Purification and properties of the assimilatory nitrite reductase from barley *Hordeum vulgare* leaves

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The assimilatory nitrite reductase (ferredoxin:nitrite oxidoreductase, EC 1.7.7.1) from barley (*Hordeum vulgare* L.) leaves has been purified over 1500-fold with a recovery of 30% and a specific activity of 84 μmol of nitrite reduced/min per mg of protein. The purification procedure includes $(\text{NH}_4)_2\text{SO}_4$ fractionation, ion-exchange and molecular-sieve chromatographies and, finally, ferredoxin–Sephacryl-4B affinity chromatography. The enzyme appears homogeneous by polyacrylamide-gel electrophoresis and consists of a single polypeptide chain with an M_r of 61 000. The absorption spectrum of the pure enzyme was typical of a haem-containing protein. The enzyme showed low thermostability and was specific for ferredoxin (K_m 0.4 μM), although reduced Methyl Viologen (K_m 120 μM) was also effective. The same K_m value for nitrite (250 μM) was obtained with both electron carriers. Cyanide acted as a powerful pure competitive inhibitor of enzyme with respect to nitrite (K_i 40 μM). Thiol-blocking agents also caused considerable inhibition, but only the ferredoxin-driven activity was significantly inhibited by sulphite and hydroxylamine.

In higher plants and algae the assimilatory nitrite reductase (ferredoxin:nitrite oxidoreductase, EC 1.7.7.1) catalyses the reduction of nitrite to ammonia with a six-electron transfer at a time (see Hewitt, 1975; Losada, 1976; Guerrero *et al.*, 1981). In the last few years, a number of algal and plant nitrite reductases have been purified to electrophoretic homogeneity by polyacrylamide-gel electrophoresis using conventional procedures (Zumft, 1972; Huckleby *et al.*, 1976; Vega & Kamin, 1977). The enzyme from barley (*Hordeum vulgare* L.) seedlings (isolated from either leaf or root tissues) has also been the subject of different studies (Mifflin, 1967; Bourne & Mifflin, 1973; Ida *et al.*, 1974), although its purification to homogeneity has not been reported to date. Recently, affinity chromatography on ferredoxin–Sephacryl has been successfully employed in the purification of nitrite reductase from spinach (*Spinacia oleracea*) leaves (see Ida, 1977).

In the present paper we report the complete purification of nitrite reductase from barley leaves and discuss some relevant structural and catalytic properties of the purified enzyme in comparison with those of pure nitrite reductase isolated from other sources.

Abbreviation used: SDS, sodium dodecyl sulphate.

Experimental

Materials

All reagents and biochemicals were obtained from commercial sources and were of the highest purity available. Electron carriers, inhibitors, β -lactoglobulin, bovine serum albumin, SDS and Brilliant Blue G-250 were obtained from Sigma Chemical Co.; Methyl Viologen, sodium hypochlorite, sodium dithionite and reagents for polyacrylamide gels from BDH Chemicals; aldolase and ovalbumin from Boehringer Corp.; DEAE-cellulose (DE-52) from Whatman Biochemicals; Sephacryl S-200 and CNBr-activated Sepharose-4B from Pharmacia Fine Chemicals. All other chemicals were obtained from Merck. *Spirulina maxima* ferredoxin ($A_{423}/A_{276} > 0.5$) was isolated as described by Hall *et al.* (1972). Ferredoxin–Sephacryl-4B was prepared as described in the booklet *Affinity Chromatography* (Pharmacia Fine Chemicals, Uppsala, 1979). About 50 mg of ferredoxin were coupled to 3 g of the CNBr-activated gel.

Barley (*Hordeum vulgare* L. var. Miranda) seedlings were grown at room temperature on Perlite under continuous illumination provided by cool-white fluorescent tubes (Sylvania F65T12/CW) after germination in aerated distilled water. Plants were

watered daily for 5–7 days with distilled water, and finally with Hoagland solution (Hoagland & Arnon, 1950) (containing 30 mM-nitrate) to induce nitrite reductase activity. After 2–3 days induction period, the leaf material was harvested and stored at -20°C until enough material was collected.

Nitrite reductase assay and analytical methods

Nitrite reductase activity was routinely assayed as described by Vega *et al.* (1980). One unit of enzyme catalysed the reduction of $1\mu\text{mol}$ of nitrite/min under these conditions. Moreover, in some cases activity was determined by measuring the appearance of NH_3 . The method of Davis (1964) was used for disc electrophoresis in 7.5% (w/v) polyacrylamide. To observe protein bands, gels were stained with Coomassie Brilliant Blue G-250 as described by Reisner *et al.* (1975). Nitrite reductase activity was located on the gels by the method of Vega & Kamin (1977). The molecular weight of nitrite reductase was determined by gel filtration on a column ($2.2\text{ cm} \times 90\text{ cm}$) of Sephacryl S-200 equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-KCl, 10 mM-2-mercaptoethanol and 1 mM-EDTA, as described by Andrews (1964), with aldolase, bovine serum albumin, ovalbumin and β -lactoglobulin as molecular-weight markers. Polyacrylamide-gel electrophoresis in the presence of SDS was performed by the method of Weber *et al.* (1972). Nitrite was determined colorimetrically by the method of Snell & Snell (1949). NH_3 was determined by the phenol/hypochlorite method as described by Solórzano (1969). Protein was measured by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Fig. 1. Elution profile of nitrite reductase from barley leaves on ferredoxin-Sepharose-4B

The column ($1.6\text{ cm} \times 16\text{ cm}$) was equilibrated with 20 mM-Tris/HCl buffer, pH 8.0. Fractions (5 ml) were collected at a 60 ml/h flow rate, into tubes containing glycerol and 2-mercaptoethanol [to provide final concentrations of 15% (v/v) and 10 mM respectively] in order to minimize enzyme inactivation.

Purification of nitrite reductase

The enzyme was purified from 500 g of fresh barley leaves by a method very similar to that reported by Ida (1977), in 50 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-2-mercaptoethanol and 1 mM-EDTA. The ferredoxin-Sepharose-4B affinity chromatography was performed in the same buffer but without 2-mercaptoethanol and EDTA. The activity was eluted with a 200 ml linear gradient of KCl from 0 mM to 400 mM in buffer (Fig. 1). Those fractions containing pure enzyme, as judged by polyacrylamide-gel electrophoresis, were pooled, concentrated (Amicon ultrafilter, Diaflo PM-10 membrane) and stored at -20°C .

Results and discussion

Purification procedure and purity of enzyme

Use of affinity chromatography on ferredoxin-Sepharose-4B has greatly improved the previously reported values of specific activity and recovery of spinach nitrite reductase preparations (see Ida, 1977). In our case the purification procedure, summarized in Table 1, yielded about 4 mg of pure nitrite reductase from 500 g of barley leaves. The enzyme was purified over 1500-fold with a recovery of 30% and a specific activity of 84.4 units/mg of protein. This specific activity is comparable with that of enzyme isolated from vegetable marrow (*Cucurbita pepo*) (85.2 units/mg of protein) by Hucklesby *et al.* (1976), but much lower than those found for spinach: 110 units/mg of protein (Vega & Kamin, 1977) or 140 units/mg of protein (Ida, 1977).

The purity of enzyme was monitored by polyacrylamide-gel electrophoresis. After the last step of purification, a single band of protein coincidental with the band of activity was detected.

Physical properties of nitrite reductase

The relative molecular mass (M_r) of nitrite reductase estimated by chromatography on Sephacryl S-200 was 61 000 (± 2000). This value is the same as that reported for the enzyme isolated from barley roots (Ida *et al.*, 1974) and is in the range reported for other preparations. After heating the pure enzyme for 3 min in boiling water with 2.5% (w/v) SDS and 5% (v/v) 2-mercaptoethanol, SDS/polyacrylamide-gel electrophoresis revealed the presence of a single band of protein, indicating that the enzyme is a monomer and confirming the homogeneity of the enzyme preparation. The same M_r , as calculated by gel filtration, was obtained by comparison of its relative electrophoretic mobility with that of standard proteins.

The absorption spectrum of the pure nitrite reductase showed maxima at 278, 388, 530 and 572 nm and a shoulder at 290 nm. This spectrum is characteristic of a haem-containing protein and is similar to that reported for spinach (Vega & Kamin,

Table 1. *Purification of nitrite reductase from barley leaves*

Nitrite reductase activity is expressed as μmol of nitrite reduced/min. The reaction was performed as indicated in the Experimental section, with chemically reduced Methyl Viologen (at 5 mM) as electron donor.

Purification step	Protein (mg)	Activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
1. Crude homogenate	19 300	1083	0.056	1	100
2. DEAE-cellulose DE-52 I	975	985	1.01	18.0	91
3. 45–90%-satd. $(\text{NH}_4)_2\text{SO}_4$	214	920	4.30	76.8	85
4. DEAE-cellulose DE-52 II	120	790	6.58	117	73
5. Sephacryl S-200	22.6	628	27.8	496	58
6. Ferredoxin–Sepharose-4B	3.85	325	84.4	1507	30

1977; Ida, 1977), *C. pepo* (Hucklesby *et al.*, 1976) and the alga *Chlorella fusca* (Zumft, 1972). The absorbance ratios A_{278}/A_{388} and A_{388}/A_{572} were 2.0 and 3.4 respectively. The former value is the same as that for nitrite reductase from *C. pepo* and is similar to the value reported for the enzyme from spinach (1.8), but is lower than that from the red alga *Porphyra yezoensis* (2.5) (Ho *et al.*, 1976) and *C. fusca* (3.3).

The enzyme showed low thermostability and was inactivated above 30°C. Complete inactivation occurred after a 10 min incubation at 60°C. The presence of Methyl Viologen (at 5 mM) did not increase the thermostability of the enzyme. However, nitrite (at 5 mM) protected the enzyme to some extent, and about 10% of the initial activity was retained after 10 min at 60°C. In the presence of nitrite, total inactivation occurred only at 70°C. Similar thermostability has been reported for nitrite reductase from higher plants; however, the enzyme from the blue-green alga *Anacystis nidulans* (Guerrero *et al.*, 1974) and the diatom *Skeletonema costatum* (Llama *et al.*, 1979) are resistant to heat inactivation.

Catalytic properties of nitrite reductase

The purified enzyme catalyses the stoichiometric reduction of nitrite to NH_3 by using Methyl Viologen or ferredoxin as electron donor. No products in intermediate reduction states were found, nor was a reaction observed when nitrite or the reduced electron carrier was omitted. Dithionite-reduced Methyl Viologen was the most efficient electron donor examined. The enzyme, in common with nitrite reductases isolated from other higher plants, presented a marked specificity for reduced ferredoxin as the physiological electron donor, although only 40% of the activity obtained with Methyl Viologen was routinely measured. The pure enzyme was completely inactive with FMN, NAD^+ or NADP^+ , all reduced with dithionite. However, a slight activity (about 8% of control) was always detected with reduced FAD.

The same K_m for nitrite (250 μM) was found when *Sp. maxima* ferredoxin or Methyl Viologen,

both reduced chemically with dithionite, was used as electron donor. This value is close to that reported for the enzyme from barley roots (see Ida *et al.*, 1974) and is of the same order to that reported for the purified enzyme from spinach. However, it is much lower than that (2 mM) previously published (see Bourne & Mifflin, 1973) for nitrite reductase isolated from barley root and leaf material. The K_m values for Methyl Viologen and *Sp. maxima* ferredoxin were 120 μM and 0.4 μM respectively.

The effect of pH on the activity of the enzyme was studied by using reduced Methyl Viologen or ferredoxin as electron donor in universal buffer (see MacKenzie, 1969) and potassium phosphate buffer. In all cases an optimum pH for activity of 6.0–6.5 was obtained. This value is lower than those (between 7.0 and 8.0) for the enzyme from green algae and other higher plants.

Inhibitors of nitrite reductase

The effect of a number of inhibitors on reduced Methyl Viologen:nitrite reductase and reduced ferredoxin:nitrite reductase activities was investigated (Table 2). The metal-binding agents KCN, bathophenanthroline and NaN_3 were used at final concentrations up to 1 mM. Methyl Viologen:nitrite reductase from barley leaves was not very sensitive to these metal-complexing agents, with the exception of cyanide, suggesting that the iron was firmly bound in the native enzyme. Similar results have been noted in the enzyme from the green alga *C. fusca* (Zumft, 1972), the fungus *Neurospora crassa* (Lafferty & Garrett, 1974) and the diatom *S. costatum* (Llama *et al.*, 1979). KCN caused severe inhibition with both electron carriers tested. The inhibition by cyanide of our Methyl Viologen:nitrite reductase activity was of the competitive type ($K_i = 40 \mu\text{M}$), as determined from Dixon (1953) plots. A replot of the slopes of the lines from the primary plot against the corresponding $1/[\text{nitrite}]$ values indicated that cyanide acted as a pure competitive inhibitor with respect to nitrite. Cyanide inhibition has been also reported for the enzyme from a number of sources, including higher plants, algae and micro-organisms.

Table 2. *Inhibitors of nitrite reductase activity with Methyl Viologen or Sp. maxima ferredoxin as electron carriers*
The enzyme (2.7 µg of protein) was incubated in a shaking water-bath at 30°C for 10 min with the inhibitor in 50 mM-potassium phosphate buffer, pH 7.0. The electron carrier (Methyl Viologen at 2.5 mM or *Sp. maxima* ferredoxin at 8 µM) and sodium dithionite (at 25 mM) were then added and the residual activity was determined as described in the Experimental section. The results are average values for three separate experiments.

Inhibitor	Concn. (mM)	Electron carrier ...	Inhibition (%)	
			Methyl Viologen	<i>Sp. maxima</i> ferredoxin
KCN	0.01		11	28
	0.1		58	66
	1		79	86
Bathophenanthroline	0.1		0	33
	1		0	71
<i>p</i> -Hydroxymercuribenzoate	0.1		54	35
	1		61	44
<i>N</i> -Ethylmaleimide	0.1		5	69
	1		14	85
Hydroxylamine	0.1		0	13
	1		0	26
Na ₂ SO ₃	0.1		0	15
	1		0	28

Bathophenanthroline only caused significant inhibition when ferredoxin was used as electron mediator. This result could be explained if this iron-complexing agent exerted its effect on the ferredoxin rather than on the native enzyme, or, if the presumed complexed iron atom(s) of the native enzyme was not essential for activity when reduced Methyl Viologen was the electron donor. NaN₃ did not cause any inhibitory effect of nitrite reductase, even at 2.5 mM. The thiol-blocking reagents *p*-hydroxymercuribenzoate and *N*-ethylmaleimide caused significant inhibition of both activities, suggesting that there are exposed cysteine residues essential to nitrite reductase function.

It is noteworthy that hydroxylamine and Na₂SO₃ (both at 1 mM) did not inhibit Methyl Viologen: nitrite reductase activity. However, both compounds were able to produce considerable inhibition of enzyme when reduced ferredoxin was used as electron donor. Purified preparations of higher-plant and algal nitrite reductases catalysed the reduction of hydroxylamine to NH₃, although at lower rates than nitrite. However, to date no sulphite reductase activity has been reported in purified nitrite reductase preparations.

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