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Threonine-insensitive Homoserine Dehydrogenase from Soybean

GENOMIC ORGANIZATION, KINETIC MECHANISM, AND IN VIVO ACTIVITY[*] [S]

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Aspartate kinase (AK) and homoserine dehydrogenase (HSD) function as key regulatory enzymes at branch points in the aspartate amino acid pathway and are feedback-inhibited by threonine. In plants the biochemical features of AK and bifunctional AK-HSD enzymes have been characterized, but the molecular properties of the monofunctional HSD remain unexamined. To investigate the role of HSD, we have cloned the cDNA and gene encoding the monofunctional HSD (GmHSD) from soybean. Using heterologously expressed and purified GmHSD, initial velocity and product inhibition studies support an ordered bi bi kinetic mechanism in which nicotinamide cofactor binds first and leaves last in the reaction sequence. Threonine inhibition of GmHSD occurs at concentrations (K_i = 160–240 mM) more than 1000-fold above physiological levels. This is in contrast to the two AK-HSD isoforms in soybean that are sensitive to threonine inhibition (K_i~150 μM). In addition, GmHSD is not inhibited by other aspartate-derived amino acids. The ratio of threonine-resistant to threonine-sensitive HSD activity in soybean tissues varies and likely reflects different demands for amino acid biosynthesis. This is the first cloning and detailed biochemical characterization of a monofunctional feedback-insensitive HSD from any plant. Threonine-resistant HSD offers a useful biotechnology tool for manipulating the aspartate amino acid pathway to increase threonine and methionine production in plants for improved nutritional content.

In plants and microorganisms, aspartate is the common precursor of the essential amino acids lysine, threonine, methionine, and isoleucine (1–2). Because this branching set of pathways is critical for central metabolism and not found in mammals, the enzymes of the aspartate family of amino acid biosynthesis present targets for the development of anti-microbial agents and herbicides (1–3) and for improving the nutritional value of food crops (4–7). In the initial step of the pathway (1), aspartate kinase (AK)[7] catalyzes the phosphorylation of aspartate (Fig. 1). Conversion of aspartyl phosphate to L-aspartate-β-semialdehyde (Asa) leads to the first branch point of the pathway, as Asa can be metabolized to either lysine or homoserine (Hse). Homoserine dehydrogenase (HSD) catalyzes the conversion of Asa to Hse in the third step of the pathway. Subsequent metabolism of Hse yields methionine and threonine. Both AK and HSD are highly regulated by feedback inhibition through threonine to control flux through the various branches of the pathway (1–2). The regulation of these enzymes by threonine depends on their structural organization in different organisms.

AK and HSD function as either monofunctional or bifunctional enzymes in bacteria, yeast, and plants. In *Escherichia coli*, AK occurs as either a threonine-resistant monofunctional enzyme or as a threonine-sensitive bifunctional AK-HSD (8–10). HSD operates either as part of a bifunctional feedback responsive AK-HSD in Gram-negative bacteria such as *E. coli* or as a monofunctional protein in Gram-positive bacteria and yeast (11, 12). In the Gram-positive bacteria *Corynebacterium glutamicum* and *Bacillus subtilis*, threonine inhibits the monofunctional HSD, but the enzyme from yeast (*Saccharomyces cerevisiae*) is resistant to the amino acid (13, 14). Differences in the C-terminal region in the yeast HSD compared with the bacteria enzyme prevent inhibition by threonine (1, 14). Plants utilize a similar complement of enzymes for the synthesis of other amino acids derived from aspartate. A family of genes encodes three monofunctional AKs in *Arabidopsis thaliana* (thale cress) (15, 16). To date, the only HSD cloned from plants, including *A. thaliana*, maize (*Zea mays*), and soybean (*Glycine max*), are those fused with AK as a bifunctional enzyme (17–

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ172918.1

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[7] The abbreviations used are: AK, aspartate kinase (E.C. 2.7.2.4); Asa, L-aspartate-β-semialdehyde; GmAK, *G. max* (soybean) aspartate kinase; HSD, homoserine dehydrogenase (E.C. 1.1.1.3); GmAK-HSD, *G. max* (soybean) AK-HSD; GmHSD, *G. max* (soybean) HSD; Hse, homoserine.

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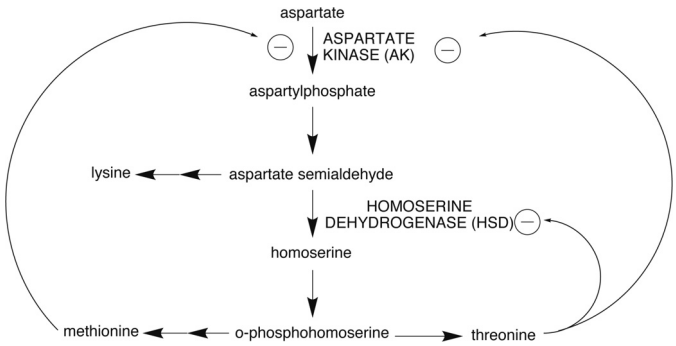

FIGURE 1. **Overview of the aspartate branch of amino acid biosynthesis.** The core pathway from aspartate is shown in detail. Multiple steps are required to produce lysine and methionine from the central reactions. Feedback inhibition of AK and HSD by downstream products is indicated by a *long dash*.

21). The AK-HSD isoforms from *Arabidopsis* and maize are sensitive to inhibition by threonine (17–19, 21), but the enzymes from soybean remain biochemically uncharacterized (20). In both bacterial and plant AK-HSD, the linker region between each enzyme domain is responsible for the regulatory effect of threonine (13, 21). Interestingly, plants also appear to use monofunctional HSD, as the activity of a threonine-resistant HSD in maize extracts was reported (22, 23), but no molecular details described.

To investigate the role of a threonine-resistant HSD in the aspartate amino acid pathway of plants, we have cloned the cDNA and gene encoding the enzyme (GmHSD) from soybean. Biochemical analysis of GmHSD using initial velocity and product inhibition studies suggest that the enzyme follows an ordered bi bi kinetic mechanism. Importantly, GmHSD is not inhibited by physiological concentrations of threonine. In contrast, kinetic analysis of the two previously uncharacterized GmAK-HSDs demonstrates that threonine tightly regulates each isoform. The ratio of threonine-resistant to threonine-sensitive HSD in soybean tissues varies and may have a role in meeting amino acid demands in different tissues. This work is the first reported isolation and molecular characterization of a monofunctional HSD from any plant. The isolation of a threonine-resistant HSD from soybean may provide a useful biotechnology tool for manipulating the aspartate amino acid pathway to increase threonine and methionine production in plants for improved nutritional content.

EXPERIMENTAL PROCEDURES

Materials—Five days after sowing, seedlings of soybean cv. Williams 82 were used to isolate RNA. Preparation of Asa was performed by ozonolysis of L-allyl glycine (24). The ozonolysis product was purified using a cation exchange resin (Dowex 50WX8–400; column: 1.5 × 10 cm). Immediately before use, the product was neutralized with $NaHCO_3$. All other chemical reagents were purchased from Sigma.

Isolation of GmHSD cDNA and Genomic Clones—Total RNA from seedling hypocotyls was isolated through a lithium chloride precipitation procedure (25). Primers (forward, 5′-dGCTGGTCTTCCTGTGATAGCATCA-3′; reverse, 5′-dCCATTTGAAGCTGCCTTCTCAACTC-3′) were synthesized based on sequence information obtained from an EST clone (GenBank™ accession number BM308805) for use as probes to screen cDNA libraries. A 394-bp fragment was amplified by reverse transcriptase-PCR (Stratagene). This fragment was cloned into a sequencing vector pGEMT-EASY (Promega). After confirming the sequence, the fragment was purified by agarose gel electrophoresis and radiolabeled with [^{32}P]dCTP using a random labeling kit (Takara Bio). The probe was used to screen a seedling cDNA library (courtesy of Dr. Joseph Polacco, University of Missouri). Two positive clones were isolated after colony hybridization with a radiolabeled probe following the standard method for three times (26). One of the recovered clones was sequenced and showed a full-length cDNA (deposited in GenBank™ accession number as DQ172918.1; supplemental Fig. 1).

The same probe used to screen the cDNA library was used for screening a commercially available soybean genomic library (Stratagene). Seven positive clones were isolated through colony hybridization with a radiolabeled probe. Hybridization was performed three consecutive times (26). DNA restriction followed by Southern analysis of one of the seven clones revealed hybridization with two HindIII inserts (3.2 and 4.2 kb) and one EcoRI insert (0.6 kb). These inserts were gel-isolated, purified, and subcloned into an intermediate vector pBlueScript before sequencing.

Southern Blot Analysis—Soybean genomic DNA was isolated from the leaves of the Williams 82 cultivar using the cetyltrimethyl ammonium bromide method (27). After quantification, 8 μg of DNA was digested along with different restriction enzymes overnight at 37 °C. The digested DNA was separated on a 0.8% agarose gel and transferred to nylon membranes by a capillary transfer using 0.4 M of NaOH. The same DNA fragment used to screen the cDNA library was labeled with [^{32}P]dCTP using the Ladderman labeling kit (PanVera) then employed as a probe for Southern analysis. After 6 h of prehybridization, overnight hybridization was done at 65 °C using 6× SSPE buffer (1× SSPE is 100 mM NaCl, 10 mM Na_2PO_4, and 1 mM EDTA), 5× Denhardt's solution, 0.5% (w/v) SDS, and 50 μg ml^{-1} salmon sperm DNA. After hybridization, the membranes were washed 3 times in wash solution I (2× SSPE and 0.5% (w/v) SDS) for 10 min at room temperature before 2 washes in wash solution II (0.1× SSPE and 0.1% (w/v) SDS) at 65 °C for 30 min. After washing, the nylon membrane was exposed to x-ray film overnight at −80 °C.

Protein Expression and Purification—The coding region of GmHSD was amplified by PCR, and the resulting product was digested with NdeI and XhoI and ligated into the corresponding restriction sites of pET-28a (Novagen). The pET28a-GmHSD expression construct was transformed into *E. coli* Rosetta(DE3) (Novagen) cells. Transformed cells were grown at 37 °C in Terrific broth containing 50 μg ml^{-1} kanamycin and 34 μg ml^{-1} chloramphenicol until A_{600nm} = 0.8–1.2. After induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside, the cultures were grown for 6–8 h at 18 °C. Cells were pelleted by centrifugation (10,000 × *g*; 10 min) and resuspended in lysis buffer (50 mM Tris (pH 8.0), 500 mM NaCl, 25 mM imidazole, 10% (v/v) glycerol, and 1% (v/v) Tween 20). After sonication and centrifugation (48,000 × *g*; 60 min), the supernatant was passed over a Ni^{2+}-nitriloacetic acid-agarose (Qiagen) column.

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The column was washed with lysis buffer without Tween 20, and the His-tagged protein eluted with elution buffer (wash buffer containing 250 mM imidazole). Size-exclusion chromatography used a Superdex-200 16/20 fast protein liquid chromatography column (Amersham Biosciences) equilibrated with 50 mM Tris (pH 8.0) and 250 mM NaCl. Protein concentration was determined using Bradford reagent (Bio-Rad) with bovine serum albumin as the standard. Purified protein was stored at −80 °C.

HSD Assays—A standard reaction mixture (0.5 ml; 25 °C) for spectrophotometrically monitoring GmHSD activity contained 0.1 M Tris (pH 8.0), 5 mM Asa, and 2 mM NAD(P)H in the forward reaction or 0.1 M Tris (pH 8.0), 50 mM Hse, and 2 mM $NAD(P)^+$ in the reverse reaction. Initial velocities were determined by following the change in $A_{340\ nm}$. For determination of steady-state kinetic parameters, reaction rates were measured under standard assay conditions with a matrix of varied substrate concentrations. Curve fitting in SigmaPlot (Systat Software, Inc.) was used to model the kinetic data to the equation for a sequential mechanism, $v = (V_{max}[A][B])/(K_{ia}K_b + K_a[B] + K_B[A] + [A][B])$, where v is the initial velocity, V_{max} is the maximum velocity, K_A and K_B are the K_m values for substrates A and B, respectively, and K_{ia} is the substrate inhibition constant. The same assays were used to monitor activity of monofunctional and bifunctional HSD.

For product and feedback inhibition assays, enzymatic activity was measured in reactions under standard assay conditions containing inhibitor and varied concentrations of either substrate. The fitting analysis of data used SigmaPlot to fit data to the equations for either competitive, $v = (V_{max}[S])/((K_m(1 + [I]/K_{is}) + [S])$, or non-competitive, $v = (V_{max}[S])/((K_m(1 + [I]/K_{is})) + ([S](1 + [I]/K_{ii})))$, inhibition, where I is inhibitor concentration.

Assays of HSD activity in extracts of soybean tissues were performed using the standard assay system. Tissue extracts were prepared in 100 mM Tris (pH 8.5), 2 mM dithiothreitol, 1 mM EDTA. Homogenates were centrifuged at 4 °C, and the supernatant was collected. Assays contained 10–100 μg of total protein.

Expression and Purification of GmAK and GmAK-HSD—Soybean AK (GmAK; GenBank™ accession number AAD41796.1) was amplified from the seedling cDNA library and cloned into pCR-Blunt-II TOPO vector. Sequencing confirmed the fidelity of the clone. GmAK was subcloned into pET-28a for bacterial expression using Nhe1 and NotI sites. The two bifunctional AK-HSDs in soybean (GmAK-HSD-1, GenBank™ accession numbers AAC05983; GmAK-HSD-2, GenBank™ accession number AAC05981 (20)) were also amplified from the same library, cloned into pCR-Blunt-II TOPO vector, sequenced, and subcloned into pET-28a using NheI and NotI sites. Protein expression and purification of His-tagged GmAK, GmAK-HSD-1, and GmAK-HSD-2 used the same procedure as described for GmHSD.

AK Assays—AK activity of GmAK, GmAK-HSD-1, and GmAK-HSD-2 was determined spectrophotometrically at 25 °C by measuring the rate of ADP formation using a coupled assay with pyruvate kinase and lactate dehydrogenase (28). A standard reaction mixture (0.5 ml) contained 100 mM Tris (pH 8.0), 100 mM KCl, 2.5 mM ATP, 10 mM aspartate, 5 mM $MgCl_2$, 2 mM sodium phosphoenolpyruvate, 0.2 mM NADH, 5 units of pyruvate kinase, and 10 units of lactate dehydrogenase. Kinetic parameters and inhibition constants were determined using the equations described above.

RESULTS

Cloning and Genome Analysis of GmHSD—Screening a soybean seedling cDNA library with an EST sequence (accession number BM308805) as the probe revealed a full-length cDNA clone. The putative cDNA clone was sequenced, and analysis of the sequence revealed a 1472-bp DNA insert containing a single open reading frame of 1128 bp (deposited in GenBank™ accession number as DQ172918.1; supplemental Fig. 1). The cDNA has a 90-bp 5′-untranslated region (UTR) and a 251-bp 3′-UTR. The predicted open reading frame encodes a protein of 376 amino acids with a calculated molecular mass of 40.6 kDa and predicted isoelectric point of 6.19. BLAST analysis showed that this sequence shares 40 and 25% amino acid sequence identity with the monofunctional HSD from *S. cerevisiae* and *B. subtilis*, respectively.

To determine the gene organization of GmHSD, sequencing of two HindIII (3.2 and 4.2 kb) fragments and one EcoRI (0.6 kb) fragment from 1 of the 7 clones obtained from genomic library screening revealed the entire coding region of GmHSD (deposited in GenBank™ accession number as DQ788566.1). Of the 7997 bp sequenced, the cDNA of GmHSD begins at position 1461 and ends at position 7763. The start codon of the cDNA maps to position 1537, and the stop codon is located at 7760 in the genomic sequence. The nucleotide sequence of the genomic clone along with the deduced amino acid sequence from the full-length DNA, was used to determine the position and span of introns and exons (Fig. 2*A*). The GmHSD coding sequence is interrupted by 11 introns that vary in length from 107 to 1637 bp. Southern analysis with six different restriction enzymes was carried out to determine the copy number of HSD in the soybean genome (Fig. 2*B*). Except for EcoRI, strong hybridization was seen for one band, whereas the other bands showed weak hybridization. The obtained results indicate that GmHSD is encoded by a single low copy gene but that hybridization to other HSD-related genes occurs most likely the AK-HSD isoforms in soybean (20).

Expression and Purification of GmHSD—To verify that the isolated cDNA encoded a functional HSD, recombinant protein was overexpressed in *E. coli* Rosetta(DE3) as a hexahistidine-tagged fusion protein and purified to apparent homogeneity using Ni^{2+}-affinity and size-exclusion chromatography (Fig. 3). By SDS-PAGE analysis, the purified protein migrated with a monomer weight of ~40 kDa, which corresponds to the predicted molecular weight (40.6 kDa). GmHSD eluted from the size-exclusion column as a 70-kDa species representing a dimer.

Kinetic Analysis of GmHSD—Purified protein was first analyzed for HSD and AK activity. GmHSD displayed a specific activity of 13 μmol min^{-1} mg of $protein^{-1}$ using Asa and NADH as substrates and exhibited no detectable AK activity. A complete steady-state kinetic analysis of the forward and reverse reactions of the enzyme was performed to understand

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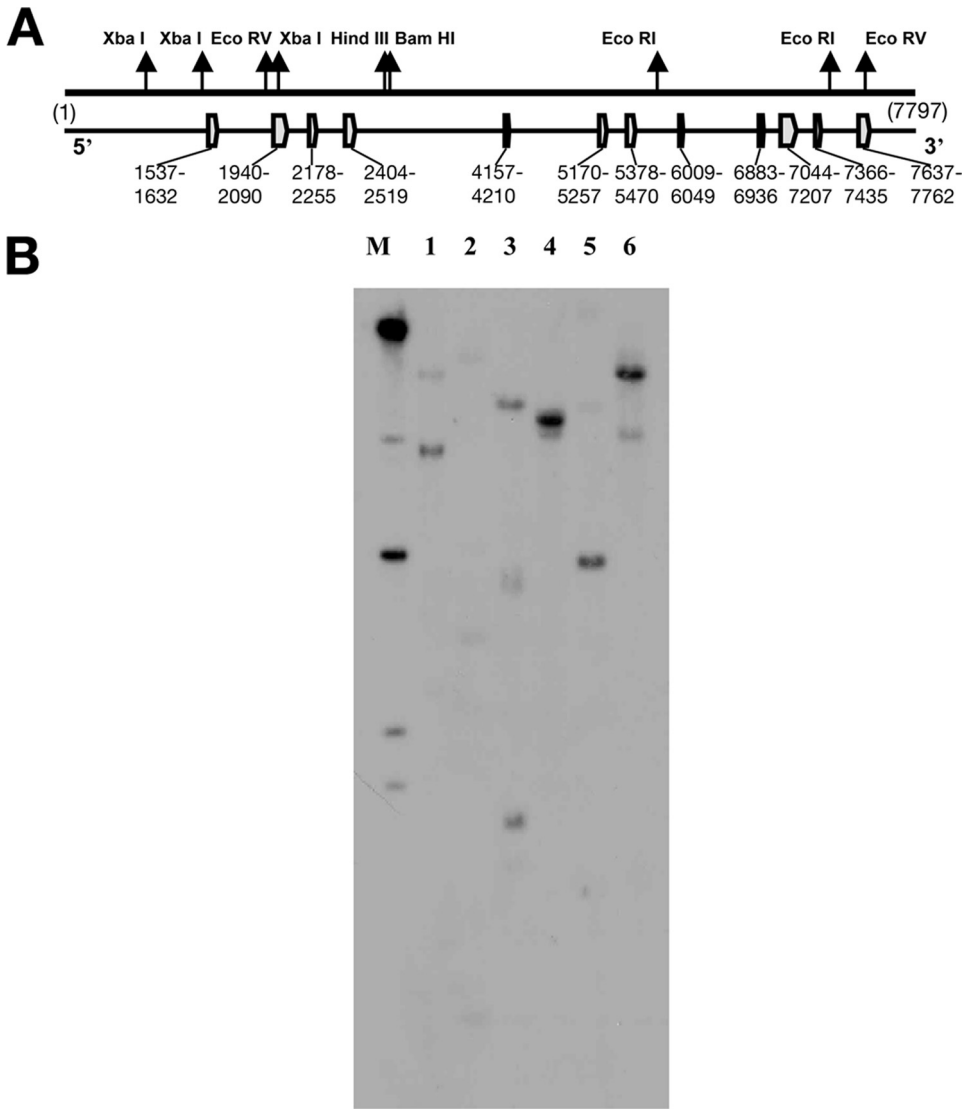

FIGURE 2. **GmHSD genomic organization and Southern blot analysis.** *A*, a partial restriction map of the GmHSD genomic clone and its corresponding gene structure is shown. The *boxed portions* on the line marked 5′ to 3′ indicate exons with nucleotide numbering as indicated. The *connecting lines* represent introns. *B*, Southern analysis of GmHSD is shown. Eight μg of soybean genomic DNA was used for each digestion as follows: *lane m*, λ-HindIII DNA marker; *lane 1*, BamHI; *lane 2*, DraI; *lane 3*, EcoRI; *lane 4*, EcoRV; *lane 5*, HindIII; *lane 6*, XbaI. The gel was blotted to nitrocellulose membrane followed by hybridization with ^{32}P-labeled GmHSD cDNA.

the equilibrium of this metabolic step and to determine nucleotide cofactor specificity. Kinetic parameters for GmHSD were determined for the forward (Asa/NADH and Asa/NADPH) and reverse (Hse/NAD$^+$ and Hse/NADP$^+$) reactions. Initial velocity data were generated by varying both substrates and fitting to the equation for a sequential bi bi mechanism (Fig. 4 and Table 1). Comparison of catalytic efficiencies for the forward and reverse reactions shows that the equilibrium favors production of Hse using either nucleotide cofactor. Although GmHSD displays a 1.6-fold preference for NADPH as the cofactor in the forward reaction, both cofactors are efficiently used for catalysis. In the reverse reaction NADP$^+$ is favored nearly 4-fold as the cofactor.

Kinetic Mechanism of GmHSD—The reciprocal plots of Asa *versus* NAD(P)H and Hse *versus* NAD(P)$^+$ (Fig. 4) showed intersecting patterns, suggesting a sequential bi bi kinetic mechanism rather than a ping-pong type of mechanism. To distinguish between ordered and random bi bi mechanisms, product inhibition studies were performed. For these experiments, NADPH and NADP$^+$ were used as the nucleotide cofactors because these are preferred by GmHSD. Reactions were performed in the presence of a product inhibitor with one substrate at varied concentrations and the other at an unsaturating concentration. In the forward reaction Hse was a non-competitive inhibitor *versus* both Asa and NADPH, and NADP$^+$ showed non-competitive and competitive inhibition patterns *versus* Asa and NADPH, respectively (Table 2). Similar product inhibition patterns were observed for the reverse reaction. Asa was a non-competitive inhibitor *versus* both substrates, and NADPH displayed non-competitive inhibition *versus* Hse but was competitive *versus* NADP$^+$. The competitive inhibition observed in each direction with the nicotinamide cofactors indicates that they bind to the same form of the enzyme in an ordered mechanism.

Feedback Inhibition of GmHSD by Aspartate-branch Amino Acids—HSD activity in plants is reported to be sensitive to inhibition by downstream products of the aspartate-derived amino acid pathway. To examine the effect of the aspartate-derived amino acids (aspartate, lysine, methionine, isoleucine, and threonine) on GmHSD, enzyme assays were performed in the presence of each amino acid. Isoleucine (100 mM) had no effect on GmHSD activity. Aspartate, lysine, and methionine showed slight inhibition (*i.e.* less than 10% at 100 mM). Threonine (100 mM) inhibited GmHSD ~40%. Detailed analysis of threonine inhibition of GmHSD (Fig. 5) showed the amino acid to be a competitive inhibitor *versus* Asa (K_i = 163 ± 30 mM) and a non-competitive inhibitor *versus* NADPH (K_i = 239 ± 36 mM). Importantly, the concentrations of threonine (and other aspartate-derived products) are significantly higher than physiological levels, suggesting that GmHSD is insensitive to feedback inhibition.

HSD Activity in Soybean—To determine the physiologic level of threonine inhibition of HSD activity *in vivo*, we measured enzymatic activity with and without exogenous threonine in seed, root, stem, and leaf tissues from soybean (Fig. 6). Measurement of HSD activity showed the highest activity in mature

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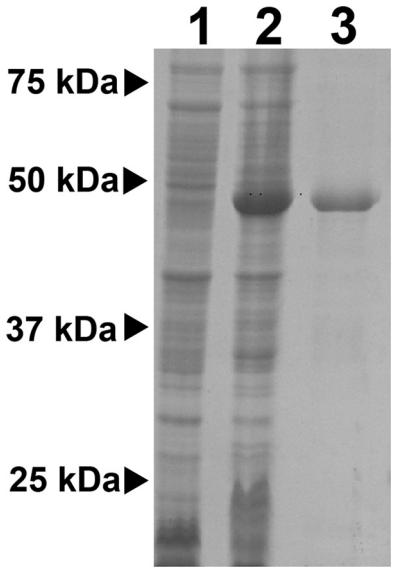

FIGURE 3. **Analysis of expression and purification of GmHSD from *E. coli*.** An SDS-PAGE of samples stained for total protein with Coomassie Blue is shown. *Arrows* correspond to molecular mass markers as indicated. *Lanes 1–3* are as follows: *lane 1*, 50 μg of *E. coli* sonicate before induction; *lane 2*, 50 μg of *E. coli* sonicate 8 h after induction with IPTG; *lane 3*, 3 μg of purified GmHSD after nickel-affinity and size-exclusion chromatography.

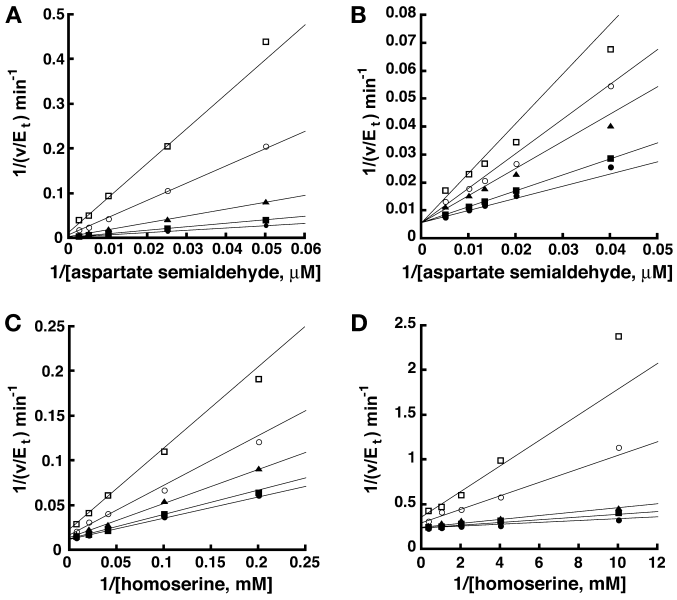

FIGURE 4. **Initial velocity data for GmHSD.** Enzyme assays were performed as described under "Experimental Procedures." Initial velocity data are shown as symbols in the reciprocal plots. All assays were performed in triplicate with less than 5% error between measurements. *Lines* represent the global fit of data to a sequential bi bi kinetic mechanism. *A*, shown is a double-reciprocal plot of $1/(v/E_t)$ *versus* 1/[aspartate semialdehyde] at 20, 40, 100, 200, and 300 μM NADH (from *top* to *bottom*). *B*, shown is a double-reciprocal plot of $1/(v/E_t)$ *versus* 1/[aspartate semialdehyde] at 25, 50, 75, 100, and 200 μM NADPH (from *top* to *bottom*). *C*, shown is a double-reciprocal plot of $1/(v/E_t)$ *versus* 1/[homoserine] at 2.5, 5, 10, 25, and 50 mM NAD^+ (from *top* to *bottom*). *D*, shown is a double-reciprocal plot of $1/(v/E_t)$ *versus* 1/[homoserine] at 0.1, 0.25, 0.5, 1, and 3 mM $NADP^+$ (from *top* to *bottom*).

leaves and stems with the lowest activity in roots. The addition of threonine to the extracts inhibited HSD activity in all tissues but to varying degrees. Protein extracts from root and stem tissues showed an approximate 50% decrease in HSD activity after threonine addition. HSD activity in leaf tissues (both young and mature) was reduced to ~70% that of the original activity. Seed protein extracts displayed a slight 5% decrease in HSD activity. These results suggest that the monofunctional HSD is most active in seeds followed by roots and stems and then leaves. Moreover, the differences in HSD activity indicate the presence of threonine-sensitive HSD, most likely bifunctional AK-HSD proteins, in these tissues.

Kinetic Analysis of GmAK and GmAK-HSD—To examine the threonine inhibition of proteins encoded by reported sequences for the monofunctional AK (GenBank^{TM} accession number AAD41796.1) and bifunctional AK-HSD (GenBank^{TM} accession numbers AAC05983 and AAC05981; (20)) in soybean, each of these enzymes was cloned, expressed, and purified for kinetic characterization and analysis of threonine inhibition.

In both the forward and reverse HSD reactions, the bifunctional GmAK-HSD proteins displayed similar steady-state kinetic parameters with 2–3-fold differences in k_{cat}/K_m values (Table 3). In general, the catalytic efficiencies of the bifunctional and monofunctional HSD proteins were similar in the forward reaction (Tables 1 and 3), although GmHSD showed a consistent preference for NADPH as cofactor. The K_m for Asa with NADPH as co-substrate is 12–20-fold higher in both of the GmAK-HSD than GmHSD. In the reverse reaction the monofunctional GmHSD is generally more efficient than the bifunctional AK-HSD proteins, which show a roughly 10-fold higher K_m value for NAD^+. The AK activity of GmAK and both GmAK-HSD showed that the monofunctional protein was catalytically more efficient than the fused proteins but with a slightly higher K_m for aspartate (Table 4).

The effect of threonine on GmAK and each of the GmAK-HSDs was examined. GmAK displayed a $K_{0.5} = 1.2 \pm 0.1$ mM for threonine inhibition. AK activity in the bifunctional enzymes was inhibited by threonine, with $K_{0.5} = 23 \pm 2$ μM and $K_{0.5} = 25 \pm 1$ μM for GmAK-HSD-1 and GmAK-HSD-2, respectively. Using the forward reaction substrates (2.5 mM Asa and 50 μM NADPH), the HSD activity in GmAK-HSD-1 and GmAK-HSD-2 showed $K_{0.5}$ values for threonine of 159 ± 9 and 140 ± 17 μM, respectively. The inhibition constants for the bifunctional enzymes are nearly 1000-fold lower than those determined for GmHSD and similar to those reported for AK-HSD in *Arabidopsis* and maize (17–19).

DISCUSSION

In plants and microbes the synthesis of four essential amino acids occurs through the aspartate amino acid pathway. Within this pathway, AK and HSD are critical regulatory targets and vary in their structural organization. HSDs exist either as bifunctional enzymes in Gram-negative bacteria or as monofunctional enzymes in Gram-positive bacteria and yeast (11–14). To date, the only HSD activity to be cloned and characterized in the plant kingdom has been as part of bifunctional AK-HSD (17–21). Here we describe the first molecular and kinetic analysis of a threonine-resistant HSD from soybean, demonstrating that plants, unlike bacteria and yeast, are unique in using both monofunctional and bifunctional HSD.

Sequence analysis of GmHSD shows that the monofunctional enzyme shares 20–40% sequence identity with bacterial

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TABLE 1

Kinetic parameters of GmHSD

Assays were performed as described under "Experimental Procedures." Kinetic parameters were determined from initial velocity data as described for $n = 3$. Average values ± S.E. are shown. Asa, forward reaction; Hse, reverse reaction.

Substrate A	Substrate B	k_{cat}	K_m^A	K_m^B	k_{cat}/K_m^A	k_{cat}/K_m^B
		min^{-1}	μM	μM	$M^{-1} s^{-1}$	$M^{-1} s^{-1}$
Asa	NADH	581 ± 27	569 ± 51	158 ± 18	17,020	61,290
Asa	NADPH	168 ± 8	98.1 ± 16.6	27.6 ± 3.7	28,540	101,400
Hse	NAD^+	85.8 ± 2.4	17,400 ± 1,800	2,700 ± 400	82.2	530
Hse	$NADP^+$	4.22 ± 0.09	275 ± 57	34.0 ± 0.8	256	2,070

TABLE 2

Product inhibition patterns for GmHSD

All assays ($n = 3$) were performed as described under "Experimental Procedures." Average values ± S.E. are shown. Substrate and inhibitor abbreviations are as follows: The inhibition patterns are as follows: C, competitive; NC, noncompetitive.

Reaction	Varied substrate	Fixed substrate	Inhibitor	Inhibition pattern	K_{is}	K_{ii}
					mM	mM
Forward	Asa	NADPH (100 μM)	Hse	NC	11.5 ± 1.7	21.5 ± 3.6
Forward	NADPH	Asa (250 μM)	Hse	NC	18.8 ± 2.0	23.0 ± 3.4
Forward	Asa	NADPH (100 μM)	$NADP^+$	NC	0.68 ± 0.17	1.26 ± 0.28
Forward	NADPH	Asa (250 μM)	$NADP^+$	C	0.43 ± 0.08	
Reverse	Hse	$NADP^+$ (100 μM)	Asa	NC	0.44 ± 0.10	0.80 ± 0.16
Reverse	$NADP^+$	Hse (500 μM)	Asa	NC	0.73 ± 0.11	1.55 ± 0.27
Reverse	Hse	$NADP^+$ (100 μM)	NADPH	NC	0.42 ± 0.14	0.63 ± 0.20
Reverse	$NADP^+$	Hse (500 μM)	NADPH	C	0.38 ± 0.09	

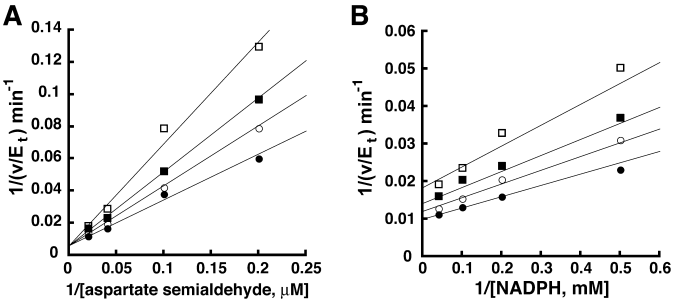

FIGURE 5. **Effect of threonine inhibition on GmHSD activity.** Enzyme assays were performed as described under "Experimental Procedures." All assays were performed in triplicate with less than a 5% error between measurements. *A*, shown is a double-reciprocal plot of $1/(v/E_t)$ *versus* 1/[aspartate semialdehyde] at 0, 50, 100, and 200 μM threonine (*top* to *bottom*). The *lines* show the fit of competitive inhibition to the data. *B*, shown is a double-reciprocal plot of $1/(v/E_t)$ *versus* 1/[NADPH] at 0, 50, 100, and 200 μM threonine (*top* to *bottom*). The *lines* show the fit of non-competitive inhibition to the data.

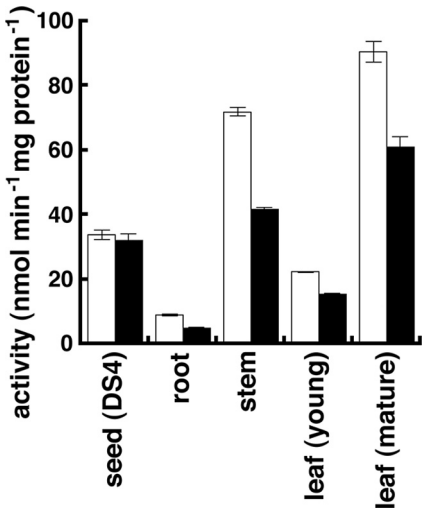

FIGURE 6. **HSD activity in soybean tissues.** HSD activity was measured using Asa and NADPH under standard assay conditions as described under "Experimental Procedures" using protein extracts from seed (developmental stage 4), roots, stems, young leaves, and mature leaves. Activity measurements were performed in the absence (*white*) and presence (*black*) of exogenous 10 mM threonine. *Bars* represent S.E. ($n = 3$).

and yeast HSD and less than 40% identity with the HSD domain of the bifunctional enzymes from *Arabidopsis*. In soybean, Southern analysis indicates strong hybridization to one gene and much weaker hybridization with other related forms (Fig. 2). Together with the two previously isolated cDNAs coding for bifunctional AK-HSD from soybean (20), there are three HSD-encoding genes in the *G. max* genome. Importantly, the presence of a monofunctional HSD in plants is not limited to soybean. A BLAST search of available databases reveals that multiple plants, including *Medicago truncatula* (barrel clover; 85%), *Populus trichocarpa* (California poplar; 76%), *Ricinus communis* (castor; 75%), *Vitis vinifera* (grape; 70%), *A. thaliana* (69%), *Z. mays* (maize; 66%), *Sorghum bicolor* (66%), and *Oryza sativa* (rice; 66%), contain sequences sharing high identity with GmHSD that are in many cases annotated as either unknown or hypothetical proteins or even incorrectly as AK. This suggests that monofunctional HSD in plants are more prevalent than previously described (22–23).

To understand the biochemical properties of GmHSD, a detailed kinetic analysis of the enzyme was performed. For examining the kinetics of the forward and reverse reactions catalyzed by GmHSD, we used both NAD(H) and NADP(H) as co-substrates. Previously, the *E. coli* HSD was only analyzed in the forward direction with NADPH, and the yeast enzyme was analyzed using NAD(H) in each direction (10–11). Likewise, kinetic studies of *Arabidopsis* AK-HSD have primarily relied on using the reverse reaction substrates Hse and $NADP^+$ (21, 29). Based on comparison of k_{cat}/K_m values for GmHSD, the forward reaction is favored by 50–200-fold over the reverse reaction (Table 1). Moreover, use of the phosphorylated nucleotides is only modestly favored. Further experiments determined the kinetic mechanism of the enzyme.

Using a combination of initial velocity and product inhibition experiments (Table 2) in both reaction directions, our data suggest an ordered bi bi mechanism for GmHSD. The intersect-

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TABLE 3

Kinetic parameters for HSD activity of GmAK-HSD-1 and GmAK-HSD-2

Assays were performed as described under "Experimental Procedures." Kinetic parameters were determined from initial velocity data as described for $n = 3$. Average values ± S.E. of fits are shown. Asa, forward reaction; Hse, reverse reaction.

Enzyme	Substrate A	Substrate B	k_{cat}	K_m^A	K_m^B	k_{cat}/K_m^A	k_{cat}/K_m^B
			min^{-1}	μM	μM	$M^{-1}\,s^{-1}$	$M^{-1}\,s^{-1}$
GmAK-HSD-1							
	Asa	NADH	1,158 ± 200	1,190 ± 231	213 ± 20	16,220	90,610
	Asa	NADPH	695 ± 79	2,190 ± 170	39.1 ± 1.4	5,290	296,200
	Hse	NAD^+	420 ± 34	9,570 ± 973	24,900 ± 3,700	731	281
	Hse	$NADP^+$	2.54 ± 0.21	1,080 ± 194	235 ± 30	39.2	180
GmAK-HSD-2							
	Asa	NADH	1,355 ± 146	845 ± 115	190 ± 22	26,730	118,900
	Asa	NADPH	779 ± 59	1,250 ± 147	39.2 ± 0.9	10,390	331,200
	Hse	NAD^+	609 ± 24	13,400 ± 1,500	24,100 ± 3,500	757	421
	Hse	$NADP^+$	3.94 ± 0.61	690 ± 65	245 ± 75	95.2	268

TABLE 4

Kinetic parameters for AK activity of GmAK-HSD-1, GmAK-HSD-2, and GmAK

Assays were performed as described under "Experimental Procedures." Kinetic parameters were determined from initial velocity data as described for $n = 3$. Average values ± S.E. of fits are shown.

	k_{cat}	K_m^{ATP}	K_m^{Asp}	k_{cat}/K_m^{ATP}	$k_{cat}K_m^{Asp}$
	min^{-1}	μM	μM	$M^{-1}\,s^{-1}$	$M^{-1}\,s^{-1}$
GmAK-HSD-1	21.1 ± 1.4	894 ± 102	5,260 ± 490	393	66.9
GmAK-HSD-2	34.7 ± 2.0	647 ± 74	6,330 ± 710	894	91.4
GmAK	138 ± 18	680 ± 109	12,100 ± 2,300	3,380	190

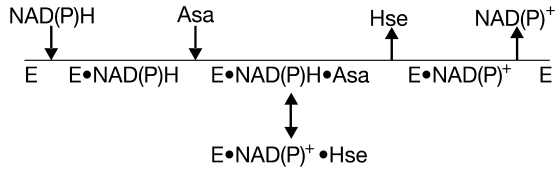

FIGURE 7. **Proposed kinetic mechanism of GmHSD.** Based on the kinetic analysis of GmHSD, NAD(P)H binds first followed by Asa. This leads to catalysis and release of Hse followed by release of $NAD(P)^+$.

ing patterns observed in the initial velocity studies (Fig. 4) eliminate a possible ping-pong mechanism, and the determined inhibition patterns (Table 2) are consistent with a reaction sequence in which nucleotide cofactor binds first and is released last (Fig. 7). The competitive inhibition pattern of product inhibition by nicotinamide cofactors in each direction indicates that the inhibitor ($NADP^+$ or NADPH) and the varied substrate (NADPH or $NADP^+$) both bind to free enzyme (Table 2). The non-competitive patterns show that Asa or Hse bind as the second substrate (Table 2). The kinetic mechanism for GmHSD is similar to that reported for the yeast enzyme (11). In contrast to the plant and yeast HSD, the *E. coli* enzyme displays either a fully ordered (10) or a preferred order random mechanism (30, 31) depending on pH of the assay conditions. Additional experiments using either isotope exchange or substrate dependence of kinetic isotope effects could conclusively distinguish between the random and ordered models (32, 33).

In contrast to the well characterized AK-HSD isoforms from plants (17–21), GmHSD is resistant to feedback inhibition by threonine (Fig. 5). The amino acid can act as an inhibitor of GmHSD but with K_i values greater than 100 mM higher than the 300 μM free threonine concentration in plants (34). For the plant and bacterial AK-HSD, the linker region between the two enzyme domains is critical for feedback regulation by threonine and for modulation of activity by other aspartate pathway amino acids (13, 21). The lack of this region in GmHSD corresponds with resistance to threonine inhibition. Moreover, a major difference between the HSD of yeast and plants *versus* the enzyme in Gram-positive bacteria is resistance to feedback inhibition by threonine (1, 14). The monofunctional enzymes from *Corynebacterium* and *Bacillus* have longer C-terminal regions implicated in feedback inhibition by threonine (35). A single amino acid change in the HSD of *Corynebacterium* was shown to be critical for inhibition by threonine (35). This residue is conserved across the HSD from Gram-positive bacteria but is absent in yeast because of a shorter C-terminal region (14). Similarly, GmHSD lacks the C-terminal extension, and biochemical analysis demonstrates that this enzyme is insensitive to inhibition by threonine at physiological levels.

Although the aspartate amino acid pathway is of major interest for metabolic engineering efforts aimed at enhancing essential amino acid content and nutritional value (4, 36), the physiological role of threonine-insensitive HSD is not well understood (37) and represents a little explored feature of this pathway in plants. Here we show that threonine-sensitive and threonine-insensitive HSD activity in soybean differs by tissue type (Fig. 6). This difference results from the soybean expressing both monofunctional HSD and bifunctional AK-HSD, which are strongly regulated by threonine levels (Table 3). In leaf, stem, and roots the percent of HSD resistant to threonine is comparable with the activity seen in maize, in which threonine-sensitive HSD accounts for ~50% of HSD activity (19). Earlier *in vivo* studies indicate that pathway activity changes in response to environmental conditions, including pH and illumination and spatial and temporal differences (19, 38). For example, in soybean seeds amino acid biosynthesis occurs before production of seed storage proteins (6, 39, 40), and the differences in the response of HSD isoforms to threonine may be a mechanism for maintaining production of amino acids derived from aspartate during early seed development. Ultimately, the levels of threonine-sensitive and -insensitive HSD in plant tissues likely reflect a need to tailor metabolism in

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response to metabolic demands. It appears that plants, unlike microbes, require a full complement of monofunctional HSD, monofunctional AK, and bifunctional AK-HSD to integrate multiple metabolic inputs for modulating the aspartate amino acid pathway. Further studies aimed at understanding the interplay of these proteins in plants are required.

The monofunctional HSD of plants may provide a useful biotechnology tool. Typically, control steps in pathways can be bypassed by inactivating the regulatory feature or by isolating inhibition-resistant forms of the target enzyme. Overexpression of threonine-insensitive HSD or reduction of its expression by RNAi or other methods may alter the flux through the branch point that balances between production of lysine *versus* threonine and methionine.

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