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ORIGINAL ARTICLE

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NADH-dependent metabolism of nitric oxide in alfalfa root cultures expressing barley hemoglobin

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Abstract Transgenic alfalfa (*Medicago sativa* L.) root cultures expressing sense and antisense barley (*Hordeum vulgare* L.) hemoglobin were examined for their ability to metabolize NO. Extracts from lines overexpressing hemoglobin had approximately twice the NO conversion rate of either control or antisense lines under normoxic conditions. Only the control line showed a significant increase in the rate of NO degradation when placed under anaerobic conditions. The decline in NO was dependent on the presence of reduced pyridine nucleotide, with the NADH-dependent rate being about 2.5 times faster than the NADPH-dependent rate. Most of the activity was found in the cytosolic fraction of the extracts, while only small amounts were found in the cell wall, mitochondria, and 105,000-*g* membrane fraction. The NADH-dependent NO conversion exhibited a broad pH optimum in the range 7–8 and a strong affinity to NADH and NADPH (K_m 3 μM for both). It was sensitive to diphenylene iodonium, an inhibitor of flavoproteins. The activity was strongly reduced by applying antibodies raised against recombinant barley hemoglobin. Extracts of *Escherichia coli* overexpressing barley hemoglobin showed a 4-fold higher rate of NO metabolism as compared to non-transformed cells. The NADH/NAD and NADPH/NADP ratios were higher in lines underexpressing hemoglobin, indicating that the presence of hemoglobin has an effect on these ratios. They were increased under hypoxia and antimycin A treatment. Alfalfa root extracts exhibited methemoglobin reductase activity, using either cytochrome *c* or recombinant barley hemoglobin as substrates. There was a correspondence between NO degradation and nitrate formation. The activity was eluted from a Superose 12 column as a single peak with molecular weight of 35 ± 4 kDa, which corresponds to the size of the hemoglobin dimer. The results are consistent with an NO dioxygenase-like activity, with hemoglobin acting in concert with a flavoprotein, to metabolize NO to nitrate utilizing NADH as the electron donor.

Keywords Hemoglobin · Hypoxia · *Medicago* · NADH/NAD ratio · Nitric oxide · NO dioxygenase

Abbreviation Hb: Hemoglobin

Introduction

Barley (class 1) hemoglobin (Hb) is induced during hypoxic stress as a result of a change in the availability of ATP in the tissue (Nie and Hill 1997). The induction of the protein appears to assist in the re-establishment of energy balance during hypoxic stress as studies with transgenic maize cell cultures have shown that cells overexpressing barley Hb maintain their energy status under hypoxic conditions in comparison to control or barley antisense lines (Sowa et al. 1998).

Hemoglobins, in addition to binding oxygen, ligate and react with a number of compounds that have similar molecular configuration. Nitric oxide, in addition to ligating with Hb, reacts with oxyH6 to form methemoglobin and nitrate. Alfalfa root cultures synthesize NO during hypoxic stress (Dordas et al. 2003a). Root cultures overexpressing Hb accumulate less NO than either control or barley antisense alfalfa root culture lines. In line with the evidence for transgenic maize cell cultures, alfalfa root cultures overexpressing Hb and with lower NO levels maintain cell energy status.

In microorganisms, NO is metabolized by NO dioxygenase, a flavohemoglobin possessing NAD(P)H-dependent enzymatic activity (Gardner et al. 1998, 2000). In animal tissues, dioxygen-dependent metabolism of NO is likely connected with heme- and flavoprotein (Gardner et al. 2001); however, a particular Hb and flavin has not been identified. In plants, class 1 Hbs

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have been implicated in NO metabolism (Hill 1998; Dordas et al. 2003a, 2003b; Seregélyes et al. 2003). In the present investigation, we report for the first time the presence of NAD(P)H-dependent NO dioxygenase-like activity in plants, and present evidence that it is connected with the function of a plant class 1 Hb. We show that this activity may be important for maintaining both low NO levels and low NADH/NAD and NADPH/NADP ratios under hypoxia.

Materials and methods

Plant material

Transgenic alfalfa (*Medicago sativa* L.) roots overexpressing and downregulating non-symbiotic Hb were obtained as described in Dordas et al. (2003a). Before hypoxic treatment, the roots were placed into syringes and flushed overnight with 40% oxygen. For hypoxic treatment, the roots were flushed for 24 h with 3% oxygen. For modeling anoxic conditions, the roots were treated with antimycin A (50 μM) under hypoxic conditions to maximize suppression of mitochondrial electron transport.

Homogenization and fractionation of the samples

Roots (100 mg) were collected, frozen in liquid nitrogen and homogenized in 1 ml of 100 mM Tris–HCl buffer (pH 7.8), containing 0.1% cysteine and 0.2% BSA. The sample was centrifuged for 5 min at 14,000 *g* and the supernatant was used for measurement of activity. For subcellular fractionation, roots were homogenized (2 g in 20 ml) in the same buffer containing 0.4 M mannitol. Cell debris was separated by centrifugation at 1,300 *g* for 5 min and then washed by repeated treatment with buffer followed by centrifugation. Mitochondria were sedimented from the supernatant at 15,000 *g* for 15 min and washed under the same conditions to remove cytosolic contaminants. Membranes were separated from the cytosol by centrifugation at 105,000 *g* for 40 min in a Beckman ultracentrifuge.

Measurement of NO conversion

NO conversion was measured using an NO electrode (NOMK2; World Precision Instruments, Sarasota, FL, USA). The medium was 50 mM sodium phosphate buffer (pH 7.4) in a 2-ml vial. For kinetic measurements, it contained 0.1 mM EDTA and 1,000 units of bovine Cu,Zn-superoxide dismutase (Sigma) to prevent formation of peroxynitrite, which itself leads to NO removal (Gardner et al. 2001). For NO production, 1 mM sodium nitroprusside was added with continuous stirring, the vial was illuminated and the NO reached a saturating concentration of 1–1.5 μM. The sample (usually 10 μl) was added followed by the addition of NADH or NADPH (0.2 mM except experiments on K_m determination). The reaction was followed until NO was depleted. There was no NO decrease with NADH or NADPH in the absence of the sample. While testing diphenylene iodonium, the sample was preincubated for 10 min with the inhibitor before adding NADH.

To identify the reaction product, NO gas (Matheson Gas Products, Toronto, Canada) was added to the phosphate buffer to yield a concentration of 50–70 μM. After adding the sample and NADH (0.2 mM), the reaction was allowed to proceed until NO was depleted, as monitored by the NO electrode. The sample was boiled for 2 min, cooled on ice and nitrate was determined using nitrate reductase from *Aspergillus* (Sigma). Nitrite formed was quantified by the Griess reagent (Granger et al. 1996).

The K_m values were determined graphically in coordinates of the ratio of NADH or NADPH concentration to the rate of NO conversion (ΔNO) and the NADH or NADPH concentration (Dixon and Webb 1979).

The molecular weight of the protein possessing NO conversion activity was determined by gel filtration on a Superose 12 HR 10/30 column (Amersham Biosciences, Canada) as described in Duff et al. (1997).

Protein was extracted from roots with 0.2 M KOH and 0.08% Triton X-100 and assayed according to Bradford (1976).

Purification of overexpressed recombinant barley Hb from *Escherichia coli*

E. coli strain DH 5α (Invitrogen, Canada) was used as a host for both recombinant and nonrecombinant pUC 19 plasmids (Invitrogen, Canada). Preparation of extracts and purification of Hb was conducted as described in Duff et al. (1997), except that Q-Sepharose and Mono-Q FPLC was substituted by DEAE-Sephacel chromatography with a 0–250 mM NaCl gradient and dithiothreitol was omitted to obtain Hb in the (ferric) methemoglobin form. Purified Hb fractions had a ratio of absorbance at 412 nm to absorbance at 280 nm in the range 2.3–2.5, which corresponds to a 70–80% degree of purification.

Binding of NO-converting protein to antibodies raised against barley (*Hordeum vulgare* L.) Hb

An IgG fraction was purified from antiserum raised against recombinant barley Hb as described in Duff et al. (1998), using Protein A-Sepharose. The IgG concentration was 1.6 mg ml^{-1}. The fraction was mixed 1:1 with the cytosolic fraction of the Hb^+(209) line (protein content 11.5 mg ml^{-1}). It was stirred for 1 h at +4°C and then loaded on a Protein A-Sepharose column (2 cm long, 0.5 cm i.d.) and eluted with 10 mM Tris–HCl buffer (pH 8.5). In a control experiment, the cytosolic Hb^+ fraction was loaded onto the same column without mixing with IgG.

Measurement of pyridine nucleotides

For measurement of pyridine nucleotides, exactly the same amounts (100 mg) of roots from each treatment were frozen in liquid nitrogen and then homogenized (Wigge et al. 1993) either in 0.2 M HCl + 1% CHAPS (for oxidized forms, NAD^+ and $NADP^+$) or in 0.2 M KOH + 0.08% Triton X-100 (for reduced forms, NADH and NADPH). Alkaline extracts were heated at 60°C for 15 min to destroy oxidized forms and then cooled on ice. Pyridine nucleotides were determined by enzymatic cycling and quantified on an LS-5 Fluorescence Spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) as described earlier (Wigge et al. 1993).

Measurements of methemoglobin reductase and malate dehydrogenase

Methemoglobin reductase was measured at 550 nm as described by Topunov et al. (1980) in 50 mM sodium phosphate buffer (pH 7.4), 50 μM cytochrome *c*, 0.2 mM NADH, 10 μM methylene blue. In some measurements, cytochrome *c* was substituted by recombinant barley Hb (20 μM) in the presence of FAD (20 μM) and in the absence of methylene blue. The spectra between 516 and 600 nm were recorded on a Hewlett Packard 8452A diode array spectrophotometer (Agilent Technologies, Canada). Malate dehydrogenase was measured at 340 nm in 50 mM Tris–HCl buffer (pH 7.5) containing 2 mM oxaloacetate and 0.2 mM NADH.

Results

NO metabolism by alfalfa root extracts

Homogenates from alfalfa roots exhibited an ability to metabolize NO at a high rate that was dependent on NADH or NADPH. Figure 1 is a profile of NO levels measured on an NO electrode. Addition of homogenate caused a slight decline in NO levels that stabilized after 2 min. Addition of either NADH or NADPH caused a steady rapid decline in NO levels that leveled off as NO approached zero. Further addition of NO re-established the rate of NO disappearance provided NADH was not limiting (data not shown). The maximal velocity with NADH was about 2.5 times greater than that of NADPH. The activity was related to the level of expression of Hb in the roots (Fig. 2). Roots overexpressing hemoglobin (Hb^+; lines 3, 209) showed approximately twice the activity of roots underexpressing hemoglobin (Hb^-; lines 24, 44). Activity in the control line (C) at 40% oxygen was similar to the underexpressing lines and significantly lower than the activity of the overexpressing lines. Only the control line showed a significantly increased activity in the presence of 3% oxygen and antimycin A. Hypoxic treatment or antimycin A alone each gave results similar to the combined treatment.

Soluble protein levels varied slightly between lines, in a range of 8–12 mg g^{-1} FW, with the control line having a lower soluble protein level (data not shown). Based on soluble protein quantity, the Hb^+ lines had significantly higher NO-metabolizing activity than the control line, which in turn had significantly higher activity than Hb^- lines.

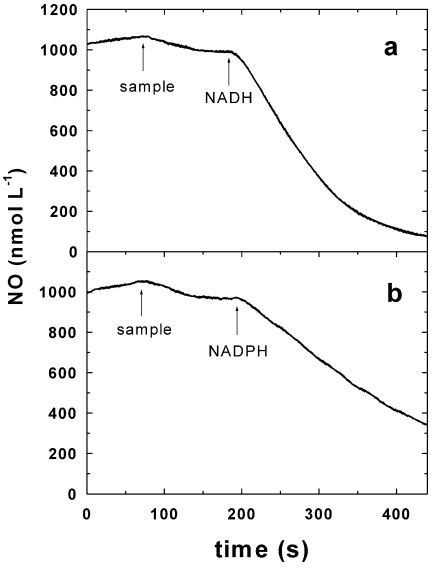

Fig. 1a,b Profile of the changes in NO concentration over time in the presence of an extract of an alfalfa (*Medicago sativa*) root culture line and NADH (**a**) or NADPH (**b**). Typical curves obtained in many similar experiments

Properties of the NAD(P)H-dependent NO conversion activity

Homogenates of the alfalfa root culture lines were fractionated in order to determine whether the observed NAD(P)H-dependent NO conversion activity was localized in a particular subcellular fraction (Table 1). Most of the activity was localized in the 105,000-*g* supernatant fraction. The activity was low in the crude pellet, mitochondria and membrane fractions. In the mitochondrial fraction, significant activity independent of NADH was observed which ceased after a period of time and could not be revived by addition of NADH (Fig. 3).

The NO conversion activity had a pH optimum at 7.4–8.0 (Fig. 4) either with NADH or NADPH. At pH 6.5, the activity was about half of the value at pH 7.5. There was a correspondence between NO degradation and the formation of nitrate ion (NO_3^-), with a stoichiometry of 0.8 ± 0.3 mol of nitrate formed per mol of NO (Table 2). The stoichiometry of NO conversion relative to NADH and NADPH oxidation was difficult to determine because of the high NADH and NADPH oxidation in the extracts in the absence of NO.

The NO degradation activity showed a very high affinity for NADH and NADPH (K_m 3 ± 1 μM for both pyridine nucleotides; Fig. 5). It was not possible to measure the K_m for NO because it was below the sensitivity of the electrode. Diphenylene iodonium (DPI, an

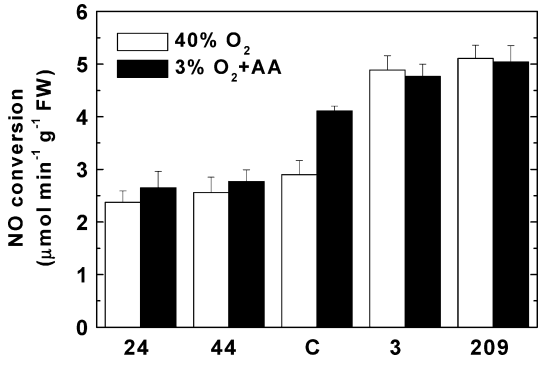

Fig. 2 Rates of NADH-dependent NO conversion in extracts of alfalfa root lines that had been exposed to two oxygen atmospheres. *AA* Antimycin A: *24, 44* lines downregulating Hb; *C* control line; *3, 209* lines overexpressing Hb. The values are averages of four independent measurements and standard errors

Table 1 Subcellular localization of NADH-dependent NO conversion activity

Fraction	NO conversion rate (μmol min^{-1} g^{-1} FW)
Crude extract (1,300 *g* supernatant)	3.21 ± 0.05
Crude pellet (1,300 *g*)	0.88 ± 0.04
Cytosol (105,000 *g* supernatant)	2.47 ± 0.08
Membranes (105,000 *g* pellet)	0.40 ± 0.01
Mitochondria (15,000 *g* pellet)	0.41 ± 0.02

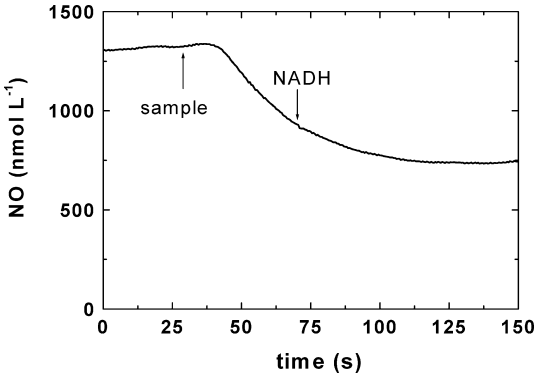

Fig. 3 NADH-independent NO conversion in the mitochondrial fraction of alfalfa root cultures. One typical curve from three repetitions

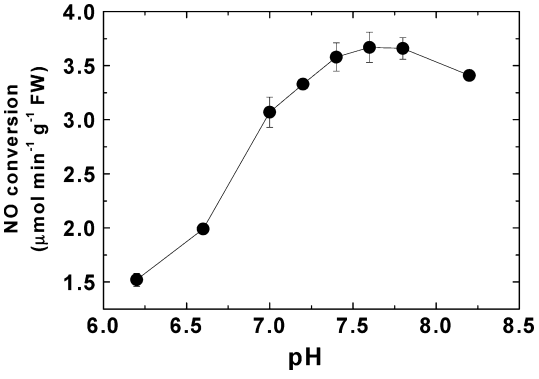

Fig. 4 pH dependence of NO conversion in the cytosolic fraction of alfalfa roots (line 209). Average values of three independent measurements and standard errors

Table 2 Nitrate formation from NO by the cytosolic fraction of the alfalfa (*Medicago sativa*) line 209

	NO decline (nmol)	NO_3^- detected (nmol)
– NADH	1 ± 1	12 ± 2
+ NADH	49 ± 8	48 ± 6
Difference	48 ± 9	36 ± 8

inhibitor of flavoproteins) demonstrated an increasing inhibition of NO conversion as concentration of the inhibitor increased, yielding an approximately 50% inhibition at 0.1 mM (Fig. 6).

Several observations suggest that Hb is an integral component in the observed NO dioxygenase activity. Firstly, a protein exhibiting NADH-dependent NO conversion activity was eluted from a Superose 12 HR 10/30 column at a molecular weight of 35 ± 4 kDa (Fig. 7). Secondly, NO dioxygenase activity is reduced when plant extracts are treated with an IgG fraction of antibodies raised against recombinant barley Hb and the mixture passed through a Protein A-Sepharose column (Table 3). Finally, extracts of *E. coli* overexpressing

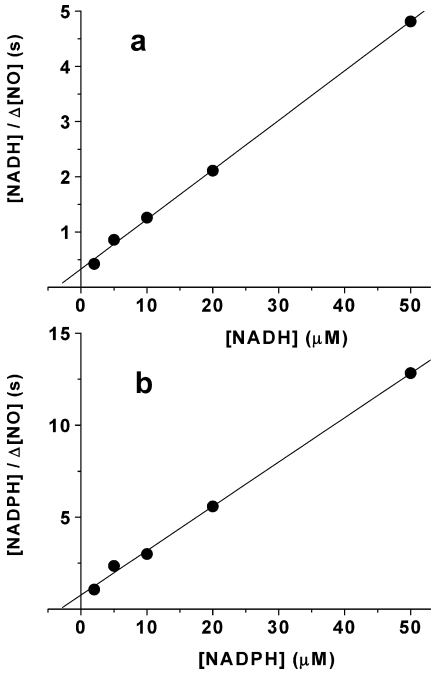

Fig. 5a,b Determination of K_m for NO conversion with NADH (**a**) and NADPH (**b**). Data from one of three representative experiments used to calculate the values for K_m

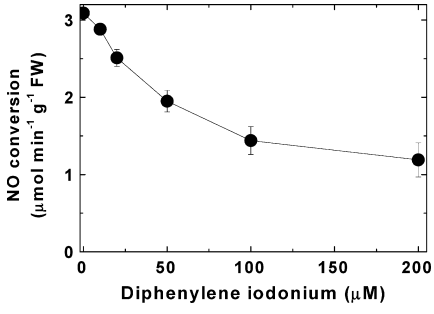

Fig. 6 The effect of diphenylene iodonium on NADH-dependent NO conversion by an alfalfa root extract (line 209). Average values from three measurements and standard errors

barley Hb exhibited more than 4-fold higher NADH-dependent NO conversion activity as compared to *E. coli* extracts not expressing barley Hb (Table 4).

Pyridine nucleotide levels in alfalfa roots

The relationship between NAD(P)H-dependent NO metabolism and the type of transgenic alfalfa root culture line led to investigations to determine whether the root cultures varied in their pyridine nucleotide profiles. The levels of NADH + NAD and NADPH + NADP in alfalfa roots did not differ significantly in any of the lines, being 92 ± 7 nmol g^{-1} FW for NAD + NADH and 74 ± 9 nmol g^{-1} FW for NADP + NADPH. The ratios of NADH/NAD and NADPH/NADP, however, varied between the lines (Fig. 8a). Under 40% oxygen,

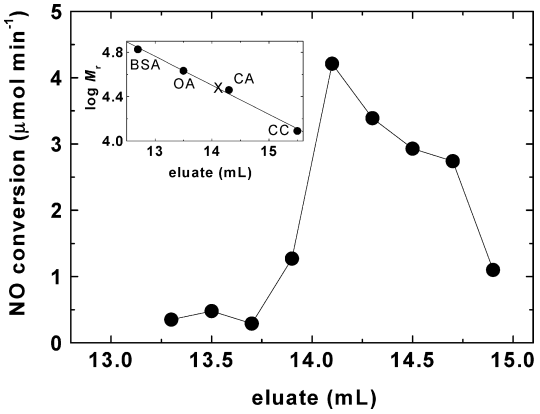

Fig. 7 Elution profile of NADH-dependent NO conversion activity from the Superose 12 column and calculation of molecular weight of the protein catalyzing this activity (*inserted graph*). *BSA* Bovine serum albumin, *OA* ovalbumin, *CA* carbonic anhydrase, *CC* cytochrome *c*, *X* location of the peak of NADH-dependent NO conversion activity

Table 3 Binding of a protein possessing NADH-dependent NO conversion activity to the antibodies raised against barley (*Hordeum vulgare*) Hb

Fraction	NO conversion rate	
	μmol min^{-1} g^{-1} FW	%
Cytosolic fraction	2.33 ± 0.22	100
Cytosolic fraction after Protein A-Sepharose	2.14 ± 0.17	92
Mixed (IgG + cytosol) fraction after Protein A-Sepharose	0.68 ± 0.22	29

Table 4 NADH-dependent NO conversion in the extracts of *Escherichia coli* non-expressing and overexpressing barley Hb

Strain	NO conversion rate (nmol min^{-1} mg^{-1} protein)
Non-expressing Hb	127 ± 26
Expressing Hb	564 ± 94

the control line and the Hb^- lines had significantly higher NADH/NAD than the Hb^+ lines, while line 24 had a significantly higher NADH/NAD ratio than the control (Fig. 8a). In the presence of 3% oxygen and antimycin A, all the lines showed a significant increase in the NADH/NAD ratios, with the exception of Hb^+(3). The Hb^- lines and the control line had increases from 50 to 70% in the ratio, while the Hb^+ lines had a 20% increase. Application of either hypoxia or antimycin A treatments alone led to an intermediate increase (20%) of the ratios in Hb^- lines.

There were similar trends in the NADPH/NADP ratios although there was greater variability in the results (Fig. 8b). Under 40% oxygen, Hb^-(24) had a significantly higher NADPH/NADP ratio than the control and Hb^+ lines, while the other lines were not significantly different from one another. In the presence of 3% oxygen and antimycin A, the control and Hb^- lines showed a 40–50% increase in the NADPH/NADP ratio while the Hb^+ lines showed an increase of 5–10%. The Hb^- lines had significantly higher NADPH/NADP ratios than the control and Hb^+ lines.

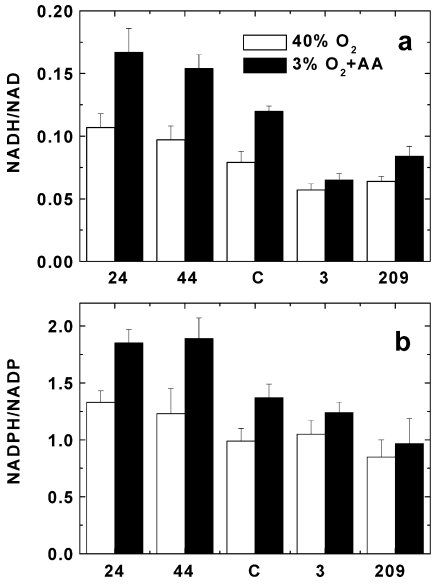

Fig. 8a,b NADH/NAD (**a**) and NADPH/NADP (**b**) ratios in alfalfa root cultures under 40% O_2 and 3% O_2 + antimycin A. Abbreviations as in Fig. 2

Methemoglobin reductase and malate dehydrogenase in alfalfa roots

It has been suggested that NO detoxification involving Hb and methemoglobin reductase may be a primary function of non-mammalian Hbs (Dordas et al. 2003b). To determine whether variations in Hb expression in alfalfa root cultures had an effect on methemoglobin reductase activity, its activity was measured in the transgenic alfalfa root culture lines. Using cytochrome *c* as a substrate, the methemoglobin reductase activity was higher in Hb^+ lines than in a control line, which had significantly higher activity than Hb^- lines (Fig. 9a). There was an approximately 2-fold increase in activity between the Hb^- lines and the Hb^+ lines. The activity was also observed with recombinant barley methemoglobin as a substrate, which was gradually reduced by root extracts to Hb and then immediately converted to the oxyhemoglobin form (Fig. 10).

NO is an effective inhibitor of cytochrome oxidase in the mitochondrial electron transport chain (Zottini et al. 2002). Increased capacity to remove NO might be expected to influence turnover of NAD(P)H through enzymes such as malate dehydrogenase. The malate dehydrogenase activity was significantly higher in Hb^+ lines and the control line compared to the Hb^- lines under aerobic conditions (Fig. 9b). There was no

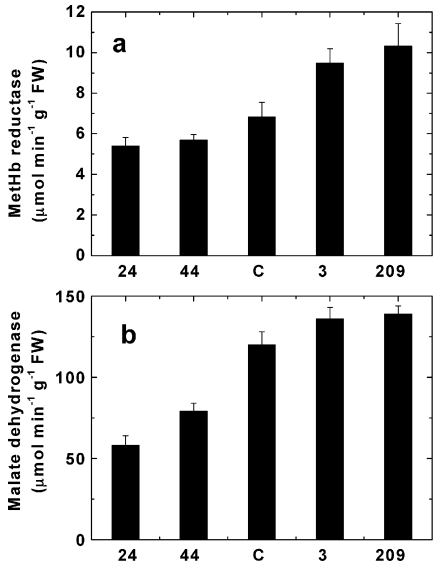

Fig. 9a,b Methemoglobin reductase activity with cytochrome *c* as a substrate (**a**) and malate dehydrogenase (**b**) activity in aerobically grown alfalfa root culture extracts. Data from three independent measurements and standard errors

significant difference in the activity of the control line compared to the Hb^+ lines. Hb^-(44) had a significantly higher malate dehydrogenase activity than Hb^-(24). There was no significant effect of a treatment with 3% oxygen plus antimycin A on the malate dehydrogenase activity of the various lines.

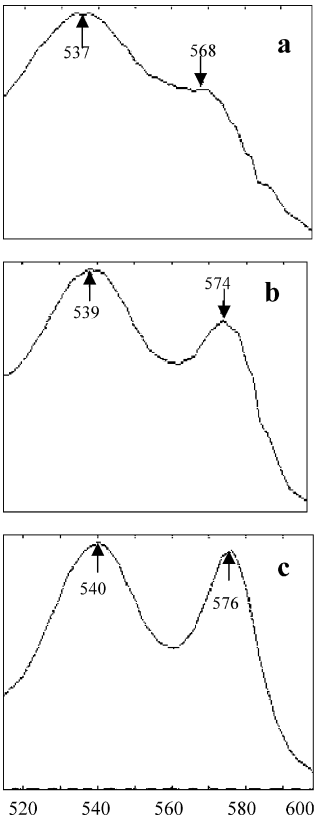

Fig. 10a–c Spectra recorded in the range 516—600 nm of purified recombinant barley (*Hordeum vulgare*) Hb: **a** in ferric methemoglobin form, **b** as the mixture of methemoglobin and oxyhemoglobin after addition of the cytosolic extract from line 209, **c** after complete reduction of Hb to ferrous oxyhemoglobin form

Discussion

Alfalfa root cultures had a high capacity for NO metabolism that was dependent on reduced pyridine nucleotide (Fig. 1). The rate of NO metabolism varied with the level of Hb expression in the cell lines (Dordas et al. 2003a), with Hb-overexpressing lines having significantly higher activity than the other lines tested (Fig. 2). Only the control line showed an increase in NO metabolism under 3% oxygen and antimycin A, a further indication of the possible involvement of Hb in the reaction as Hb expression has been shown to increase during hypoxia (Taylor et al. 1994). The differences in Hb content between overexpressing and downregulating lines (Dordas et al. 2003a) were shown to be even higher than observed differences in NADH-dependent NO-conversion rates. This may indicate that no linear relationship exists between Hb concentration and NO-consuming activity. Since flavoproteins have been shown to be involved in other systems displaying NO dioxygenase activity (Gardner et al. 2001), it is possible that a flavoprotein may be involved in reducing methemoglobin in the alfalfa root extracts and its concentration may not vary in the same proportion as the Hb in the various lines.

A protein possessing NADH-dependent NO-converting activity has a molecular weight very close to that of the barley Hb dimer (Fig. 7). It is possible that the activity resides in the Hb dimer itself or the activity may result from a sequential reaction of Hb and another (flavo?) protein of similar molecular weight. The protein participating in catalysis of NADH-dependent NO-converting activity binds to antibodies raised against barley Hb (Table 3). Extracts of *E. coli* overexpressing barley Hb exhibit a 4-fold higher NO-converting activity (Table 4). This all suggests that Hb is involved in catalysis of NADH-dependent NO degradation.

The reaction consuming NO had characteristics of a dioxygenase-like activity, because it proceeds aerobically, it depends on reduced pyridine nucleotide, and nitrate appears to be the product of the reaction. The affinity to NADH was slightly lower than that of NO dioxygenase of *E. coli*, but was higher than that of *Saccharomyces cerevisiae* or *Alcaligenes eutrophus* (Gardner et al. 2000). Both *E. coli* and *S. cerevisiae* possess NO dioxygenase with lower (1–2 orders of magnitude) affinity to NADPH (and only slightly lower V_{max}), while *A. eutrophus* dioxygenase exhibits almost no activity with NADPH. In the case of the observed activity in alfalfa root cultures, the K_m of the two pyridine nucleotides was about the same, while the V_{max} was 2.5 times higher for NADH. The inhibition of NO conversion by diphenylene iodonium may indicate

participation of flavin in catalyzing the reaction. This inhibitor was effective in suppressing NO dioxygenase activity in animal tissues (Gardner et al. 2001).

The majority of the NO degradation activity was located in the soluble, cytosolic fraction (Table 1) which, based on sequence data (Taylor et al. 1994; Arredondo-Peter et al. 1997; Trevaskis et al. 1997), is the probable location of the class 1 plant Hbs. An alfalfa class 1 Hb has been shown to locate in the cell nucleus and cytosol (Seregélyes et al. 2000).

The pH optimum of the NAD(P)H-dependent NO metabolism is broad, and the activity is still high at a pH below 7. The activity at acidic pH would be an important consideration for functioning activity during hypoxia, where acidification is a factor. It is not clear whether NO production in hypoxic conditions can be comparable to the activity rates measured in our experiments. It was shown earlier that NO accumulates in roots under hypoxia (Dordas et al. 2003a). NO-producing activity of nitrate reductase with nitrite may exceed (Dean and Harper 1988) or be comparable (Yamazaki and Sakihama 2000; Sakihama et al. 2002) to the rate of nitrate reduction.

NAD(P)H dependent NO conversion is comparable (in Hb^+ lines) to the activity of alcohol dehydrogenase induced under hypoxic treatment, which is 0.3–0.5 μmol min^{-1} mg^{-1} protein or 3–5 μmol min^{-1} g^{-1} FW (Dordas et al. 2003a). This indicates that, together with NADH removal by nitrate reductase, the capacity for NADH oxidation by the NO-conversion system is comparable to that of alcohol dehydrogenase, without formation of a toxic product (ethanol). The glycolytic flux rates during anoxia are usually close to the maximal activity of alcohol dehydrogenase (Bouny and Saglio 1996), and NADH removal during NO scavenging can substitute for alcohol dehydrogenase activity in providing sufficient glycolytic rates. The presence of NO degradation activity would relieve inhibition of the mitochondrial electron transport by NO (Zottini et al. 2002). The presence of higher malate dehydrogenase activity in plants overexpressing Hb (Fig. 9b) indicates that the cells are capable of more rapid NADH turnover.

It was previously shown that under hypoxic conditions, maize cells overexpressing Hb exhibit a lower alcohol dehydrogenase activity compared to control and to the lines underexpressing Hb (Sowa et al. 1998). One potential reason for this could be the more intensive operation of the NO-scavenging cycle, which uses NADH to reduce methemoglobin (Dordas et al. 2003b). The lower NADH/NAD and NADPH/NADP ratios in plants overexpressing Hb (Fig. 8) support the operation of such a cycle, which to some extent substitutes alcohol dehydrogenase activity for recycling NADH. Neither hypoxia nor suppression of the mitochondrial electron transport affects these ratios in overexpressing lines, while in Hb^- lines the ratios increase substantially upon low oxygen and antimycin treatment. In line with the maintenance of energy status (ATP levels) during hypoxia, Hb may participate in the maintenance of redox (NADH/NAD and NADPH/NADP) balance. The evidence suggests that Hb helps plants cope with increased NO production and with inhibition of mitochondrial electron transport caused by NO production and O_2 deficiency.

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