(2) $(NH_4)_2SO_4$ precipitation. For each 100 ml of the combined active fractions 51.6 g of solid $(NH_4)_2SO_4$ was added, maintaining the pH at 7.5 by the addition of 0.1 M Tris solution. After 30 min the precipitate was collected by centrifugation for 15 min at 30 000 × *g*. The supernatant was discarded and the sediment carefully resuspended in 50 ml of 55% $(NH_4)_2SO_4$ solution (pH 7.0). After immediate centrifugation the sediment was extracted for 30 min with 50 ml of 40% neutral $(NH_4)_2SO_4$. The bulk of nitrite reductase was recovered from this 40% extraction after a third centrifugation. The wash fraction and the dissolved 40% sediment contained negligible enzymatic activity. The enzyme extract was concentrated again by precipitation with $(NH_4)_2SO_4$ and was dialyzed against 10 mM Tris (pH 7.5) made 80 mM with respect to NaCl.

(3) Chromatography on Sephadex G-100. The enzyme solution was passed through a Sephadex G-100 column (2.5 cm × 95 cm) equilibrated with the dialysis buffer from Step 2. The elution pattern of the enzyme is shown in Fig. 1. Enzyme fractions were pooled up to 1 activity unit per ml and the solution diluted with the same volume of 10 mM Tris (pH 7.5).

(4) Second DEAE-cellulose chromatography. Nitrite reductase from Step 3 was adsorbed on a cellulose column (2.5 cm × 40 cm) and further purified by gradient elution at a rate of 55 ml/h. The gradient volume was 1000 ml and its NaCl molarity varied linearly from 0.04 to 0.4 in Tris buffer. Fractions of 5 ml were collected and the enzyme taken within a margin of 2 activity units per ml. The protein solution obtained was concentrated to about 7 ml by ultrafiltration.

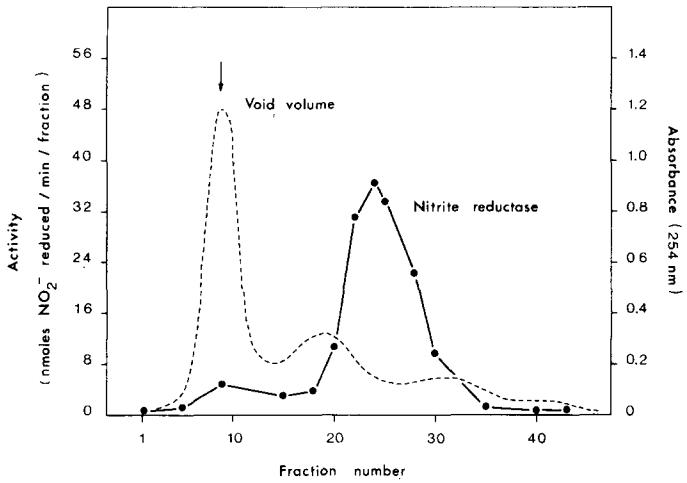

Fig. 1. Elution profile of nitrite reductase from a Sephadex G-100 column (2.5 cm × 95 cm). ●—●, enzymatic activity; — — —, ultraviolet absorption at 254 nm. Elution with 80 mM NaCl in 10 mM Tris (pH 7.5) at 30 ml/h.

(5) Separation of two nitrite reducing proteins. The nitrite reductase from the previous step consists of six major protein components when analyzed by polyacrylamide gel electrophoresis (Fig. 2a). The pattern of the stained gel shows a striking twin band. Analysis for enzymatic activity revealed that both proteins of the twin band reduce nitrite. For preparative separation of both enzymes the gel chamber of the ultraphor electrophoresis apparatus was filled 7 cm high with separation gel (acrylamide concentration 7.5%, pH 8.9) and overlayered with 3 cm of spacer gel.

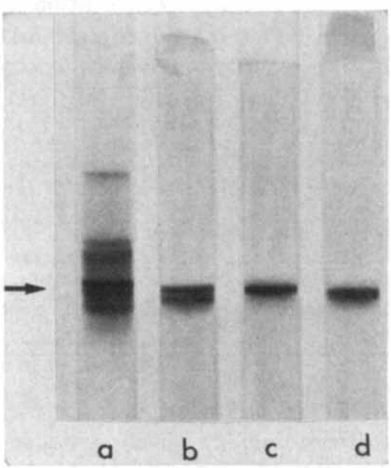

Fig. 2. Polyacrylamide gel electrophoresis of nitrite reductase. The discontinuous gel system of Jovin *et al.*[15] was used with 7.5% acrylamide at pH 8.9. The samples show the protein pattern after staining with coomassie blue[23] of (a) 250 μg of nitrite reductase after the second DEAE-cellulose chromatography (Step 4), the arrow indicates the two nitrite reducing enzymes; (b) 25 μg of a mixture of nitrite reductase I and II after its separation; (c) 50 μg of nitrite reductase I and (d) 50 μg of nitrite reductase II. Current 6 mA for 45 min per gel.

The enzyme was incubated 1 h at 6 °C with 400 units of DNAase I and 100 units of RNAase T_1 per ml of enzyme solution to eliminate traces of nucleic acids usually severely disturbing the electrophoretic separation[16,17]. The solution was made 10% in sucrose followed by addition of 0.2 ml of a 0.05% bromophenol blue solution. The amount of protein applied to the gel ranged from 1–4 mg/cm². The current was kept at 50 mA throughout the separation with a corresponding voltage of 200–300 V. When the bromophenol reached the elution chamber the fraction collector was started. Fractions of 1.7 ml were collected and analyzed for nitrite reductase activity. One run takes approx. 10 h.

Fig. 3 shows the protein and activity profiles for this separation procedure. The second and third protein peaks coincide with the two nitrite reducing enzymes. The separation is not complete because of the very small difference in their electrophoretic mobility. However, one can see a marked trough in the elution profile yielding on both sides two different homogeneous fractions when analyzed electrophoretically

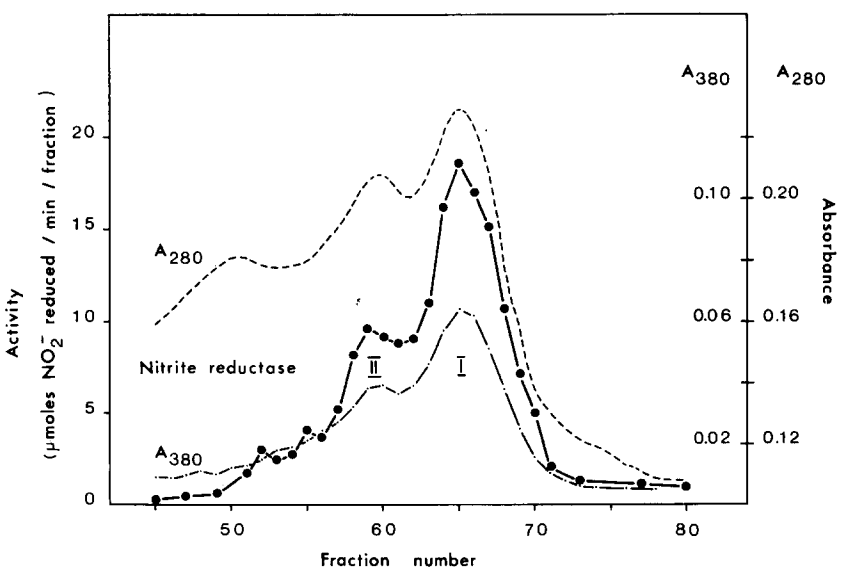

Fig. 3. Elution profile of nitrite reductase I and II from preparative polyacrylamide gel electrophoresis. The enzyme preparation corresponds to Fig. 2a. The protein profile is given by its ultraviolet absorption (— — —); nitrite reductase is measured enzymatically (●—●) or by its specific absorbance at 380 nm (· — ·). Nitrite reductase II elutes first closely followed by enzyme I (main nitrite reductase). The further elution of other proteins is not monitored. Elution rate 55 ml/h of 0.12 M Tris (pH 8.1); counter elution chamber 20 ml/h of 0.37 M Tris (pH 8.1).

(Figs 2c and 2d). Each enzyme retains its nitrite reducing activity and now migrates as a single band. This proves the twin band is not an artifact of the electrophoretic procedure. However, a twin band is again obtained when both enzymes are mixed and applied to a gel (Fig. 2b). The nitrite reductase with the lower electrophoretic mobility represents about 2/3 of the total nitrite reductase and will be identified in further discussion with suffix I. The other enzyme will have suffix II.

The enzyme preparation obtained by this method has usually a specific activity of more than 40 units per min per mg of protein. The best value determined for nitrite

reductase I was 51.7 units. Table I summarises the purification procedure. During storage at 4 °C 30% of the activity is lost within 3 weeks. The same loss is observed after a 4 months storage at −24 °C. Nitrite reductase is unstable in crude extracts and is rapidly inactivated in solutions below pH 7.

Purification of ferredoxin and flavodoxin

Ferredoxin and flavodoxin are recovered from the first DEAE-cellulose bed of the purification of nitrite reductase. After elution of nitrite reductase the column is washed with 0.6 M NaCl in 10 mM Tris (pH 8). The batches of five preparations are combined and freed from chloride by dialysis. Both electron carriers are purified by gradient elution (0.175 to 0.5 M NaCl) from a DE-52 column (2.5 cm × 15 cm). The reddish ferredoxin and flavodoxin containing fractions are treated with a neutral 2% protamine sulfate solution. The precipitated proteins are redissolved by dialysis against 0.8 M NaCl in 10 mM Tris (pH 8). The protein solution is diluted 8-fold, centrigufed and chromatographied on DEAE-cellulose as above. Ferredoxin separates now from flavodoxin and some residual cytochrome *f*. Final passage through Sephadex G-100 (column dimensions 2.5 cm × 85 cm) gives approx. 50 mg of ferredoxin with an absorption ratio $A_{420\ nm}/A_{275\ nm}$ of 0.54. Flavodoxin can be obtained in higher yield from iron deficient *Chlorella* cultures as previously described[29].

Properties of nitrite reductase

Metal content. During our study of the metal components of the nitrate reducing system from *Chlorella* we could demonstrate a close association of radioactive ^{59}Fe with nitrite reductase throughout the purification procedure, strongly suggesting that iron is an integral constituent of the enzyme[2]. Atomic absorption spectroscopy of both nitrite reductases shows the presence of two atoms of iron per enzyme

TABLE I

PURIFICATION OF NITRITE REDUCTASE FROM *Chlorella fusca*

Preparation	*Vol. (ml)*	*Total protein (mg)*	*Total act. (units)*	*Spec. act. (units/mg protein)*	*Enzyme recovery (%)*	*Protein recovery (%)*
Crude extract	1110	9800	920	0.094	100	100
1st DE-52 eluate	140	700	602	0.86	65.5	7.15
$(NH_4)_2SO_4$ ppt.	8.3	246	482	1.96	52.4	2.5
Gel filtrate	59	83	410	4.94	44.6	0.85
2nd DE-52 eluate	58	23.6	330	14.0	35.9	0.24
Effluent from polyacrylamide electrophoresis*	25	4.3	180	42.0	19.5	0.044

* Values calculated for both enzymes together.

molecule. Table II compares the iron content of nitrite reductase determined by atomic absorption with a colorimetric assay after heat treatment of the enzyme. The heat treatment removes two atoms of iron from the enzyme molecule, whereas the redissolved protein precipitate was found to be free of iron by atomic absorption. Suggestions that manganese and copper might be involved in nitrite reduction[30,31]

TABLE II

IRON CONTENT OF NITRITE REDUCTASE (MOL. WT 63 000)

Expt	*Protein concn (nmoles/ml)*	*Iron concn (nmoles/ml)*	*Fe/molecule*
1	1.8	3.5*	1.94
2	2.2	3.9*	1.77
3	9.5	19.0**	2.00
4	11.7	22.2**	1.89

* Determined by atomic absorption.

** Determined by the *o*-phenanthroline method[22], after heating the protein for 20 min at 60 °C.

are presumably not valid for the purified enzyme because of a complete absence of atomic absorption signals for these two metals.

Inhibitors. Inhibitor studies with metal complexing agents indicate that iron is involved in enzymatic activity. Methyl viologen dependent nitrite reductase activity is inhibited 90% by 1 mM cyanide, 15% by 5 mM *o*-phenanthroline and 7% by 5 mM 8-hydroxyquinoline. α,α'-Bipyridyl or EDTA at these concentrations do not inhibit enzyme activity, but dialysis against $1 \cdot 10^{-4}$ M EDTA-solution for several hours inactivates the enzyme and its activity cannot be restored by the addition of iron salts. A 70% inhibition of the enzyme is also observed after prolonged incubation with carbon monoxide.

Spectral properties. Purified nitrite reductase is reddish-brown. The visible spectrum of the enzyme has two intense absorption bands at 384 nm and 573 nm

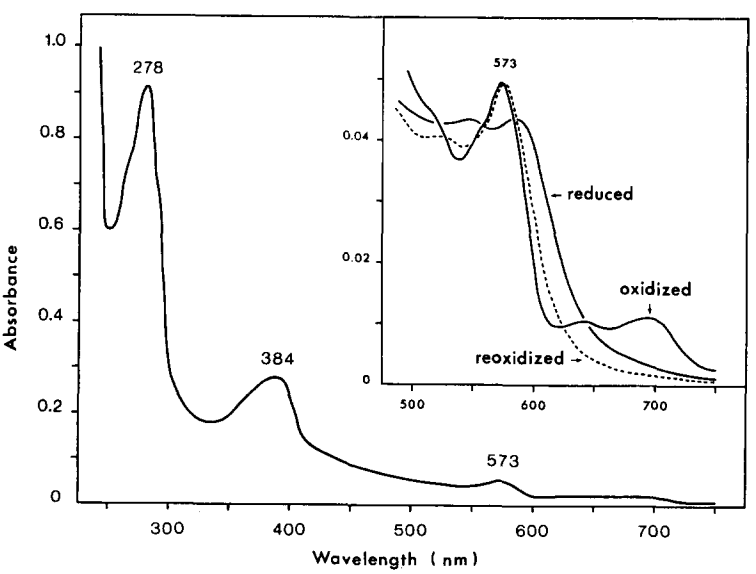

Fig. 4. Absorption spectrum of purified nitrite reductase in 10 mM Tris (pH 7.5). The insert shows the absorption beyond 500 nm in greater detail. The curve of the reduced enzyme is obtained on anaerobic addition of dithionite, the curve of the reoxidized enzyme after flushing the cuvette with oxygen and adding nitrite.

and two bands of very low absorbance at 635 nm and 692 nm. Shoulders were found at 530 nm and 560 nm (Fig. 4). The ratio of the protein absorbance at 278 nm to the most prominent visible absorbance at 384 nm is 0.34 for the oxidized from. The extinction coefficient at 384 nm is 22 $mM^{-1} \cdot cm^{-1}$ and the ratio of the absorption bands $A_{384\ nm}/A_{573\ nm}$ is 4.1. No significant spectral difference has been found between nitrite reductase I and II. However preparations of nitrite reductase II, although electrophoretically pure, have sometimes a lower specific activity. This activity loss is accompanied by a decreased absorbance at the 384 nm and 573 nm band.

Anaerobic addition of dithionite to the enzyme results in the loss of the 573 nm band and the appearance of two small peaks at 555 nm and 585 nm. The 635 nm and 692 nm maxima disappear. The original state of the enzyme after addition of dithionite cannot be restored when the enzyme is flushed with oxygen and/or nitrite is added. Only the 573 nm peak reappears, whereas the 555 nm peak is shifted to 525 nm and the absorbance beyond 600 nm is not restored. The absorption spectrum obtained after complexing the enzyme with pyridine or cyanide does not show pronounced absorption maxima and is very unlike a hemochrome spectrum, but HCl–butanone treatment releases a red iron containing pigment from the enzyme.

Substrate and cofactor specificity. Table III shows the ferredoxin dependent reduction of nitrite in a reconstituted enzymatic system. The system uses NADPH as primary electron donor, transferring its reduction equivalents via NADP reductase to ferredoxin. Reduced ferredoxin is oxidized by nitrite reductase catalyzing concomitantly the stoichiometric reduction of nitrite to ammonia. For 1 mole of nitrite reduced, 3 moles of NADPH are oxidized. It must be emphasized that in the absence of NADP reductase or ferredoxin no reduction of nitrite takes place with NADPH. Crude extracts which were selectively freed from ferredoxin by DEAE-cellulose treatment are not able to reduce nitrite either with NADPH or NADH. Flavodoxin, a physiological substitute for ferredoxin under conditions of limited iron supply can catalyze nitrite reduction when reduced by illuminated spinach chloroplasts (H. Spiller and W. G. Zumft, unpublished).

Purified *Chlorella* ferredoxin has in its oxidized form absorption maxima at 275, 330, 420 and 463 nm. 2 gram atoms of iron per mole of protein were found by atomic absorption spectroscopy. The molecular weight was determined by gel chromatography to be 11 700.

TABLE III

COFACTOR REQUIREMENT OF NITRITE REDUCTION IN A RECONSTITUTED ENZYMATIC SYSTEM

The reaction was carried out in Warburg vessels under N_2 atmosphere at 26 °C. The complete system contained in a total volume of 2 ml 120 μmoles Tris (pH 7.5), 4 μmoles $NaNO_2$, 6 μmoles NADPH, 0.5 mg NADP reductase, 1 mg *Chlorella* ferredoxin and 4 μg nitrite reductase. After 15 min NADPH was determined spectrophotometrically, nitrite and ammonium colorimetrically as described in Methods.

System	*NADPH oxidized (μmoles)*	*NO_2^- reduced (μmoles)*	*NH_4^+ formed (μmoles)*
Complete	4.1	1.34	1.3
Nitrite reductase omitted	0.2	0.1	0.1
NADP reductase omitted	0	0	0.1
Ferredoxin omitted	0	0	0.1

In addition to nitrite both nitrite reductases also reduce hydroxylamine to ammonia. To demonstrate this ,an anaerobic system has to be used and the oxidation of reduced methyl viologen is followed spectrophotometrically. The reduction rate of hydroxylamine is 6-fold lower than that of nitrite. Under the same conditions sulfite cannot be reduced by *Chlorella* nitrite reductase. The addition of bovine serum albumin or cysteine, required for a spinach sulfite reductase preparation[32], does not make nitrite reductase reduce sulfite.

Molecular weight. We have previously estimated the molecular weight of nitrite reductase by gel chromatography to be 63 000[5]. Since it was not clear at that time that the preparation contains two electrophoretically separable forms of the enzyme, the molecular weights of nitrite reductase I and II were reexamined by dodecyl sulfate electrophoresis in polyacrylamide gel. In order to subject all proteins to the same conditions and to detect the smallest variation in their electrophoretic mobility the split-gel technique was used[33]. Both nitrite reductases migrate the same distance from the origin when applied to a split-gel and application of a mixture of the enzymes gives only a single band and not a twin band as in usual electrophoresis. These results indicate an identical molecular weight for nitrite reductase I and II, as well as the fact that both forms cannot be separated by gel chromatography. Nitrite reductase has an electrophoretic mobility of 0.215 related to bromophenol blue. This corresponds to a molecular weight of 63 000 (Fig. 5), a value also obtained by gel chromatography.

Amino acid composition. Table IV gives the amino acid composition of nitrite

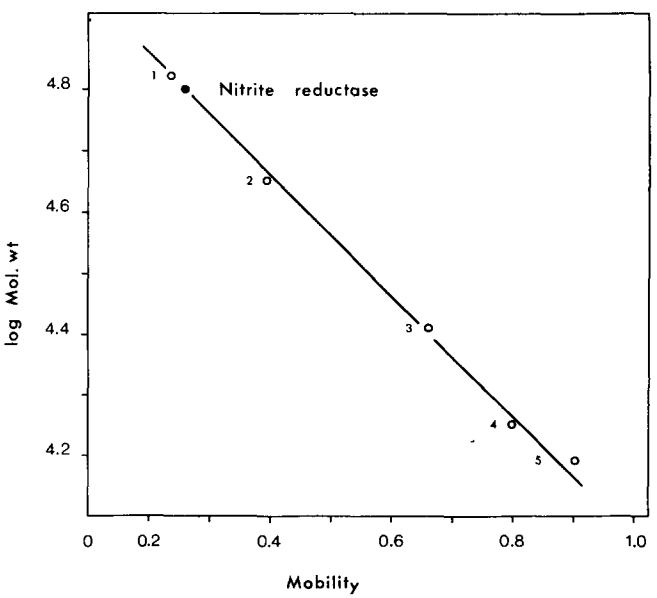

Fig. 5. Molecular weight determination of nitrite reductase. The enzyme and protein standards were dialysed 3 h against 10 mM phosphate buffer (pH 7.0) containing 1% dodecyl sulfate and 1% mercaptoethanol; dialysis was continued overnight against the same buffer with 0.1% dodecyl sulfate and 0.1% mercaptoethanol. To one half of a split-gel 25 μg of enzyme were applied and to the other half a mixture of 5 μg of each of the following proteins: (1) bovine serum albumin, mol. wt 67 000; (2) ovalbumin, mol. wt 45 000; (3) chymotrypsinogen, mol. wt 25 700; (4) myoglobin, mol. wt 17 000 and (5) hemoglobin, mol. wt 15 500.

TABLE IV

AMINO ACID COMPOSITION OF NITRITE REDUCTASE

Amino acid	*Number of residues per molecule* (mol. wt 63 000)*
Asp	61
Thr**	34
Ser**	27
Glu	63
Pro	36
Gly	56
Ala	60
Cys	10
Val***	39
Met	13
Ile***	31
Leu	49
Tyr	14
Phe	23
His	13
Lys	38
Arg	29
Trp†	4
Total	600

* Calculated values for 20 and 70 h hydrolysis.
** Extrapolated to zero time hydrolysis.
*** Extrapolated to 100 h hydrolysis.
† Determined by hydrolysis with 2% thioglycolic acid[27].

reductase I as average values from 20 and 70 h hydrolysis. Serine and threonine are corrected by extrapolation to zero time hydrolysis whereas valine and isoleucine are extrapolated to 100 h hydrolysis. Four residues of tryptophan were determined by hydrolysis in thioglycolic acid[27]. Its presence is consistent with a shoulder at 290 nm in the ultraviolet absorption spectrum of the enzyme. The protein shows a slight predominance of acidic amino acids in accordance with an isoelectric point of around five. The enzyme contains ten residues of cysteine whose sulfhydryl groups most probably participate in the electron transfer as enzymatic activity is completely blocked by 0.1 mM *p*-chloromercuribenzoate. The large number of cysteine residues might account for its possible role in binding the two iron atoms to the protein moiety.

DISCUSSION

Work on nitrite reduction with higher plants and algae has been reviewed by Kessler[34] and more recently by Beevers and Hageman[35]. As the latter authors have pointed out, nitrite reductase has not been purified completely from any organism. The procedure described here, allows a 500-fold purification of *Chlorella* nitrite reductase to a homogeneous state with approx. 20% recovery. The specific activity of 51.7 μmoles NO_2^- reduced per min per mg protein is the highest value reported so far. Hucklesby and Hewitt[9] recently purified marrow nitrite reductase to a specific activity of 46 units but their recovery was low and no physicochemical characteriza-

tion of the enzyme was possible. The method described in this paper achieves the separation of two nitrite reducing proteins, designated enzyme I and II, by electrophoresis in polyacrylamide gel. Enzyme I has a slightly lower electrophoretic mobility and is present in *Chlorella* in approximately twice the concentration of enzyme II. It remains to be established if the difference between both enzymes is because of a different primary structure, *i.e.* that they are isoenzymes, or if one enzyme is an artifact derived from the other one in an early step of purification. Hucklesby and Hewitt[9] have also observed the occurence of a marked twin band in the electrophoretic pattern of purified spinach nitrite reductase. Although theses authors did not identify this twin band as the two enzymes described here, this points strongly to a common phenomenon in the behavior of plant nitrite reductases.

Ferredoxin was found to be the natural cofactor of nitrite reductase, reduced chemically, photosynthetically or by reconstituted enzymatic systems[6,36–38]. For *Chlorella* it was demonstrated here that in a reconstituted enzymatic system with chloroplast NADP reductase nitrite is stoichiometrically reduced to ammonia in a ferredoxin dependent reaction. Recently, a low potential electron transfer protein flavodoxin was isolated from *Chlorella*[29,39]. As already demonstrated by Bothe[89] with *Anacystis* flavodoxin (= phytoflavin) and spinach nitrite reductase, this flavoprotein can substitute for ferredoxin. Thus there are two natural ocurring cofactors ferredoxin and flavodoxin available for electron transport to nitrite reductase. The close relation of nitrite reductase to the photosynthetic apparatus was concluded several years ago by Kessler[34] from physiological work. The same author and his coworkers proposed from inhibitor studies with uncoupling agents a requirement of ATP for nitrite reduction[34,404,41]. However in the systems described here no stimulatory effect of ATP could be observed.

Measurements of the molecular weight by either gel chromatography[5] or dodecyl sulfate electrophoresis in polyacrylamide gel gave the same value, 63 000. It can be assumed therefore, that nitrite reductase occurs under native conditions also in a monomeric form and consists only of a single polypeptide chain. Analysis of the C-terminal and N-terminal amino acids should provide supporting evidence for this assumption. The visible spectrum of *Chlorella* nitrite reductase has no maxima or shoulders but a rather low absorbance in the 450 nm region. This fact suggests that the enzyme has no flavin prosthetic group. Conclusive evidence, however, has to await a flavin analysis of the purified enzyme. The absorption spectrum slightly resembles that of a heme-protein. Indeed a red iron-pigment is released from nitrite reductase upon acid treatment, the enzyme-bound iron is not accessible to α,α'-bipyridyl and complexes of nitrite reductase with cyanide, pyridine and carbon monoxide are formed. These complexes however, are unlike those of the corresponding hemochromes and the $\varepsilon_{384\ nm}$ of 22 $mM^{-1} \cdot cm^{-1}$ for 2 atoms of iron is too low to account for heme groups. Iron is easily removed from the protein by the mild heat treatment used for the colorimetric iron determination and preliminary evidence shows the presence of acid labile sulfide. These facts do not favor the presence of a protoheme in the enzyme.

Although *Chlorella* nitrite reductase is unable to reduce sulfite there exists a striking similarity between this enzyme or nitrite reductase from spinach[7,42] and sulfite reductase from spinach[13,32] (A. Schmidt, unpublished), yeast[43,44] and *E. coli*[45]. As shown in Table V nitrite and sulfite reductases obviously contain a common

TABLE V

COMPARISON OF NITRITE REDUCTASE AND SULFITE REDUCTASE

	Chlorella nitrite reductase	*Spinacea nitrite reductase*	*Spinacea sulfite reductase*	*Saccharomyces sulfite reductase*	*E. coli sulfite reductase*
Absorption maxima	384, 573, >600	380, 570	385, 404, 589, >700	386, 587*	386, 587, >600*
Absorption ratio	4.1	4.0	4.1	4.2	4.4
Mol. wt	63 000	63 000	83–85 000	350 000	760 000
Iron	+	+	+	+	+
Flavin	−	−	−	+	+
Electron acceptors	NO_2^- NH_2OH	NO_2^- NH_2OH	SO_3^{3-}, NO_2^- NH_2OH	SO_3^{2-}, NO_2^- NH_2OH	SO_3^{2-}, NO_2^- NH_2OH
Electron donors	Fd, MV	Fd, MV	Fd, MV	MV, NADPH	MV, NADPH
Complexes with CO or bases	+	?**	+	+	+
Refs	5, this paper	6, 7, 9, 42	13, 32	43	45

* Flavin absorption bands not given for simplicity.
** Nitrite reductase from *Cucurbita* was found to be CO sensitive[46].

chromophore absorbing around 380 nm and 570 nm with a corresponding ratio close to 4. All these enzymes contain iron, form complexes with bases or carbon monoxide and are rather similar in their electron donor and acceptor properties. When NADPH instead of ferredoxin is the electron donor as for the sulfite reductase from yeast and *E. coli* the enzymes contain in addition a flavin dependent diaphorase. In that case the molecular weight is considerably higher than that of the ferredoxin dependent enzymes. Moreover, Yoshimoto and Sato[44] obtained a methyl viologen dependent sulfite reductase from a yeast mutant which had lost its NADPH-dependent reactions. This enzyme had a sedimentation coefficient of 5.1 S compared to 14.8 S of the wild type and was devoid of flavin. The light absorption properties of this protein are almost identical to *Chlorella* nitrite reductase. Siegel and Kamin[45] concluded for *E. coli* sulfite reductase the presence of a heme-like high-spin ferric iron mainly from an EPR signal at $g = 5.25$ and 6.7. More experimental evidence is required to reveal the nature of this iron chromophore as well as to establish its proposed identity in nitrite and sulfite reductase.

ACKNOWLEDGEMENTS

My thanks to Prof. E. Kessler for his support during this work. I am indebted to Miss Inge Wieschollek for excellent assistance and Mrs Rita Hellmann for maintaining the *Chlorella* cultures. I wish to thank Dr L. Gürtler for amino acid analysis and Mr W. Schmidt for a gift of RNAase T_1. Supported by the Deutsche Forschungsgemeinschaft and the Stiftung Volkswagenwerk.

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