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Hypoxically inducible barley lactate dehydrogenase: cDNA cloning and molecular analysis

(*Hordeum vulgare* L./protein purification/amino acid sequence/mRNA induction)

DAVID HONDRED* AND ANDREW D. HANSON*†‡

*Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824; and †Institut botanique de l'Université de Montréal, 4101 rue Sherbrooke Est, Montréal, PQ H1X 2B2, Canada

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ABSTRACT **In the roots of barley and other cereals, hypoxia induces a set of five isozymes of L-lactate dehydrogenase [LDH; (*S*)-lactate:NADH oxidoreductase, EC 1.1.1.27]. Biochemical and genetic data indicate that the five LDH isozymes are tetramers that arise from random association of the products of two *Ldh* loci. To investigate this system, cDNA clones of LDH were isolated from a λgt11 cDNA library derived from hypoxically treated barley roots. The library was screened with antiserum raised against barley LDH purified ≈3000-fold by an improved three-step procedure. Immunopositive clones were rescreened with a cDNA probe synthesized by the polymerase chain reaction using primers modeled from the amino acid sequences of two tryptic LDH peptides. Two types of LDH clones were found. Nucleotide sequence analysis of one representative insert of each type (respectively, 1305 and 1166 base pairs) revealed open reading frames encoding 10 peptide fragments of LDH. The 1305-base-pair insert included the entire coding region of a 356-residue LDH monomer. The nucleotide sequences of the two LDH cDNAs were 92% identical in the coding region, but highly divergent in the 3′ noncoding region, and thus probably correspond to the two postulated *Ldh* loci. The deduced amino acid sequences of the two barley LDHs were 96% identical to each other and very similar to those from vertebrate and bacterial LDHs. RNA blot hybridization showed a single mRNA band of 1.5 kilobases whose level rose about 8-fold in roots during hypoxic induction, as did the level of translatable LDH message.**

In hypoxic or anaerobic conditions, root and seed tissues of many higher plants produce both ethanol and L-lactate as glycolytic end products (1, 2). Ethanol glycolysis predominates under long-term anaerobiosis in laboratory conditions for most plant tissues, and has received much more study than lactate glycolysis. However, lactate is typically a prominent end product during the transition phase from aerobic to anaerobic metabolism (3–5), and it has been proposed that intracellular lactate accumulation lowers cytoplasmic pH to the range required for ethanol glycolysis to begin (6). *In vivo* NMR studies of intracellular pH support the latter idea (5). Such studies also indicate that excessive lactate glycolysis can result in cell death from cytoplasmic acidosis (7), leading to the view that survival during short-term anaerobiosis depends on the balanced operation of lactate glycolysis and ethanol glycolysis. The role of lactate glycolysis in long-term survival is less clear; although little lactate may be produced, the necessary enzyme is often present at high levels (8, 9).

Lactate synthesis from pyruvate requires one enzyme, L-lactate dehydrogenase (LDH; EC 1.1.1.27), and ethanol synthesis requires two, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). These enzymes as well as other glycolysis-related enzymes are induced in cereal roots during oxygen deprivation (8–13). In the cases of ADH and PDC, accompanying increases in mRNA have been demonstrated (14–15), and for ADH this has been shown to reflect increased transcription (16). Anaerobic regulatory elements have been identified in the promoter region of *Adh* genes and it has been suggested that these elements are conserved among all anaerobically induced genes (17), allowing them to function as a coordinately induced group.

We are interested in understanding both the role of inducible LDH in plant tolerance to oxygen deprivation and the anaerobic regulation of *Ldh* genes. To further these investigations, we have cloned and characterized cDNAs encoding barley LDH.§ We have also compared the deduced amino acid sequences for barley LDH with those of vertebrate and bacterial LDHs.

MATERIALS AND METHODS

Plant Material. For LDH protein purification, barley (*Hordeum vulgare* L. cv. Robust) plants were grown and hypoxically treated in greenhouse conditions (9). For RNA isolation, cv. Himalaya plants were grown hydroponically in a growth chamber and hypoxically treated for 0, 2, 4, and 6 days (8).

Protein Purification and Antiserum Production. LDH activity was measured spectrophotometrically (8). Protein was assayed by the method of Bradford (18). Purity of LDH was monitored by SDS/PAGE (19). LDH was extracted from lyophilized barley roots (15 g dry weight) and applied to an Affi-Gel Blue agarose column (Bio-Rad) as described previously (9) except that the column volume was reduced from 40 ml to 4 ml. The column was washed with low-salt buffer (40 mM Tris acetate, pH 8.2/1 mM EDTA/0.5 mM dithiothreitol) until absorbance at 280 nm was <0.03, and bound dehydrogenases were eluted with low-salt buffer containing NADH (1 mg/ml). Eluted LDH activity (typically in 80 ml) was loaded directly onto a Mono Q HR5/5 FPLC column (Pharmacia-LKB) equilibrated with low-salt buffer; the column was washed with this buffer until absorbance at 280 nm was <0.01, and bound material was eluted with a linear KCl gradient in low-salt buffer. Fractions (between 200 and 300 mM KCl) containing LDH activity were pooled, concentrated, and chromatographed on an oxamate-agarose column (Sigma) as described (9). LDH peptides were prepared by trypsin digestion or CNBr cleavage and separated by HPLC. Amino acid sequences of individual peptides were deter-

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Abbreviations: ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; PCR, polymerase chain reaction; PDC, pyruvate decarboxylase.

‡To whom reprint requests should be addressed at: Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824.

§The sequences reported in this paper have been deposited in the EMBL/GenBank data base (accession nos. M31479 for LDHA and M31478 for LDHB).

Table 1. Purification of hypoxically induced LDH from barley roots

Fraction	Yield, %	Specific activity,* unit(s)/mg	Fold purification
Crude extract	100	0.13	1
Affi-Gel Blue agarose	40	14.4	110
Mono Q	30	120	920
Oxamate-agarose	18	425	3270

*One unit converts 1 μmol of substrate per min.

mined by automated Edman degradation either at the University of Michigan Protein Sequencing Facility, Ann Arbor, or the National Research Council Biotechnology Research Institute, Montréal. Anti-LDH serum was raised in rabbits (20) and was tested for specificity by immunoblot analysis (21).

RNA Isolation and *in Vitro* Translation. Total RNA was isolated by the method of Hall *et al.* (22). Poly(A)$^+$ RNA was purified by chromatography on poly(U)-Sephadex (BRL) (23) and was translated in a rabbit reticulocyte lysate system (Promega) according to the manufacturer's instructions. *In vitro* synthesized LDH was immunoprecipitated as described (24).

Polymerase Chain Reaction (PCR). Oligonucleotide primers were synthesized based on the amino acid sequences of two tryptic peptides. The amino acid sequence Gln-His-Ala$_3$-Phe was used to model the oligodeoxynucleotide pool 5′-CARCAYGCNGCNGCNTT-3′ (256-fold degenerate), and Asp-Leu-Val-Ile-Val-Thr-Ala-Gly-Ala-Arg to model 5′-CKIGCICCIGCIGTIACDATIACIARRTC-3′ (24-fold degenerate, plus 7 deoxyinosine residues). Poly(A)$^+$ RNA (from roots treated hypoxically for 4 days) was reverse-transcribed using avian myeloblastosis virus reverse transcriptase (Life Sciences) as described by Kimmel and Berger (25). The single-stranded DNA was amplified using a DNA amplification kit and a thermal cycler (Perkin-Elmer/Cetus). Reaction mixtures contained 100 pg of DNA, 100 pmol of each oligonucleotide pool, and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase in a volume of 100 μl. One thermal cycle consisted of 94°C for 3 min, 50°C for 2 min, and 72°C for 3 min. A total of 30 cycles was performed. The gel-purified PCR-synthesized product [≈100 base pairs (bp)] was used as a PCR template to synthesize radiolabeled probe (26).

Preparation and Screening of the cDNA Library. Poly(A)$^+$ RNA (from roots treated hypoxically for 2 days) was used to construct a cDNA library in phage λgt11 (Promega). The library was size-selected for inserts >1.0 kilobase (kb) and was amplified. Anti-LDH serum (1:500 dilution) was used to screen 540,000 plaques (50% recombinants) of the amplified library (27). Immunoselected plaques were transferred to duplicate nitrocellulose filters and hybridized to radiolabeled PCR-synthesized probe (total radioactivity, 2.7×10^8 cpm; specific activity, 7.6×10^9 cpm/μg). Plaque hybridizations were performed for 18 hr at 60°C in 50 ml of hybridization buffer [5× SSC (28) containing 10× Denhardt's solution (28), 50 mM sodium phosphate (pH 7.0), 0.1% (wt/vol) SDS, and tRNA (brewer's yeast; Boehringer Mannheim) at 100 μg/ml]. Hybridized filters were washed in 5× SSC/0.1% sodium pyrophosphate/0.1% SDS at 30°C for 20 min and at 60°C for 10 min.

A single immunopositive cDNA clone hybridized with the PCR-generated probe. As the LDH insert of this clone was too short to encode the entire protein, it was ^{32}P-labeled by the random primer method (29) and used to screen a further 540,000 plaques for clones with longer inserts. Plaque hybridizations (total radioactivity, 5.9×10^8 cpm; specific activity, 3.9×10^9 cpm/μg) were performed at 60°C for 18 hr in 120 ml of hybridization buffer containing polyadenylic acid (Boehringer Mannheim) at 50 μg/ml. Hybridized filters were washed as before, with a subsequent wash at 65°C in 0.1× SSC/0.1% sodium pyrophosphate/0.1% SDS for 30 min.

Characterization of cDNA Clones. *Eco*RI inserts of selected λgt11 phage were cloned in both orientations into plasmid pUC119. Single-stranded plasmid DNA was prepared (30). Series of nested-deletion subclones were generated (31). Sequencing of DNA was by the dideoxynucleotide chain-termination method (32).

RNA Blot Analysis. Poly(A)$^+$ RNA was denatured and electrophoresed (1 μg per lane) in 1.5% agarose gels containing formaldehyde (33) and then was transferred to nitrocellulose. Filters were hybridized to insert DNA labeled with ^{32}P by the random primer method.

RESULTS

Protein Purification and Antibody Production. Monospecific anti-LDH serum was needed to immunologically screen the λgt11 expression library. As a result, an improved LDH purification scheme was developed (Table 1, Fig. 1). This scheme yielded LDH of specific activity almost double that previously reported: 425 units/mg of protein compared to 250 units/mg (Table 1 and ref. 9). Purified enzyme activity was unstable, with a half-life of 12 hr at 4°C. Only one polypeptide band ($M_r \approx 40{,}000$) was detected after SDS/PAGE (Fig. 1). Nondenaturing PAGE (data not shown) revealed that purified LDH contained the five isozymes reported previously (8). The anti-LDH serum recognized all five LDH isozymes but did not react with any other proteins in native or SDS-denatured preparations of total soluble root proteins (Fig. 1).

Effect of Hypoxia on Translatable LDH mRNA Level. Following 2 days of oxygen deprivation, levels of LDH mRNA increased 8-fold (Fig. 2). During the same time, enzyme activity was accumulating at maximum rates (data not shown, see ref. 8). Based on these data, poly(A)$^+$ RNA isolated from barley roots subjected to 2 days of hypoxia was used for cDNA synthesis.

Isolation of cDNA Clones. When 270,000 recombinants were screened with anti-LDH serum, only one immunopositive clone was found that also hybridized with the ≈100-bp probe synthesized by PCR. The insert of this clone did not encode a full-length LDH protein. When this insert was used to screen a further 270,000 recombinants, 10 clones were obtained, of which 9 fell into the same class with respect to restriction fragment patterns given by *Sal* I, *Pst* I, and *Xma*

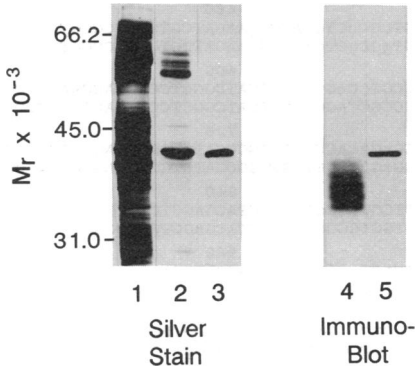

FIG. 1. Purification of barley LDH and specificity of anti-LDH serum. Lanes 1–3 are silver-stained SDS/PAGE profiles from the following purification steps: lane 1, Affi-Gel Blue agarose; lane 2, Mono Q; lane 3, Oxamate-agarose (3 μg of protein). Lanes 4 and 5 are immunoblots of total soluble proteins (≈10 μg per lane) from barley roots, separated by nondenaturing PAGE (lane 4) or SDS/PAGE (lane 5) and probed with anti-LDH serum. Positions of SDS molecular weight markers are shown at left.

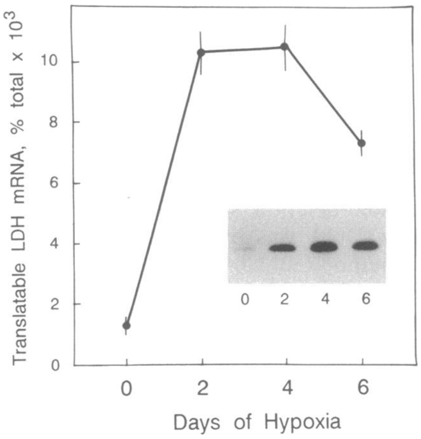

FIG. 2. Effect of hypoxia on the level of translatable LDH message in barley roots. Poly(A)$^+$ RNA obtained from roots hypoxically treated for the indicated times was translated *in vitro*. The translated LDH (M_r ≈40,000) was immunoprecipitated, subjected to SDS/PAGE, and visualized by fluorography (*Inset*). Levels of LDH mRNA were estimated from the amount of radioactive LDH synthesized and are expressed relative to the total trichloroacetic acid-precipitable radioactivity. Data points are means of 4–6 translations (± SE).

I. One clone was distinct. The nucleotide sequence of the longest insert from the class of 9, along with that of the distinct clone, is shown in Fig. 3. The former (designated LDHA) has a 1305-bp insert with an open reading frame comprising 356 amino acids (Figs. 3 and 4). The predicted ATG initiation codon occurs 65 bp from the *Eco*RI adaptor site. The latter clone (LDHB) has a 1166-bp insert with a long open reading frame, which lacks a start codon near the 5′ terminus and so is presumably truncated (Figs. 3 and 4). Both clones have putative polyadenylylation signals (AATAAA) 10–20 bp from their 3′ termini. The nucleotide sequences of the coding regions of LDHA and LDHB show 92% identity.

Deduced Amino Acid Sequences. The deduced amino acid sequences of LDHA and LDHB are 96% identical, and both include ten amino acid sequences determined from barley LDH peptides (Fig. 4). LDHA was calculated to encode a peptide of 37.7 kDa, which is close to experimental values from SDS/PAGE (Fig. 1 and ref. 9). The derived LDH amino acid sequences were compared with the National Biomedical Research Foundation Protein Sequence Data Bank (release 13.0, September 1987) by using the computer program FASTP (34). To identify particularly conserved regions, the amino acid sequences of LDHA and LDHB are aligned with those of representative vertebrate and bacterial LDHs in Fig. 4. From residue 45 onwards, both LDHA and LDHB exhibit ≈50% and ≈40% overall identity with vertebrate and bacterial LDHs, respectively.

Expression of LDH mRNA. When the LDHA cDNA insert was hybridized to RNA blots, a single 1.5-kb band was detected (Fig. 5). Because the LDHA probe recognizes LDHB as well as LDHA sequences, it is probable that the 1.5-kb band is a composite of LDHA and LDHB messages. Hypoxic treatment caused LDH mRNA to accumulate in roots; after 2 days the mRNA level was ≈8.5-fold higher than in well-aerated roots (Fig. 5). This agrees well with the 8-fold rise in translatable LDH message level shown in Fig. 2.

DISCUSSION

Biochemical data indicate that barley LDH is a tetramer that exists as a set of five isozymes produced by random association of two subunit types that differ in charge (8, 9). The inheritance pattern of barley isozyme profiles can be accounted for by a two-locus model (35) similar to that for LDH isozymes in vertebrate somatic cells, where the M (muscle) and H (heart) LDH subunits are the products of separate genes (36). The genetic data for barley also imply that the two

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                                                 30                                                60                                                90
        LDHA     CCGCATTTGATCGGACCAGCGTCCCATTCCACCCCCTTCCCACCCCGTCGAGGATGCACAAGGCGTCGTCGCTGTCGGAGCTCGGCTTCGA
        LDHB

                                     120                                               150                                               180                                               210
CGCCGGGGgCGCCTCGTCGGGCTTCTTCCGgcCGGTGGCcGACGGCTGCCCGgcCACcCcgACCTCCTcCgCCGtCCCGCACCGCCGCCTCACCAAGaTcTCCGTCATCGGgGCCGGCAA
CGCCGGGGaCGCCTCGTCGGGCTTCTTCCGcgCGGTGGCgGACGGCTGCCCGatCACgCacACCTCCTgCtCCGcCCCGCACCGCCGCCTCACCAAGgTgTCCGTCATCGGcGCCGGCAA
                                     240                                               270                                               300                                               330
CGTGGGCATGGCCATCGCGCAGACCATCCTCACcCAGAACCTGGCcGACGAGATCGCGCTcGTcGACGCgCTCCCCGACAAGCTCCGCGGCGAGGCGCTCGACCTGCAGCACGCCGCCGC
CGTGGGCATGGCCATCGCGCAGACCATCCTCACgCAGAACCTGGCgGACGAGATCGCGCTgGTgGACGCcCTCCCCGACAAGCTCCGCGGCGAGGCGCTCGACCTGCAGCACGCCGCCGC
                                     360                                               390                                               420                                               450
cTTCCTCCCGCGCGTCCGCA  TCTCCGGCACcGACGCCGCCGTCACCAAGAACTCcGACCTCgTCaTCGTCACGGCcGGgGCCAGGCAGATCCCgGGcGAGACCAGGCTCAACCTGCT
gTTCCTCCCGCGCGTCCGCAtcgTCTCCGGCACgGACGCCGCCGTCACCAAGAACTCgGACCTCaTCgTCGTCACGGCgGGcGCCAGGCAGATCCCcGGgGAGACCAGGCTCAACCTGCT
                                     480                                               510                                               540                                               570
GCAGAGGAACGTcGCcCTcTACCGCAAGATCGTGCCGCCCGTcGCCGAGCACTCCCCGGACGCGCTGCTGCTCGTCGTcTCCAACCCCGTcGACGTCCTCACCTACGTCGCcTGGAAGCT
GCAGAGGAACGTgGCgCTgTACCGCAAGATCGTGCCGCCCGTgGCCGAGCACTCCCCGGACGCGCTGCTGCTCGTCGTgTCCAACCCCGTgGACGTCCTCACCTACGTCGCgTGGAAGCT
                                     600                                               630                                               660                                               690
cTCcGGcTTCCCGGCCAGCCGCGTCATCGGCTCCGGCACCAACCTCGACTCCTCCcGcTTCAGGTTCCTCaTCGCCGAGCACCTCGACGTcAaCGCGCAGGACGTGCAGGCaTACATGGT
gTCgGGgTTCCCGGCCAGCCGCGTCATCGGCTCCGGCACCAACCTCGACTCCTCCaGgTTCAGGTTCCTCgTCGCCGAGCACCTCGACGTgAgCGCGCAGGACGTGCAGGCgTACATGGT
                                     720                                               750                                               780                                               810
GGGTGAGCACGGtGACAGCTCGGTGGCGATcTGGTCGAGCATCAGCGTGGGCGGgATGCCGGCGtTCAAGTCGCTgCGcGACAGCCACCGGAGCTTCGACGAGGCGGCGCTGGAGGGCAT
GGGTGAGCACGGcGACAGCTCGGTGGCGATaTGGTCGAGCATCAGCGTGGGCGGcATGCCGGCGcTCAAGTCGCTcCGgGACAGCCACCGGAGCTTCGACGAGGCGGCGCTGGAGGGCAT
                                     840                                               870                                               900                                               930
CCGGCGCGCCGTGGTGGGcGGCGCCTACGAGGTGATCGGCCTCAAGGGGTACACcTCtTGGGCcATtGGCTACTCtGTcGCtAGCCTCGCcgCtTCCCTcCTCCGCGACCAGCGCCGCGT
CCGGCGCGCCGTGGTGGGgGGCGCCTACGAGGTGATCGGCCTCAAGGGGTACACaTCcTGGGCaATcGGCTACTCcGTgGCcAGCCTCGCgaCaTCCCTgCTCCGCGACCAGCGCCGCGT
                                     960                                               990                                              1020                                              1050
GCACCCtGTcTCtGTcCTtGCCtCGGGCTTCCAtGGCATCTCaGACGGCCACGAGGTGTTCCTCAGCCTGCCtGCCCGcCTaGGCCGtGgCGGCaTCCTCGGtGTtGCcGAGATGGAcCT
GCACCCgGTgTCcGTgCTcGCCgCGGGCTTCCAcGGCATCTCcGACGGCCACGAGGTGTTCCTCAGCCTGCCcGCCCGgCTcGGCCGcGcCGGCgTCCTCGGcGTcGCtGAGATGGAtCT
                                    1080                                              1110                                              1140                                              1170
CACCGAGGCtGAGGCCGCcCAGCTCCGgCGcTCCGCCAAGACGCTCTGGGAGAACTGCCAGCTtCTcGaCCTCTGAGcGAggaacgccagttgatctacttgctgtaaaatcatgaagca
CACCGAGGCcGAGGCCGCgCAGCTCCGcCGgTCCGCCAAGACGCTCTGGGAGAACTGCCAGCTcCTtGgCCTCTGAG GA
                                    1200                                              1230                                              1260                                              1290
tttgcaagaaCTCTAgTaCTATAT  GTTGTtCTgTTccT      GCTcCCaCaCg                AATTTgGGAgTGtTTTCTTAGgGaTGatGTAgTaatgtaacgccTcTTAaTTT
            tCTCTAcTgCTATATatGTTGTgCTaTTaaTataacGCTgCCtCtCaaatgcttgtgtAATTTtGGAcTGaTTTCTTAGaGcTGgaGTAcT                 ggTaTTAgTTT
                                    1310
caTGAAATAAAATGcGATTTgGgcCGACAAAAAAA      LDHA
ggTGAAATAAAATGaGATTTtGtgC                LDHB
```

FIG. 3. Nucleotide sequences of the LDHA and LDHB cDNA clones. The putative initiation codon, stop codons, and polyadenylylation signals are underlined. The sequences corresponding to the oligodeoxynucleotide pools used to prime the PCR reaction are highlighted. Lowercase letters denote differences in nucleotide sequence between the two clones.

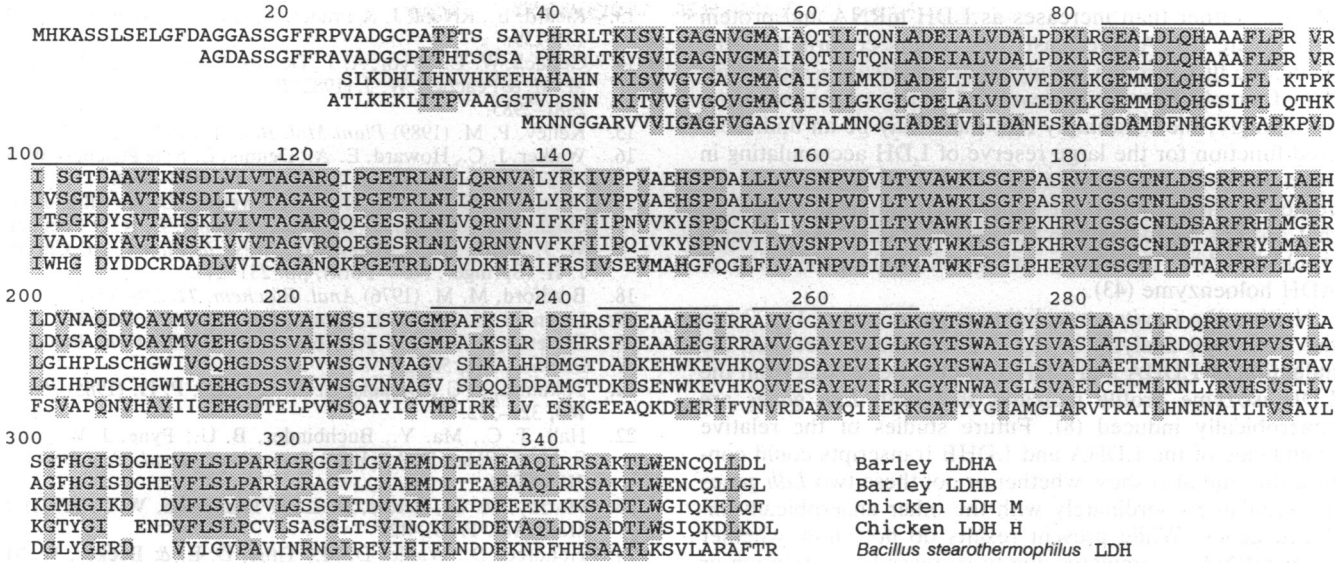

FIG. 4. Deduced amino acid sequences of barley LDHA and LDHB, aligned with the amino acid sequences of representative vertebrate muscle (M) and heart (H) LDH forms and a bacterial LDH. Highlighted residues are those common to barley LDHA or LDHB and one or more of the other LDHs. Amino acid residue numbers are those of barley LDHA. Vertebrate and bacterial LDH amino acid sequences were obtained from the National Biomedical Research Foundation Protein Sequence Data Bank as described in the text.

Ldh loci are tightly linked, perhaps reflecting a relatively recent gene-duplication event (35). Our finding of two similar but distinct types of LDH cDNA clone is clearly in accord with the two-locus model. That the two types of cDNA exhibit only minor nucleotide sequence divergence (8%) in their coding regions is consistent with the hypothesis of a recent gene duplication. For comparison, the two principal vertebrate *Ldh* loci, which are functionally diverged and may be on different chromosomes, typically show severalfold more nucleotide sequence divergence and are considered to be the result of an ancient duplication event (36, 37).

The deduced amino acid sequence of barley LDHA is longer than vertebrate and bacterial LDHs by approximately 20 and 35 residues, respectively. Alignment of primary structures shows that these size differences reflect variation at the N terminus. We cannot exclude the possibility that barley LDHs undergo a processing event of some kind, such as cleavage of a transit peptide. However, any substantial modification seems unlikely for two reasons. First, the *in vitro* translation product of LDH mRNA has the same apparent molecular mass on SDS/PAGE as purified LDH. Second, like other glycolytic enzymes, LDH is considered to be cytosolic (1). Moreover, among vertebrate LDHs, the N-terminal portion of the peptide is quite variable; it forms an arm that extends from the body of the subunit and participates in subunit interaction rather than catalysis (37–39). From approximately residue 45 onwards, the barley LDH chains show considerable similarity to vertebrate and bacterial LDHs. By analogy with vertebrate LDH (37, 38), this region can be divided into three domains: the coenzyme-binding domain (residues 45–117 and 140–185), the substrate-binding domain (residues 186–356), and the loop region (residues 118–139). As among vertebrates (37), the substrate-binding domain is somewhat less conserved than the two others. It is interesting that amino acid residues which are directly involved in the catalytic mechanism in other LDHs (38, 40) have precise counterparts in barley LDH. In barley, these include Gln-122, Arg-128, Asp-188, His-215, Asp-217, and Thr-270. Localized, highly conserved regions surround these catalytically important residues. The sequence of one of these regions has been reported for potato LDH (41) and is identical to barley LDH residues 180–191.

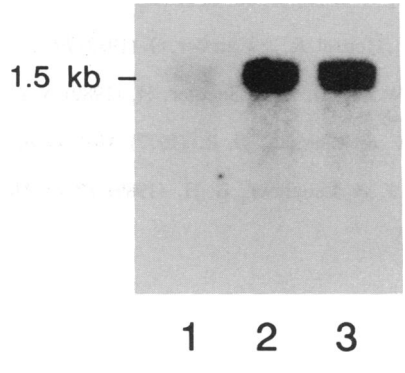

FIG. 5. RNA blot analysis of LDH mRNA levels in well-aerated roots (lane 1) and roots exposed to hypoxic conditions for 2 days (lane 2) or 4 days (lane 3). The size of the hybridizing RNA (1.5 kb) is indicated.

Plant LDHs have much potential for investigating evolutionary relationships among higher plant taxa and between plants and other organisms. Evolutionary trends among animal LDHs have been studied extensively (36, 37), and there is hence a large data base for comparisons. As a marker of evolutionary divergence between plants and other groups, LDH offers the simplifying feature that not only its biochemical function but also its main physiological role in fermentative metabolism have remained unaltered over time. This is not always so for glycolytic and other enzymes common to plants and other organisms. For instance, in plants ADH is primarily used during ethanol fermentation to reduce acetaldehyde, whereas in animals it serves to oxidize alcohols. Other glycolytic enzymes, such as glyceraldehyde phosphate dehydrogenase, a well-studied evolutionary marker in plants (42), have both a cytoplasmic form that functions in glycolysis and a chloroplastic form that participates in photosynthesis.

The data reported here demonstrate that the anaerobic induction of LDH activity is accompanied by a large increase in the abundance of LDH mRNA, so that LDH can be classified as an anaerobic protein as defined by Sachs *et al.* (10). As is the case with LDH enzyme levels (8–9), the LDH message accumulates to high levels during periods of hypoxia. These results are surprising inasmuch as the contribution of lactate glycolysis to total glycolytic carbon flux

declines rather than increases as LDH mRNA and protein accumulate (8). At the least, our results confirm that the metabolic flux through the glycolytic pathways is not determined simply by relative levels of gene expression. More intriguingly, the data imply that there may be an undiscovered function for the large reserve of LDH accumulating in hypoxic roots. One possibility is that lactate glycolysis becomes far more important under environmental conditions that are not reproduced in laboratory studies. An example would be Zn^{2+} deficiency, which can limit production of ADH holoenzyme (43).

Neither the *in vitro* translation experiments of Fig. 2 nor the RNA blot analysis of Fig. 5 discriminates between the induction of LDHA and LDHB. Indirect evidence from the LDH isozyme profile indicates that both *Ldh* genes are anaerobically induced (8). Future studies of the relative abundance of the LDHA and LDHB transcripts could confirm this and also show whether or not these two *Ldh* genes are regulated coordinately with the other anaerobically induced genes. While present results do not show whether LDH mRNA accumulates due to increased transcription or decreased degradation, the former is probable inasmuch as the induction of ADH and other anaerobic proteins involves enhanced transcription (16, 17, 44). The availability of cDNA clones for barley LDH will make possible further study of the anaerobic induction mechanism; it will be of particular interest to determine whether cis-acting regulatory elements such as the proposed anaerobic regulatory elements of maize (17) are to be found in LDH genes.

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1. Davies, D. D. (1980) in *The Biochemistry of Plants*, ed. Davies, D. D. (Academic, New York), Vol. 2, pp. 581–611.
2. Hanson, A. D., Hoffman, N. E., Hondred, D., Brown, A. H. D. & Alexander, D. C. (1987) in *Plant Gene Systems and Their Biology*, eds. Key, J. L. & McIntosh, L. (Liss, New York), pp. 121–129.
3. Smith, A. M. & ap Rees, T. (1979) *Phytochemistry* **18**, 1453–1458.
4. Smith, A. M. & ap Rees, T. (1979) *Planta* **146**, 327–334.
5. Roberts, J. K. M., Callis, J., Wemmer, D., Walbot, V. & Jardetzky, O. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3379–3383.
6. Davies, D. D., Grego, S. & Kenworthy, P. (1974) *Planta* **118**, 297–310.
7. Roberts, J. K. M., Callis, J., Jardetzky, O., Walbot, V. & Freeling, M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6029–6033.
8. Hoffman, N. E., Bent, A. F. & Hanson, A. D. (1986) *Plant Physiol.* **82**, 658–663.
9. Hoffman, N. E. & Hanson, A. D. (1986) *Plant Physiol.* **82**, 664–670.
10. Sachs, M. M., Freeling, M. & Okimoto, R. (1980) *Cell* **20**, 761–767.
11. Laszlo, A. & St. Lawrence, P. (1983) *Mol. Gen. Genet.* **192**, 110–117.
12. Bailey-Serres, J., Kloeckener-Gruissem, B. & Freeling, M. (1988) *Plant Cell Environ.* **11**, 351–357.
13. Ricard, B., Rivoal, J. & Pradet, A. (1989) *Plant Mol. Biol.* **12**, 131–139.
14. Gerlach, W. L., Pryor, A. J., Dennis, E. S., Ferl, R. J., Sachs, M. M. & Peacock, W. J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2981–2985.
15. Kelley, P. M. (1989) *Plant Mol. Biol.* **13**, 213–222.
16. Walker, J. C., Howard, E. A., Dennis, E. S. & Peacock, W. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6624–6628.
17. Dennis, E. S., Walker, J. C., Llewellyn, D. J., Ellis, J. G., Singh, K., Tokuhisa, J. G., Wolstenholme, D. R. & Peacock, W. J. (1989) in *Environmental Stress in Plants*, ed. Cherry, J. H. (Springer, New York), pp. 231–245.
18. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
19. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
20. Weretilnyk, E. A. & Hanson, A. D. (1989) *Arch. Biochem. Biophys.* **271**, 56–63.
21. Tokuhisa, J. G., Daniels, S. M. & Quail, P. H. (1985) *Planta* **164**, 321–332.
22. Hall, T. C., Ma, Y., Buchbinder, B. U., Pyne, J. W., Sun, S. M. & Bliss, F. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3196–3200.
23. Murray, M. G., Peters, D. L. & Thompson, W. F. (1981) *J. Mol. Evol.* **17**, 31–42.
24. Hondred, D., Wadle, D.-M., Titus, D. E. & Becker, W. M. (1987) *Plant Mol. Biol.* **9**, 259–279.
25. Kimmel, A. R. & Berger, S. L. (1987) *Methods Enzymol.* **152**, 307–325.
26. Schowalter, D. B. & Sommer, S. S. (1989) *Anal. Biochem.* **177**, 90–94.
27. Mierendorf, R. D., Percy, C. & Young, R. A. (1987) *Methods Enzymol.* **152**, 458–469.
28. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
29. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13.
30. Vieira, J. & Messing, J. (1987) *Methods Enzymol.* **153**, 3–11.
31. Dale, R. M. K. & Arrow, A. (1987) *Methods Enzymol.* **155**, 204–214.
32. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
33. Hershey, H. P., Colbert, J. T., Lissemore, J. L., Barker, R. F. & Quail, P. H. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2332–2336.
34. Lipman, D. & Pearson, W. T. (1985) *Science* **227**, 1435–1441.
35. Hoffman, N. E., Hondred, D., Hanson, A. D. & Brown, A. H. D. (1988) *J. Hered.* **79**, 110–114.
36. Markert, C. L., Shaklee, J. B. & Whitt, G. S. (1975) *Science* **189**, 102–114.
37. Li, S. S.-L., Fitch, W. M., Pan, Y.-C. E. & Sharief, F. S. (1983) *J. Biol. Chem.* **258**, 7029–7032.
38. Holbrook, J. J., Liljas, A., Steindel, S. J. & Rossman, M. G. (1975) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), Vol. XIA, pp. 191–292.
39. Eventoff, W., Rossman, M. G., Taylor, S. S., Torff, H.-J., Meyer, H., Keil, W. & Kiltz, H.-H. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2677–2681.
40. Clarke, A. R., Wigley, D. B., Chia, W. N., Barstow, D., Atkinson, T. & Holbrook, J. J. (1986) *Nature (London)* **324**, 699–702.
41. Mayr, U., Hensel, R. & Kandler, O. (1982) *Phytochemistry* **21**, 627–631.
42. Martin, W., Gierl, A. & Saedler, H. (1989) *Nature (London)* **339**, 46–48.
43. Pryor, A. & Marshall, D. R. (1977) *Mol. Gen. Genet.* **157**, 47–51.
44. Ferl, R. J. & Laughner, B. H. (1989) *Plant Mol. Biol.* **12**, 357–366.