Purification and Characterization of Soybean Root Nodule Ferric Leghemoglobin Reductase

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ABSTRACT

A ferric leghemoglobin reductase from the cytosol of soybean (Glycine max) root nodules was purified to homogeneity and partially characterized. The enzyme is a flavoprotein with flavin adenine dinucleotide as the prosthetic group and consists of two identical subunits, each having a molecular mass of 54 kilodaltons. The pure enzyme shows a high activity for ferric leghemoglobin reduction with NADH as the reductant in the absence of any exogenous mediators. The enzyme also exhibits NADH-dependent 2,6-dichloroindophenol reductase activity. A sequence of the first 50 N-terminal amino acids of the purified protein was obtained. Comparisons with known protein sequences have shown that the sequence of the ferric leghemoglobin reductase is highly related to those of the flavin-nucleotide disulfide oxidoreductases, especially dihydrolipoamide dehydrogenase of the pyruvate dehydrogenase complex.

Lb plays an important role in symbiotic nitrogen fixation because it facilitates diffusion of O2 to the N2-fixing bacteroids and buffers O2 concentration within the infected cells of nodules (2, 3). The role of Lb as an O2 carrier, like that of other heme-based O2-binding proteins such as myoglobin or hemoglobin, requires that the molecule be in the ferrous oxidation state (Lb2). Because Lb3 is the predominant form of Lb in nodules (12), mechanisms must exist in vivo for maintaining Lb in the functional ferrous state.

Apelby (1) showed that anaerobic suspensions of washed soybean bacteroids could reduce Lb2 to Lb3 slowly without added electron carriers, suggesting that an "enzyme" was responsible for such Lb3 reduction. Kretovich et al. (11) were the first to report that a Lb3 reductase was present in the lupin nodules. Since then, several authors (4, 15, 19, 20) have reported a variety of NAD(P)H-dependent Lb3 reductases from the soybean nodule cytosol. These reductases have been proposed to be flavoproteins or metalloflavoproteins. All have low affinity for Lb3, low Lb3 reductase specific activity, and usually require for activity some exogenous electron carriers such as DCIP, methylene blue, or Cyt c. Their ability to catalyze Lb3 reduction in vivo is doubtful.

Klucsa and colleagues (9, 16, 17) identified a protein with Fb+ activity from soybean nodule cytosol. This enzyme showed a high activity for Lb3 reduction with NAD(P)H as reductant in the absence of exogenous electron mediators. In this study the Fb+ was purified to homogeneity. The enzyme was partially characterized and the sequence of N-terminal amino acids was determined. Based on the amount enzymatic activity and the apparent high affinity for Lb3 of the purified enzyme, it seems likely that this enzyme may play a physiological role in maintaining Lb in the functional ferrous state in soybean nodules.

MATERIALS AND METHODS

Plant Materials

Soybean (Glycine max [L.] Merr. cv Hobbit) seeds were inoculated with Bradyrhizobium japonicum strains SR, 123, or 89, just prior to planting in Turface (Loveland Grass Co., Omaha, NE) or sand. The seeds were planted in 18 cm diameter pots (3-4 seeds per pot) and grown in a greenhouse or growth chamber. Plants were harvested after 30 to 35 d, and nodules were removed from the roots, washed, and stored at -70°C. Soybean root nodules (cv Pella; inoculated with B. japonicum strain 122DE) and nodule cytosol, provided by Dr. Arp at the University of California, Riverside, were also used for the purification of Fb+.

Extraction of Soybean Nodule Cytosol

The whole extraction procedure was carried out at 4°C. Frozen nodules (50 g) were homogenized in a Sorvall Omni-Mixer (Sorvall, Norwalk, CT) in the presence of 150 mL of 0.1 mM potassium phosphate buffer (pH 6.8) and 15 g of polystyrolpolypropyridine for 1 min, cooled on ice and homogenized again for 1 min. The homogenate was filtered through eight layers of cheesecloth. The filtrate was centrifuged at 15,000g for 15 min. The supernatant was collected and precipitated with ammonium sulfate between 30 and 85% saturation. The precipitate was collected by centrifugation at 15,000g for 15 min and redissolved in about 20 mL of 50 mM potassium phosphate buffer (pH 7.0).
Purification of FLbR

FLbR was purified by several steps involving conventional column chromatography and FPLC. Gel filtration with Sephadex G-25 (Pharmacia, Piscataway, NJ) was used to remove small molecules and desalt the enzyme fraction. A column (2.5 × 75 cm) of Sephadex G-25 was equilibrated with 50 mM potassium phosphate buffer (pH 7.0), containing 2 mM MgCl₂ and 2 mM 2-mercaptoethanol. Approximately 20 mL of extract from the ammonium sulfate precipitation step was loaded onto the column and the column was eluted at a flow rate of 1 mL min⁻¹. Protein fractions were monitored by A₂₅₀ of the eluate. The first peak fraction eluting between 100 to 160 mL was collected and centrifuged at 27,000g for 20 min. The supernatant was applied to a hydroxylapatite (HPT, Bio-Rad) column.

The HPT column (2.5 × 30 cm) was equilibrated with 10 mM potassium phosphate buffer (pH 7.0), containing 2 mM MgCl₂ and 2 mM 2-mercaptoethanol. The column was eluted first with 10 mM phosphate buffer (pH 7.0), at a flow rate of 1 mL min⁻¹, which eluted most unwanted proteins, including Lb. This fraction was collected and used for further purification of Lb. The column was washed until A₂₅₀ of the eluate was near to zero. The column was then eluted with 200 mM phosphate buffer (pH 7.0), which removed >90% proteases (G. Sarath, University of Nebraska-Lincoln, personal communication). Finally, the column was eluted with 700 mM phosphate buffer (pH 7.0). This fraction (about 120 mL) contained FLbR and was concentrated to about 10 mL with a Centiprep-30 concentrator (Amicon, Beverly, MA). This concentrated fraction was desalted using a P6-DG (Bio-Rad) gel filtration column equilibrated and eluted with 20 mM Tris-HCl (pH 6.5). The desalted sample was concentrated to about 10 mL for the next FPLC separation.

The desalted protein fraction was applied to a HR5/5 Mono-Q column on a FPLC System (Pharmacia). The column was eluted with 20 mL of a linear NaCl gradient, starting from 100% buffer A (50 mM Tris-HCl [pH 6.45]) to 35% buffer B (50 mM Tris-HCl plus 1 M NaCl [pH 6.45]), at 1 mL min⁻¹. Protein fractions were monitored at A₂₅₀ and assayed for DCIP reductase activity of FLbR. Fractions containing activity were pooled (about 4 mL) and rechromatographed on the Mono-Q column under the conditions described above.

The FLbR fractions from the second Mono-Q separation step were concentrated to about 0.5 mL and applied to a Superose-12 gel filtration column (1.25 × 60 cm) on FPLC. The column was equilibrated and eluted with 50 mM Tris-HCl (pH 6.5), containing 150 mM NaCl, at 0.8 mL min⁻¹. FLbR fractions were assayed, pooled (about 30 mL), and stored at −70°C.

The FLbR was subsequently purified to homogeneity by a final fractionation using a Phenyl-Superose column. FLbR fractions from the Superose-12 were concentrated to about 0.5 mL and applied to a prepacked HR5/5 Phenyl-Superose column (Pharmacia) on FPLC. Column was eluted with 30 mL volume of a reversed linear ammonium sulfate (100-0%) gradient, starting from 100% buffer A (50 mM potassium phosphate, 1.7 mM ammonium sulfate [pH 7.0]) to 100% buffer B (50 mM potassium phosphate [pH 7.0]), at 0.5 mL min⁻¹. FLbR fractions were assayed, collected (about 2 mL), and stored at −70°C.

Measurement of M₅ and Homogeneity

Electrophoretic fractionation on a 12.5% SDS-polyacrylamide/PAGE gel (85 × 65 × 0.75 mm) was performed to estimate the size and subunit compositions, and to assess the presence of contaminating proteins. SDS-treated FLbR samples (10–20 µg) and SDS-PAGE M₅ markers (Mr, 14,400–97,400, Bio-Rad No. 161-0304) were applied to the gel. Electrophoresis was performed at 150 V for about 1 h until the tracking dye approached the end of gel. Proteins in the gel were silver-stained (Bio-Rad, Silver Stain Kit, No. 161-0443).

Gel filtration with Superose-12 on FPLC was also used to estimate the apparent Mr of the native FLbR protein. A MW-GF-200 Kit (Mr, 12,000–200,000, Sigma), the Mr markers for gel filtration chromatography, was used. The void volume of Superose-12 column was calculated using Blue Dextran (Mr, 2,000,000, Sigma No. D 4772). The procedure for use of Mr markers on gel filtration chromatography was as described by Sigma (Technical Bulletin No. GF-3, 1986).

IEF Analysis

IEF electrophoresis was performed according to a procedure described by the supplier (Bio-Rad, Mini IEF Cell, Nos. 170–2975 and 170–2976). Five percent acrylamide/PDA and 2% ampholytes (Bio-Lyte 3/5, 5/7 ampholytes) were used in the mini-IEF gel (125 × 65 × 0.4 mm). Two microliters of FLbR samples (1–2 µg) and 2 µL of IEF standards (Bio-Rad, No. 161–0310) were applied onto the gel. Electrofocusing was performed at a gradient voltage (from 100–450 V) for about 1.5 h, and the gel was silver stained as indicated before.

Identification and Determination of Flavins

Identification of the flavin bound to FLbR was carried out by digesting the enzyme with proteases and analyzing the released flavin by HPLC. The reaction mixture (1 mL) contained 0.65 M potassium phosphate buffer (pH 7.5), 10 µg FLbR, 4 µg trypsin, and 4 µg chymotrypsin. Use of a high concentration of potassium phosphate inhibited phosphatases that otherwise could have degraded flavin coenzymes to riboflavin (10). After incubation at 37°C for 2 h, the remaining proteins were precipitated with 10% (w/v) TCA, the solution was centrifuged, and the supernatant was analyzed for free flavins by HPLC. This analytical HPLC separation of flavins was performed essentially as described by Light et al. (13), using a µBondapak C-18 column (Waters, Milford, MA) and an isocratic elution with 95% A (20% methanol, 5 mM ammonium acetate [pH 6.0]):5% B (100% methanol) at 2 mL min⁻¹. Flavins were detected by fluorescence with excitation at 445 nm and emission at 520 nm. Identity of the released flavins was ascertained by its retention time and coelution with standards. Standards of FMN and FAD were treated the same way as the enzyme to ensure that no degradation of any of coenzymes to riboflavin occurred during the incubation and analysis of the sample.
The flavin content of the purified FLbR was determined according to Yagi (24), using a Perkin-Elmer fluorescence spectrophotometer (model MPF 44A) with excitation 450 nm and emission 525 nm, and slit width 2.5 nm for both.

**Assays of DCIP Reductase and FLbR Activities**

The DCIP reductase activity of FLbR was assayed by measuring the reduction of DCIP with a Cary-219 spectrophotometer (Varian, Palo Alto, CA). The reaction contained 100 μL of 10 mM NADH, 100 μL of 5 mM EDTA, 20 μL of 1.25 mM DCIP, 10 to 100 μL FLbR sample, and 20 mM Tris buffer (pH 8.1) to a total volume of 1 mL. The absorbance change at 600 nm with time measured at 23°C and the specific activity was calculated by using an extinction coefficient of 600 nm of 21.0 mm⁻¹ cm⁻¹. Specific activity was expressed as nmol DCIP reduced min⁻¹ mg⁻¹ of protein.

The FLbR activity was assayed by measuring the change of absorbance at 574 nm (16). The assay mixture contained 0.8 mM NADH, 30 to 40 μM Lb⁺, 35 mM potassium phosphate buffer (pH 7.0), 1 to 3 μg FLbR in a total of 1 mL. The initial rate of Lb⁺O₂ production was determined with a Spectronic 3000 Array (Milton Roy, Rochester, NY) interfaced to a computer with kinetic acquisition software. The specific activity of FLbR was calculated by using an extinction coefficient at 574 nm of 15.1 mm⁻¹ cm⁻¹. Specific activity was expressed as nmol Lb⁺O₂ formed min⁻¹ mg⁻¹ of protein.

**Partial Sequence of FLbR**

Automated protein sequencing was performed on a Milligen/Biosearch 6000 series ProSequencer at the University of Nebraska-Lincoln Center for Biotechnology, Protein Core Facility. A sequence of the first 50 N-terminal amino acid residues of FLbR was obtained. This partial protein sequence was compared for homology to known protein sequences in the protein sequence bank (Micro Genie program, Beckman Inc., 1988).

**Other Methods**

Lb and Lb⁺ were prepared by a procedure described by Saari (16). Concentration of Lb⁺ was measured using an extinction coefficient at 495 nm of 8.5 mm⁻¹ cm⁻¹. The protein concentration was assayed by the Bio-Rad protein microassay procedure (Cat. No. 500-0001, 500-0006), using BSA as a standard.

**RESULTS AND DISCUSSION**

**Purification of FLbR**

FLbR was purified to homogeneity from the cytosolic extract of soybean root nodules by several chromatographic steps. Prepacked Superose-12 and Phenyl-Superose column separations on FPLC were used as the final steps. Specific activities of DCIP reductase and FLbR from each purification step were measured (Table 1). Each purification step yielded increases in specific activity for both DCIP reductase and FLbR. Specific activities for both DCIP reductase and FLbR were averaged from 10 preparations. The FLbR preparation from the final Phenyl-Superose step (Fig. 1) was highly purified, with a specific activity about 2500 times higher than that obtained from the Sephadex G-25 step. The ratio of DCIP reductase activity to FLbR activity decreased from 780 to 10. About 100 to 200 μg of purified protein was obtained from 50 g of frozen soybean nodules. The whole purification procedure was accomplished in 2 d and represented a significant improvement compared to the method previously reported by Saari and Klucas (17), in which a purification of 1100-fold and a specific activity of 218 were obtained.

**Molecular Mass and Homogeneity**

The FLbR preparation from the final Superose-12 and the Phenyl-Superose separation steps were subjected to Superose-12 gel filtration and SDS-PAGE to determine the Mᵣ and to check protein homogeneity. The Mᵣ of the native FLbR estimated by gel filtration was about 110,000. The electropherogram of the silver-stained gel is shown in Figure 2. The enzyme appeared to be composed of two identical subunits of approximately 54,000. The enzyme fraction from the Phenyl-Superose separation had only a single distinct band on the silver-stained gel, while the protein fraction after the Superose-12 purification step exhibited additional protein bands. The distinct band on the silver-stained gel and the sharp peak on the profile indicated that the protein from this purification was highly pure and homogeneous (Figs. 1 and 2).

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**Table 1. Purification and Specific Activities of FLbR from Soybean Nodule Cytosol**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total Protein mg</th>
<th>DCIP Reductase(Å) Specific Activity units/mg</th>
<th>FLbR(Å) Specific Activity</th>
<th>Total FLbR Activity</th>
<th>FLbR Yield</th>
<th>Purification of FLbR</th>
<th>Ratio² A/B</th>
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<tr>
<td>Sephadex G-25</td>
<td>1810.00</td>
<td>148</td>
<td>0.2</td>
<td>345</td>
<td>100</td>
<td>1</td>
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<tr>
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<td>32.00</td>
<td>124</td>
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<td>233</td>
<td>68</td>
<td>37</td>
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<td>Mono-Q-1</td>
<td>1.56</td>
<td>1030</td>
<td>82.0</td>
<td>128</td>
<td>37</td>
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<td>12.6</td>
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<tr>
<td>Mono-Q-2</td>
<td>0.56</td>
<td>1510</td>
<td>135.0</td>
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<td>22</td>
<td>675</td>
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<td>Superose-12</td>
<td>0.38</td>
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<td>20</td>
<td>1210</td>
<td>10.0</td>
</tr>
<tr>
<td>Phenyl-Superose</td>
<td>0.10</td>
<td>4890</td>
<td>500.0</td>
<td>50</td>
<td>15</td>
<td>2500</td>
<td>9.9</td>
</tr>
</tbody>
</table>

* One unit is defined as 1 nmol DCIP reduced min⁻¹. ² One unit is defined as 1 nmol Lb⁺O₂ formed min⁻¹. ³ This is the ratio of specific DCIP reductase activity to specific FLbR activity.
purified quite different proteins were components had the partial protein. Their IEF in gel methylene-bis-acrylamide IEF, mini-IEF gel. Phenyl-Superose separation the represent different forms or pi values. Figure 1. Profile of the final purification step of FLbR on a HR5/5 Phenyl-Superose column with FPLC.

**pl Values and Possible Isoforms of FLbR**

The purified proteins from the final Superose-12 and the Phenyl-Superose separation steps were electrofocused on the mini-IEF gel. The cross-linking agent, PDA, was substituted for methylene-bis-acrylamide (bis) in polyacrylamide gel for IEF, which reduced the background of gels after silver staining, increased gel strength, and improved resolution. Three distinct bands with nearly equal density were observed on the gel (Fig. 3), with pi values of 5.5, 5.6, and 5.7, respectively. Because the denatured protein revealed only one band on SDS-PAGE gel (Fig. 2), it is unlikely that these three bands represent different kinds of proteins, but they might be isoforms or modifications of the protein.

Multiple components of Lb\(^{-2}\) reductase were also observed in lupin nodules by Topunov et al. (20) using IEF technique. Their IEF fractions consisted of four components and all components had the NADH-diaphorase activity. Since their preparations were impure, the components were probably quite different proteins rather than isozymes of the reductase.

**Partial Sequences of N-Terminal Amino Acids**

A sequence of the first 50 N-terminal amino acids from the purified FLbR protein was obtained. This partial protein sequence (soybean fragment) was then matched to known sequences in sequence bank using a computer program (Micro Genie, Beckman, 1988) to identify sequence homology. The sequence was highly homologous to an enzyme family of the flavin-nucleotide disulfide oxidoreductases, and especially, to lipoamide dehydrogenase (E\(_2\)), a common component of three \(\alpha\)-ketoacid dehydrogenase complexes oxidizing pyruvate, \(\alpha\)-ketoglutarate, and branched-chain \(\alpha\)-ketoacids. Table II shows the alignment of the first 50 N-terminal amino acid sequences of lipoamide dehydrogenases (E\(_2\)) from *Escherichia coli* (18), *Saccharomyces cerevisiae* (yeast) (5), pig heart, and human liver (14). This partial protein sequence of FLbR has a 54, 70, 78, and 78% identity with the sequences of *E. coli*, yeast, pig heart, and human liver E\(_2\), respectively. A comparison of amino acid sequences around the redox-active thiol center (or the disulfide active site) and FAD-binding domain at the N-terminus was done between the soybean fragment and the known flavoproteins (Table II; and also see ref. 6). Remarkable homology was observed with the disulfide active site of *E. coli*, yeast, pig heart, human liver, human glutathione reductase, and other flavin proteins. The sequenced FLbR fragment contains all the necessary amino acid residues for the disulfide active site within this limited partial sequence. A comparison of the four amino acid sequences between the two cysteine residues of the active disulfide bridge also shows remarkable enzyme-dependent homology in that leucine-45 is present in all E\(_2\)s, whereas it is replaced by valine in glutathione reductases and in mercuric reductase. Since the soybean fragment has a leucine residue at this position, FLbR

![Figure 2](image-url)
may be classified in the E₃ group and not in glutathione and mercuric reductases groups.

The soybean fragment also has, at least within this partial sequence, all the conservative amino residues in the FAD-binding domain. The homology in FAD-binding domain provides molecular evidence that flavins may exist in FLbR as coenzymes. The analytical HPLC separation of flavin demonstrated that the FLbR contained FAD instead of FMN. The fluorescence microdetermination of flavin yielded a molar ratio of 1.7 ± 0.5 (se, n = 2) of flavin to FLbR. All of these results indicated that the FLbR is a FAD-containing enzyme.

Although comparisons did not give definite descriptions of the structure and function of the FLbR because of the limitation of the sequence, it provided us with many important clues for understanding the enzymatic reaction mechanism and some structural aspects. E₃ is a multifunctional enzyme that catalyzes a variety of NADH-linked reactions using different acceptor substrates and it has been referred to as a transhydrogenase and DCIP diaphorase (8). Recently, the enzyme E₃ has been shown to have oxidase (22) and nitroreductase activities (21). These multiple catalytic properties are probably not specific for E₃ because free flavins can facilitate these reactions (23). The established metabolic role of E₃ as a component of various multienzyme systems, but evidence is accumulating to suggest that the enzyme may serve as yet undefined additional functions because it is associated with the cellular plasma membrane of a diverse range of organisms (7). Considering the similarity of the structure and function between the FLbR isolated from soybean nodules and E₃ from different sources, it will be necessary to explore inter- and intracellular locations of the FLbR in nodules, to analyze its molecular structure, and to investigate in detail the relationship between the enzyme and its prosthetic groups as well as

![Figure 3. IEF of FLbR purified from soybean nodule cytosol. Five percent acrylamide/PDA and 2% ampholytes (Biolyte 3/5, 5/7 ampholytes) were used in this mini-IEF gel (125 × 65 × 0.4 mm). Two micrograms of Bio-Rad IEF standards (lane 1) and 2 μg of pure FLbR from the final Phenyl-Superose purification step (lane 2) were applied on the IEF gel, respectively, and the gel was silver stained.](image)

### Table II. Comparison of Partial Amino Acid Sequences of Soybean Nodule FLbR with E. coli, Yeast, Pig Heart, and Human Liver E₃s

Alignment of amino acid sequences of *E. coli* (18), yeast (5), pig heart, and human liver (14) E₃s and the soybean FLbR fragment were determined by a computer program, Micro Genie (Beckman, Palo Alto, CA, 1988). For the E₃ conservative sequence, the capital letters represent conservative amino acids among all species, the lower case letters represent variable amino acid residues, and the periods represent amino acids that are species specific. 'X' represents unidentified residue. The identical sequences are presented in the box.

<table>
<thead>
<tr>
<th>Enzyme/Species</th>
<th>Amino Acids</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₃ conservative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>1–25</td>
<td>A d q p i d a D V . v I G . G P g G Y V A A I K A</td>
</tr>
<tr>
<td>Yeast</td>
<td>1–24</td>
<td>T I N K S H D V I I G G P A G Y V A A I K A</td>
</tr>
<tr>
<td>Pig heart</td>
<td>1–25</td>
<td>A D Q P I D A D V T V I G S G P G Y V A A I K A</td>
</tr>
<tr>
<td>Human liver</td>
<td>1–25</td>
<td>A D Q P I D A D V T V I G S G P G Y V A A I K A</td>
</tr>
<tr>
<td>Soybean FLbR</td>
<td>1–25</td>
<td>A S G S D E N D V 1 V I G G P G Y V A A I K A</td>
</tr>
<tr>
<td>E₃ conservative</td>
<td>26–50</td>
<td>A Q L G f k T v c i E K n e t L G G T C L N V G C</td>
</tr>
<tr>
<td>E. coli</td>
<td>25–49</td>
<td>A D L G L E T V I V E R Y N T L G G V C L N V G C</td>
</tr>
<tr>
<td>Pig heart</td>
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<td>26–50</td>
<td>A Q L G L K T T X I E K N Y T L G G T C L N V G C</td>
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cofactors. These studies will give insights into catalytic mechanisms and biological functions of FLbR.

ACKNOWLEDGMENTS

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LITERATURE CITED