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Mechanism of Control of *Arabidopsis thaliana* Aspartate Kinase-Homoserine Dehydrogenase by Threonine*

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The regulatory domain of the bifunctional threonine-sensitive aspartate kinase homoserine dehydrogenase contains two homologous subdomains defined by a common loop- α helix-loop- β strand-loop- β strand motif. This motif is homologous with that found in the two subdomains of the biosynthetic threonine-deaminase regulatory domain. Comparisons of the primary and secondary structures of the two enzymes allowed us to predict the location and identity of the amino acid residues potentially involved in two threonine-binding sites of *Arabidopsis thaliana* aspartate kinase-homoserine dehydrogenase. These amino acids were then mutated and activity measurements were carried out to test this hypothesis. Steady-state kinetic experiments on the wild-type and mutant enzymes demonstrated that each regulatory domain of the monomers of aspartate kinase-homoserine dehydrogenase possesses two nonequivalent threonine-binding sites constituted in part by Gln⁴⁴³ and Gln⁵²⁴. Our results also demonstrated that threonine interaction with Gln⁴⁴³ leads to inhibition of aspartate kinase activity and facilitates the binding of a second threonine on Gln⁵²⁴. Interaction of this second threonine with Gln⁵²⁴ leads to inhibition of homoserine dehydrogenase activity.

In plants and bacteria, the first and the third steps of methionine and threonine biosynthesis are catalyzed by isoforms of the bifunctional enzyme, aspartate kinase (AK)¹ (EC 2.7.2.4)-homoserine dehydrogenase (HSDH) (EC 1.1.1.3) (1, 2). In *Escherichia coli*, the expression of one of these bifunctional enzymes is repressed by methionine (3), whereas the expression of the other enzyme is repressed by threonine (4). The activity of this later bifunctional isoform is also inhibited by threonine. The threonine-sensitive isoform has been extensively characterized in bacteria (5, 6). It is a homotetramer containing two threonine-binding sites per monomer (5, 6). Although the two threonine-binding sites of the bacterial enzyme have not yet been identified, preliminary work has demonstrated that the regulatory domain of the bacterial enzyme is located in the intermediary region (amino acids segment 316–447) between the AK catalytic domain (amino acids segment

1–315) and the HSDH catalytic domain (amino acids segment 448–812) (7–11) (Fig. 1). Furthermore, internal sequence comparisons carried out on the *E. coli* enzyme indicated that the intermediary region corresponding to the regulatory domain is composed of two homologous subdomains (316–366 and 397–447) sharing 47% of amino acid identities (9) (Fig. 1).

In plants, two genes encoding bifunctional AK-HSDH were also found (12–15). The corresponding isoforms exhibit high homologies with the bacterial threonine-sensitive AK-HSDH. In *Arabidopsis thaliana*, the genes encoding the two AK-HSDH are located on chromosome 1 (13) (GenBankTM accession number trEMBL Q9SA18) and on chromosome 4 (GenBankTM accession number trEMBL O81852). As a first step toward a structural characterization of the bifunctional threonine-sensitive AK-HSDH in plants, we reported previously (16) on the purification to homogeneity of one isoform from *A. thaliana* (trEMBL O81852). In the present paper the organization of the regulatory domain of *A. thaliana* AK-HSDH is examined. Comparisons of predicted secondary structures of the biosynthetic threonine deaminase (TD) and AK-HSDH as well as the knowledge of TD three-dimensional structure (17, 18) allowed us to point to two amino acids in the AK-HSDH regulatory domain sequence potentially involved in the two threonine-binding sites of the enzyme. These amino acids were mutated, and the kinetic behavior of the mutants was determined and compared with that of the wild-type enzyme. Results indicate that the position of the amino acid in the threonine-binding sites was predicted correctly. Moreover, steady-state kinetics allow us to propose a structural model for the mechanism of control of AK and HSDH activities by threonine.

EXPERIMENTAL PROCEDURES

Materials—New England Biolabs supplied restriction endonucleases. Isopropyl β -D-thiogalactoside was supplied by Roche Molecular Biochemicals and amino acids by Sigma. Oligonucleotides used for PCR amplifications and site-directed mutageneses were obtained from Genome Express (Meylan, France). Chicken liver malic enzyme (EC 1.1.1.40) was purchased from Sigma.

Site-directed Mutageneses—Site-directed mutageneses were carried out on the previously constructed pET23/AK-HSDH vector (16) using the QuickChangeTM site-directed mutagenesis kit (Stratagene). Oligonucleotides were designed to replace Gln⁴⁴³ and Gln⁵²⁴ of the *A. thaliana* AK-HSDH by alanine and to modify the restriction enzyme digestion profile for identification of mutants. Sequencing of the mutants was performed (Genome Express) and showed no mutations other than those desired.

Overproduction and Purification of AK-HSDH—Overproduction and purification of the *A. thaliana* mutants were carried out as published previously for the wild-type AK-HSDH enzyme (16).

In Vitro Assays of AK—AK activity was assayed in the forward direction by the hydroxamate method (19). Enzyme activity was expressed as micromoles of aspartyl-P produced per min⁻¹ per mg⁻¹ of protein using an $\epsilon_{505\text{ nm}}$ of 750 M⁻¹cm⁻¹. The assay mixture contained 100 mM Tris-HCl, pH 8.0, 400 mM hydroxylamine-KOH, pH 8.0, 0–50 mM aspartate-KOH, pH 8.0, 0–40 mM ATP-KOH, pH 8.0, 20 mM MgCl₂,

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¹ The abbreviations used are: AK, aspartate kinase; TD, threonine deaminase; HSDH, homoserine dehydrogenase.

and 150 mM KCl. The reaction was initiated by the addition of AK-HSDH enzyme (final volume, 1 ml) and was carried out at 37 °C for 10 min. The reaction was terminated by the addition of a 0.5-ml solution of 0.37 M FeCl₃, 20% trichloroacetic acid, and 0.72 M HCl. The mixture was centrifuged for 15 min at 10,000 × g, and the absorbance of the supernatant was measured at 505 nm.

In Vitro Assays of the Reverse Reaction of HSDH—Aspartate semialdehyde is not available as a commercial product. Therefore, HSDH activity was first assayed in the nonphysiological direction (aspartate semialdehyde synthesis from homoserine and NADP⁺). Enzyme activity was expressed as micromoles of NADPH produced per min⁻¹ per mg⁻¹ of protein using an $\epsilon_{340\text{ nm}}$ of 6250 M⁻¹cm⁻¹. The mixture contained 100 mM Tris-HCl, pH 8.0, 150 mM KCl, 0–1 mM NADP⁺, and 0–50 mM homoserine. The reaction was initiated by the addition of AK-HSDH enzyme (final volume, 1 ml) and was carried out at 37 °C. Enzyme activity was visualized by monitoring absorbance changes at 340 nm.

Production of Aspartate Semialdehyde—HSDH activity was also measured in the forward direction. In this case, aspartate semialdehyde was produced from aspartate and ATP using monofunctional aspartate kinase and aspartate semialdehyde dehydrogenase (EC 1.2.1.11). Aspartate semialdehyde was produced extemporaneously in a medium containing 50 mM Tris-HCl, pH 8.0, 100 mM aspartate-KOH, pH 8.0, 50 mM ATP-KOH, pH 8.0, 50 mM MgCl₂, 50 mM KCl, 4 mM NADPH, and 100 mM L-malate-KOH, pH 8.0. Production of aspartate semialdehyde was initiated by the addition of 30 µg of pure *A. thaliana* monofunctional AK (overproduced and purified in our laboratory)² and 70 µg of pure *A. thaliana* aspartate semialdehyde dehydrogenase (20). Regeneration of NADPH was driven by the addition of 25 µg of commercial chicken liver malic enzyme (EC 1.1.1.40). The reaction was allowed to proceed for 2 h at 37 °C in a volume of 250 µl. Protein was then eliminated by centrifugation on Nanosep 10K (Pall Filtron). Aspartate semialdehyde concentration in the eluate was then determined enzymatically with an excess of NADPH and AK-HSDH.

In Vitro Assays of HSDH in the Forward Direction—Enzyme activity was expressed as micromoles of NADPH transformed per min⁻¹ per mg⁻¹ of protein using an $\epsilon_{340\text{ nm}}$ of 6250 M⁻¹cm⁻¹. The mixture contained 100 mM Tris-HCl, pH 8.0, 150 mM KCl, 0–1 mM NADPH, and 0–5 mM aspartate semialdehyde. The reaction was initiated by the addition of AK-HSDH enzyme (final volume, 250 µl) and was carried out at 37 °C. Enzyme activity was visualized by monitoring absorbance changes at 340 nm.

Gel Filtration Experiments—Molecular mass determinations were carried out on a HiLoad 16/60 Superdex S 200 (Amersham Biosciences) column equilibrated in 50 mM Hepes-KOH, pH 7.5, 150 mM KCl, and 10% glycerol (v/v) with (5 mM) or without threonine.

Electrophoresis and Protein Determination—SDS-PAGE were performed according to Chua (21). Protein concentration was measured either by the method of Bradford (22) (for crude extracts only) with bovine γ -globulin as standard or by measuring A_{205} as described by Scopes (23).

Kinetic Data Analyses—Kinetic data were fitted with the appropriate theoretical equations by using the KaleidaGraph program (Abelbeck software).

Secondary Structure Analyses—Secondary structure analyses were carried out with the program SOPM available on the ExPASy web site (24). A search of homologies was carried out with the Pfam protein data base (25).

RESULTS

Regulatory Domain of AK-HSDH—It was previously shown by internal sequence comparison that the *E. coli* threonine-sensitive AK-HSDH regulatory domain is composed of two homologous subdomains (9). This feature is also found for *A. thaliana* threonine-sensitive AK-HSDH (Fig. 1). Indeed, subdomain 1 (residues 414–453) and subdomain 2 (residues 495–534) of the plant AK-HSDH exhibit 33% of identity at the amino acid level (Fig. 1). To characterize more deeply the regulatory domain of the plant enzyme, and in particular the sites where threonine potentially binds, we analyzed the predicted secondary structure of the subdomains. As shown in Fig. 1, each subdomain of the regulatory domain of AK-HSDH is predicted to exhibit a common loop- α helix-loop- β strand-

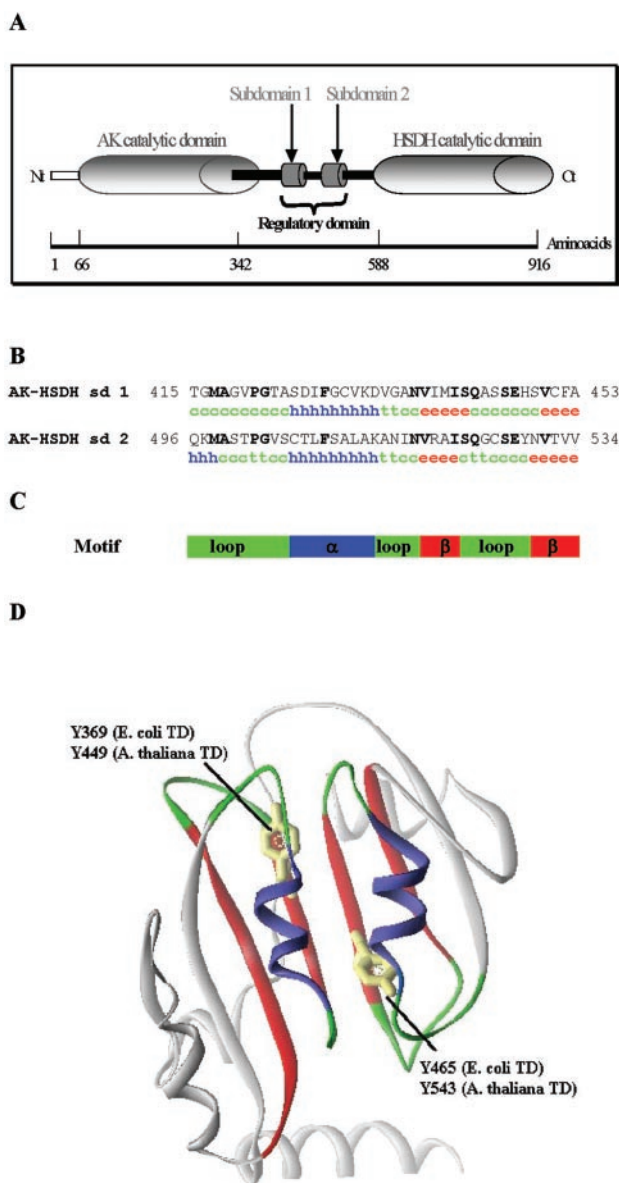


FIG. 1. Regulatory domain of AK-HSDH. A, functional domains of AK-HSDH. The drawing corresponds to the sequence of the plant enzyme. B, comparisons of the primary and secondary structures of the two subdomains of the regulatory domain of *A. thaliana* AK-HSDH. *Sd 1*, subdomain 1; *sd 2*, subdomain 2. Conserved amino acids are indicated in bold. α helices (h), β strands (e), and loops (c) or turns (t) are colored. C, motif corresponding to the secondary structure of each subdomain. D, structure of the regulatory domain of the TD enzyme from *E. coli* (1TDJ, Refs. 17 and 18). The regulatory domain is made of two homologous subdomains. The regulatory domain of the plant enzyme has a similar fold. Amino acid residues (Tyr³⁶⁹ and Tyr⁴⁶⁵ for *E. coli*, Tyr⁴⁴⁹ and Tyr⁵⁴³ for *A. thaliana*) involved in the two nonequivalent binding sites are indicated. Secondary structures found in the motif of each subdomain are colored. Parts of the regulatory domain not involved in the loop- α -loop- β -loop- β motif are represented in gray.

loop- β strand motif (loop- α -loop- β -loop- β motif).

Comparisons with TD—The primary sequence of the regulatory domain of TD does not exhibit homology with that of the regulatory domain of AK-HSDH. However, the structure of the *E. coli* TD crystallized in the absence of effector (17) clearly shows that the regulatory domain of TD is also composed by a pair of loop- α -loop- β -loop- β motifs as predicted in the AK-HSDH regulatory domain (Fig. 1). Thus, although the primary sequences of TD and AK-HSDH are different, the regulatory domains of these enzymes probably have more similar folds (Fig. 2).

² G. Curien, unpublished result.



FIG. 2. Primary and secondary structures of the subdomains of the regulatory domain of AK-HSDH and TD. A, comparison of primary and secondary structures between the two subdomains of the regulatory domain of *A. thaliana* TD. Amino acid residues (Tyr⁴⁴⁹ and Tyr⁵⁴³) involved in the two effector-binding sites of TD are underlined. Sd 1, subdomain 1; sd 2, subdomain 2. B, comparison of primary and secondary structures between the two subdomains of the regulatory domain of *A. thaliana* AK-HSDH. Amino acid residues investigated by site-directed mutagenesis are underlined. C, comparison of secondary structures of the two subdomains of the regulatory domain of AK-HSDH and TD. Conserved amino acids are indicated in bold. α helices (h), β strands (e), and loops (coil (c) or turn (t)) are indicated.

Effector-binding Sites of TD—In our study of *A. thaliana* TD we showed that each subdomain of the regulatory domain of TD possesses an effector-binding site (18). Indeed, the amino acid residue located at the C-terminal extremity of the first β strand (underlined in the motif) of each loop- α -loop- β -loop- β motif belongs to an effector-binding site (Figs. 1 and 2) (18). These amino acid residues are Tyr⁴⁴⁹ and Tyr⁵⁴³ in *A. thaliana* TD (Figs. 1 and 2) (18).

Prediction of the Amino Acid Residues Involved in AK-HSDH Threonine-binding Sites—Because of the structural similarity between TD and AK-HSDH regulatory domains, the locations of the putative amino acids involved in the threonine-binding sites in AK-HSDH regulatory domain can thus be predicted. The putative amino acids selected for mutagenesis studies were chosen as follows. First, the amino acids selected must be conserved between subdomain 1 and subdomain 2 of the regulatory domain of AK-HSDH. Second, these amino acids must be located at the end of the first β strand of the loop- α -loop- β -loop- β motif found in each subdomain. We selected at this step three amino acids in each subdomain. These amino acids were Ile⁴⁴¹, Ser⁴⁴², and Gln⁴⁴³ for subdomain 1 and Ile⁵²², Ser⁵²³, and Gln⁵²⁴ for subdomain 2. Third, the amino acids selected must be conserved between plant and bacterial threonine-sensitive AK-HSDH sequences. From this last selection, only Ile⁴⁴¹ and Gln⁴⁴³ for subdomain 1 and Ile⁵²² and Gln⁵²⁴ for subdomain 2 are conserved between various plants and bacterial threonine-sensitive AK-HSDH. Therefore, these amino acids were selected for mutagenesis and replacement by alanine residues. Mutation of Ile or Gln of each subdomain leads to large modifications of the threonine sensitivity of the mutant enzymes. However, the strongest effects were observed for Gln⁴⁴³ and Gln⁵²⁴ mutants. As a consequence, we choose to report under “Results” only data concerning the mutagenesis of Gln⁴⁴³ and Gln⁵²⁴.

Construction, Overproduction, and Purification of AK-HSDH Mutants—Site-directed mutageneses were carried out on vector pET23/AK-HSDH (16) and two mutants were constructed (pET23/AK-HSDH(Q443A) and pET23/AK-HSDH(Q524A)). Overproduction and purification to homogeneity were carried out for each mutant as described for the wild-type *A. thaliana*

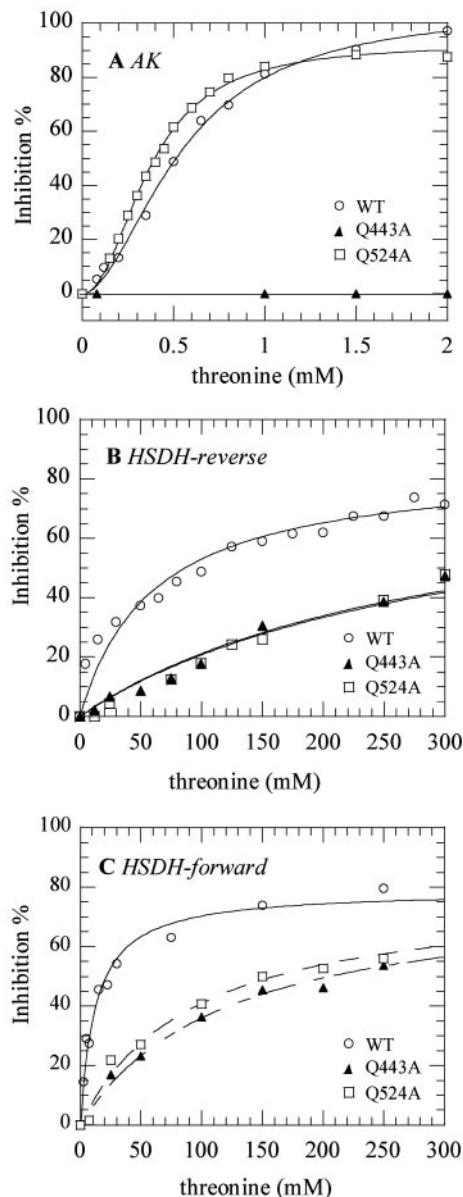


FIG. 3. Effect of threonine on AK and HSDH activities of the wild-type and mutant AK-HSDH enzymes. A, effect on AK activity. The data corresponding to the wild-type enzyme and to mutant Q524A were fitted with a Hill equation (wild type (WT) $K_{0.5} = 0.50 \pm 0.01$ mM, $n_H = 1.95 \pm 0.08$; Q524A, $K_{0.5} = 0.37 \pm 0.01$ mM, $n_H = 2.14 \pm 0.07$). Mutant Q443A is completely insensitive to threonine inhibition (up to 100 mM threonine). B, effect on the reverse reaction of HSDH. The data were fitted with a hyperbolic equation (wild type, $K_{0.5} = 60 \pm 10$ mM; Q443A, $K_{0.5} = 347 \pm 16$ mM; Q524A, $K_{0.5} = 335 \pm 16$ mM). C, effect on the forward reaction of HSDH. The data were fitted with a hyperbolic equation (wild type, $K_{0.5} = 12 \pm 1$ mM; Q443A, $K_{0.5} = 119 \pm 6$ mM; Q524A, $K_{0.5} = 92 \pm 6$ mM).

enzyme (16). Solubility and yield of the two purified mutants were similar to those obtained for the wild-type AK-HSDH (16) (not shown). For each mutant, a $K_{0.5}$ value for threonine was determined for the inhibition of AK and HSDH activities. Kinetic parameters for AK activity and both directions of HSDH reversible reaction were also determined.

Inhibition of AK Activity by Threonine—In the wild-type enzyme the inhibition of AK activity by threonine originates from an increase in the apparent K_m values for ATP and aspartate (16). At saturation of aspartate and ATP, threonine inhibits AK activity (Fig. 3A) in a cooperative manner ($K_{0.5} = 500$ μ M; n_H of 1.95). Inhibition is virtually 100% at saturation

TABLE I
Kinetic constants for HSDH activities of the wild-type and mutant AK-HSDH enzymes

Enzyme	Reverse reaction		Forward reaction ^b	
	V_m^a	Homoserine (K_m)/NADP ⁺ (K_m)	V_m^c	Aspartate semialdehyde (K_m)
Wild-type	18.8	5.2 ± 0.4 mM/166 ± 9 μM	165	1.4 ± 0.1 mM
Subdomain 1, Q443A	19.8	3.5 ± 0.3 mM/135 ± 9 μM	190	1.5 ± 0.2 mM
Subdomain 2, Q524A	16.6	5.1 ± 0.1 mM/135 ± 6 μM	196	0.8 ± 0.1 mM

^a Reverse reaction: μmol of aspartate semialdehyde produced · min⁻¹ · mg⁻¹ of protein.

^b The mixture containing aspartate semialdehyde also contained NADPH. This prevented the determination of a K_m value of NADPH for the forward reaction of HSDH.

^c Forward reaction: μmol of aspartate semialdehyde transformed · min⁻¹ · mg⁻¹ of protein.

of threonine. Fig. 3A shows that the mutation of the residue in subdomain 1 (Q443A) or in subdomain 2 (Q524A) leads to different effects on the inhibition pattern. AK activity of the mutant Q443A becomes completely insensitive to threonine inhibition (Fig. 3A). By contrast, mutation of residue Gln⁵²⁴ does not modify the $K_{0.5}$ value for inhibition of the activity of AK by threonine ($K_{0.5} = 368$ μM) (Fig. 3A).

Inhibition of HSDH Activity by Threonine in the Reverse Direction—We demonstrated previously for the wild-type enzyme (16) that the inhibition of HSDH activity by threonine in the reverse direction can be fitted by a hyperbolic equation (see Fig. 3B). The concentration of threonine required to inhibit 50% of wild-type HSDH activity in the reverse direction is high ($K_{0.5} = 60$ mM) compared with that required for 50% inhibition of wild-type AK activity ($K_{0.5} = 500$ μM). As reported previously, the inhibition of wild-type HSDH activity in the reverse direction results from an increase in the apparent K_m values for NADP⁺ and homoserine (16). At saturation of threonine a maximum inhibition of 85% of the reverse reaction of HSDH activity is reached. Fig. 3B shows that mutation of the residue in subdomain 1 (Q443A) or in subdomain 2 (Q524A) leads to similar effects on the inhibition of HSDH activity in the reverse direction by threonine. Indeed, the $K_{0.5}$ value for inhibition of the HSDH reverse reaction by threonine was increased by a factor of approximately 6 for both mutants Q443A ($K_{0.5} = 347$ mM) and Q524A ($K_{0.5} = 335$ mM) (Fig. 3B). This contrasts with the results obtained when AK activity of these mutants was measured (only mutation Q443A led to modification of the inhibition pattern). Although the $K_{0.5}$ value for inhibition by threonine of the reverse reaction of HSDH does not correspond to a physiological value, mutations of Gln⁴⁴³ and Gln⁵²⁴ led in both cases to an alteration of the apparent affinity for threonine. These results clearly demonstrate the existence of two nonequivalent threonine-binding sites per monomer.

Inhibition of HSDH Activity by Threonine in the Forward Direction—To characterize the inhibition of HSDH activity in the forward direction, aspartate semialdehyde was produced using purified monofunctional aspartate kinase and aspartate semialdehyde dehydrogenase (described under “Experimental Procedures”). Fig. 3C shows that the inhibition of the forward reaction of wild-type HSDH activity by threonine can be fitted by a hyperbolic equation. A $K_{0.5}$ value of 12 mM was calculated. This value is 5-fold lower than that obtained for the reverse reaction (Fig. 3C). As observed for HSDH activity in the reverse direction, threonine does not lead to a complete inhibition (80% of inhibition) of the forward reaction of the wild-type HSDH. Mutation of residues from subdomain 1 (Q443A) or subdomain 2 (Q524A) leads to a similar decrease in the apparent affinity for threonine. Indeed, the $K_{0.5}$ value for inhibition of HSDH activity in the forward direction was increased by a factor of approximately 10 for both mutants. A value of 119 mM was indeed calculated for the Q443A mutant and 92 mM for the Q524A mutant (Fig. 3C). The same conclusions as those given above for the reverse direction can thus be drawn from the more delicate measurements of the reaction in the forward direction.

Kinetic Parameters for AK and HSDH Activities—The effects of the mutations on the kinetic parameters (V_{max} , K_m) were also determined for each mutant. The maximal velocity of AK and HSDH reactions was not modified for mutants Q443A and Q524A compared with that determined for the wild-type enzyme (see Tables I and II). The effects of the mutations on the K_m values of substrates and cofactors of both AK and HSDH were also determined. As shown in Table I, mutation of Gln⁴⁴³ or Gln⁵²⁴ does not modify the kinetic parameters of the forward and reverse reactions of HSDH. By contrast and as shown in Table II, the kinetic properties of AK for aspartate and ATP were found to be slightly modified for mutants Q443A and Q524A compared with the wild-type enzyme. Indeed, the K_m value for aspartate was increased by factors of 2 and 5 for mutants Q443A and Q524A, respectively. The K_m value for ATP was decreased by a factor of approximately 5 for mutants Q443A and Q524A.

Gel Filtration Experiments—We observed previously that elution of the wild-type AK-HSDH on Superdex S 200 (HiLoad 1.6 × 60) is modified on addition of threonine (16). Indeed, when gel filtrations were carried out without threonine, wild-type AK-HSDH was eluted with an apparent molecular mass of 470 kDa (16) corresponding to an oligomer with a size larger than that of a tetramer (Fig. 4). However, in the presence of 5 mM threonine, the wild-type enzyme behaved as a tetramer with an apparent molecular mass of 320 kDa (16). To determine whether threonine had an effect on the quaternary structure of the AK-HSDH mutants, gel filtration experiments were carried out with the mutant enzymes in the presence or the absence of the effector. By contrast with the wild-type enzyme, elution of mutants Q443A and Q524A is not modified by the addition of threonine (5 mM) (Fig. 4). Indeed, the mutant enzymes were eluted with an apparent molecular mass of 470 kDa with or without threonine (Fig. 4).

DISCUSSION

Identification of Two Nonequivalent Threonine-binding Sites—Previous binding experiments have demonstrated that each monomer of AK-HSDH is able to bind two threonine molecules (5, 6). However, the location of the two binding sites was unknown. Our work allowed the identification of two amino acid residues (Gln⁴⁴³ and Gln⁵²⁴) belonging to two threonine-binding sites on each monomeric unit of threonine-sensitive AK-HSDH. Kinetic measurements of threonine inhibition on mutants Q443A and Q524A demonstrated clearly that these two sites are nonequivalent. Indeed, mutation of Gln⁴⁴³ changes the inhibition by threonine for both AK and HSDH activities, whereas mutation of Gln⁵²⁴ only alters the inhibition by threonine for HSDH activities.

Proposed Model for the Mechanism of Inhibition by Threonine—Kinetic experiments showed that mutation of Gln⁵²⁴ in subdomain 2 leads to an increase in the $K_{0.5}$ threonine value for inhibition of HSDH activities (forward and reverse directions). This result suggests therefore that binding of threonine on subdomain 2 is involved in the inhibition of HSDH activity

TABLE II
Kinetic constants for AK activity of the wild-type and mutant AK-HSDH enzymes

Enzyme	V_m^a	Aspartate (K_m)	ATP (K_m)
Wild-type	5.4	11.6 ± 0.6 mM	5.5 ± 0.4 mM
Subdomain 1, Q443A	5.1	24.5 ± 1.1 mM	1.2 ± 0.1 mM
Subdomain 2, Q524A	4.7	50.5 ± 1.5 mM	1.0 ± 0.1 mM

^a μmol of aspartyl phosphate produced $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein.

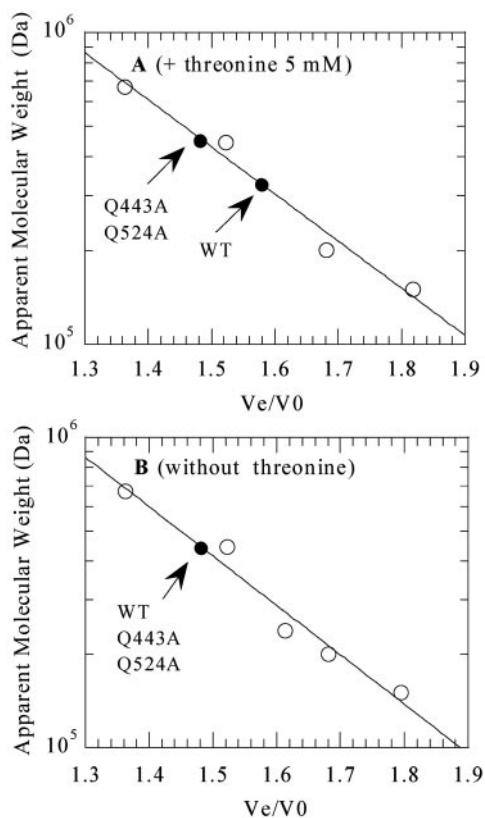


FIG. 4. Native molecular mass determination of wild-type and mutant AK-HSDH by chromatography on HiLoad 16/60 Superdex S 200 with 5 mM threonine (A) or without effector (B). A 0.5-ml sample of wild-type or mutant AK-HSDH (0.5 mg) was applied to the S200 column and eluted at 1 ml/min in a buffer containing 50 mM Hepes-KOH, 150 mM KCl, 10% glycerol and without or with 5 mM threonine. Standards used (2 mg) were thyroglobulin (669 kDa), apoferritin (443 kDa), threonine deaminase (used only for the calibration without threonine; 238 kDa), β -amylase (200 kDa), and alcohol dehydrogenase (150 kDa).

(Fig. 5). Furthermore, because the mutation of subdomain 2 does not modify the $K_{0.5}$ threonine value for inhibition of AK activity, this result also indicates that binding of threonine on subdomain 2 controls exclusively HSDH activity. Analysis of kinetic experiments carried out on mutant-modified subdomain 1 is more complex. Indeed, modification of subdomain 1 (Q443A) completely suppressed the ability of threonine to inhibit AK (Fig. 3). This result suggests that binding of threonine on subdomain 1 is responsible for inhibition of AK activity (Fig. 5). Mutagenesis of subdomain 1 also induces a modification of the $K_{0.5}$ threonine for inhibition of HSDH activities. Because HSDH inhibition was shown to be dependent on threonine binding on subdomain 2, an interpretation of this result is that in addition to preventing threonine binding on subdomain 1, mutation of subdomain 1 also hinders the binding of threonine on subdomain 2. Thus, a coupling between subdomain 1 and subdomain 2 exists, but the communication is not reciprocal. From the observations of the effect of threonine on the kinetics of wild-type and mutant enzymes, one can propose that Gln⁴⁴³

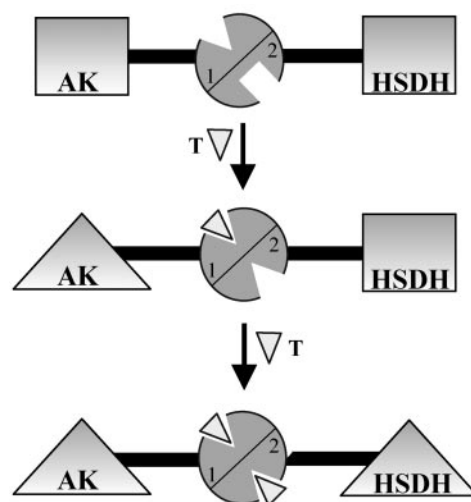


FIG. 5. Proposed model for the inhibition of AK-HSDH by threonine. Active catalytic domains of AK and HSDH are represented with squares, whereas inhibited catalytic domains are represented with triangles. Threonine binding on subdomain 1 would induce in parallel 1) conformational modifications of subdomain 2 and 2) conformational modifications of AK catalytic domain leading to AK inhibition. Conformational modification of subdomain 2 would induce the binding of a second threonine leading to conformational modifications of HSDH catalytic domain and HSDH inhibition.

is a high affinity binding site for threonine, whereas Gln⁵²⁴ is a low affinity binding site for the effector. Our results suggest therefore that interaction of threonine with the high affinity binding site Gln⁴⁴³ would lead to a loss of AK activity (Fig. 5). At the same time, threonine interaction with this high-affinity site would also induce a modification of the conformation of subdomain 2, allowing the second binding site with lower affinity (Gln⁵²⁴) to interact with a second threonine (Fig. 5). The binding of this second threonine would lead to the inhibition of HSDH activity (Fig. 5). Final validation of this model will be obtained with the determination of crystallographic structure of the wild-type and mutant enzymes with and without threonine.

Comparison with the Mechanism of Control of Threonine Deaminase by Isoleucine—In a previous study that combined kinetic and binding experiments we showed that subdomain 1 of TD (containing Tyr⁴⁴⁹) is a high affinity binding site for isoleucine, whereas subdomain 2 (containing Tyr⁵⁴³) is a low-affinity binding site for isoleucine (18). It was also demonstrated that interaction of subdomain 1 of TD with a first isoleucine induces 1) a modification of conformation of the catalytic domain of TD leading to a slight activation of enzyme activity, and 2) a conformational modification of subdomain 2 leading to an enhancement of the affinity of subdomain 2 for isoleucine. Finally, isoleucine interaction with subdomain 2 induces conformational modifications of the catalytic domain leading to final inhibition of the enzyme (18).

Our present results on AK-HSDH mutants suggest therefore that the mechanism of control of AK-HSDH by threonine is similar to the mechanism of control of TD by isoleucine as described above. Indeed, for AK-HSDH and TD, mutation of subdomain 2 leads only to a decrease in the affinity of subdomain 2 for the effector, whereas mutation of subdomain 1 leads to a decrease in the affinity of both subdomains for the effector. Thus, one can propose that in the wild-type AK-HSDH and TD, interaction of a first effector on subdomain 1 induces conformational modification leading to or facilitating the binding of a second effector on subdomain 2 (Fig. 5).

Gel Filtration Analysis—In parallel to the loss of sensitivity

toward threonine inhibition, the apparent molecular mass of mutants Q443A and Q524A also becomes insensitive to threonine addition (at the concentration of threonine used in the experiment). This result shows that binding of threonine on the wild-type AK-HSDH induces conformational modifications that cannot occur in the mutant enzymes. Because the Q524A mutant still binds threonine on subdomain 1 (Gln⁴⁴³), one can propose that binding of threonine in subdomain 2 is responsible for the shift in the apparent molecular weight observed when wild-type enzyme is gel-filtered in the presence of threonine. Further work is required to characterize the effect of threonine binding on the quaternary structure of the enzyme.

Amino Acids Involved in the Effector-binding Sites—As described above, the amino acid residues located between the C-terminal extremity of the first β strand and the last loop of the loop- α -loop- β -loop- β motif of each subdomain of AK-HSDH belong to an effector-binding site. Additional mutations (I441A and I522A) carried out in the end of the first β strand of each subdomain of AK-HSDH disclose that Ile⁴⁴¹ and Ile⁵²² also belong to the two effector-binding sites (results not shown).

As observed for mutant Q443A, mutation of Ile⁴⁴² (and not Ile⁵²²) by alanine leads to a loss of threonine sensitivity for the AK activity (results not shown). In agreement with the behavior of the mutants Q443A and Q524A, mutation of Ile⁴⁴² and Ile⁵²² by alanine leads to a decrease of threonine sensitivity for inhibition of HSDH activities (results not shown). Additional mutations and crystallographic determination of the structure in the presence of the effector will be required to define accurately all the amino acid residues conferring the specificity of the effector-binding site of each subdomain.

General Implication and Prediction—A main issue of the characterization and comparison of the different mechanisms of control of allosteric enzymes is to determine whether general mechanisms can be uncovered and therefore used to predict function from the analysis of the amino acid sequences. To determine whether the loop- α -loop- β -loop- β motif found in AK-HSDH and TD is present in other allosteric proteins, homology search was carried out using the Pfam protein families data base (25).

The search showed that this motif is annotated as an “ACT domain.” Interestingly and as described recently (26), this motif can be found in a great many allosteric proteins involved in amino acid or purine biosynthesis. As shown in the Pfam pro-

tein families data base (25) and as reported recently (26), the ACT domain can be found in one copy or in duplicate (in the case of AK-HSDH or TD). As demonstrated by the work carried out on TD (18) and AK-HSDH (this article) the existence of a pair of ACT domains would lead to the creation of two non-equivalent binding sites allowing complex regulatory patterns of various protein activities.

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