

The Central Enzymes of the Aspartate Family of Amino Acid Biosynthesis

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ABSTRACT

The aspartate pathway is responsible for the biosynthesis of lysine, threonine, isoleucine, and methionine in most plants and microorganisms. The absence of this pathway in humans and animals makes the central enzymes potential targets for inhibition, with the aim of developing new herbicides and biocides, and also for enhancement, to improve the nutritional value of crops. Our current state of knowledge of these enzymes is reviewed, including recently determined structural information and newly constructed bifunctional fusion enzymes.

Introduction

The aspartate pathway (Figure 1) uses L-aspartic acid as the precursor for the biosynthesis of the amino acids lysine, methionine, isoleucine, and threonine.¹ This is an essential pathway in plants and microorganisms involving, as it does, one-fourth of the building block amino acids that are required for protein synthesis. In addition, there are several important metabolic intermediates and products from this pathway including diaminopimelic acid, a key component required for cross-linking in bacterial cell wall biosynthesis, and dipicolinic acid, important for sporulation in Gram-positive bacteria. During evolution members of the animal kingdom have lost the enzymes that catalyze the reactions in this pathway, and therefore the amino acids that are derived from aspartic acid are essential dietary components. As a consequence of this evolutionary specialization, this pathway has become the focus for two divergent goals. Enhancement of the flux through this pathway, either by increasing the expression of these central enzymes or by altering their regulation, improves the nutritional value of important crop plants by increasing the levels of these essential amino acids, in particular the amino acid lysine. Conversely, the design of specific inhibitors of the central enzymes of this pathway will provide lead compounds for the development of new, safe herbicides, and also new biocides to

combat the growing threat from antibiotic-resistant microorganisms. My laboratory has focused on improving our knowledge of the structures and mechanisms of the central enzymes in this pathway, knowledge that will provide the basis for the achievement of each of these goals.

Overview

The organisms that utilize this pathway contain several isofunctional aspartokinases that catalyze the initial commitment step in the pathway, the phosphorylation of L-aspartic acid. In many of these organisms at least one of these enzymes is bifunctional, catalyzing both the first and, surprisingly, the third reaction in this metabolic sequence. Many bacteria contain several isoforms of aspartokinases, some of which are bifunctional.² These isofunctional enzymes are subject to differential regulation, both by feedback inhibition and by repression at the genetic level, from the end product amino acids. In *Escherichia coli* there are three aspartokinases. Isoform I is bifunctional and is both inhibited and repressed by threonine. Isoform II is also bifunctional and is repressed by methionine, while isoform III is a monofunctional enzyme inhibited by lysine.³ In *Bacillus* there are two aspartokinases, one that is regulated by diaminopimelate levels, and one that is inhibited by both threonine and lysine.⁴ As is typical in highly branched metabolic pathways, there is additional regulation, beyond this first step, at each of the branch points (Figure 2). This regulatory scheme allows exquisite control over not only the total flux through this pathway but also the relative rates of production of each of these amino acids. All of the central enzymes in the aspartate family have been purified, and their catalytic properties have been elucidated to some extent. These studies have included an examination of substrate specificity and also the identification of some alternative substrates and inhibitors. Substrate binding groups and active site catalytic groups have been identified by classical modification studies and by site-directed mutagenesis. High-resolution structural studies have provided a detailed picture of the active site for two of these enzymes, and these structures support reasonable hypotheses for catalytic mechanisms. Similar structural studies are proceeding for the other central enzymes. Finally, separation and characterization of the individual catalytic domains of the bifunctional enzyme, and gene fusion to create new bifunctional enzymes, has allowed an examination of the mechanisms of regulation of the central enzymes of the aspartate pathway.

The Aspartokinases

In *E. coli*, the bifunctional enzymes aspartokinase-homoserine dehydrogenase I and II (AK-HDH I and

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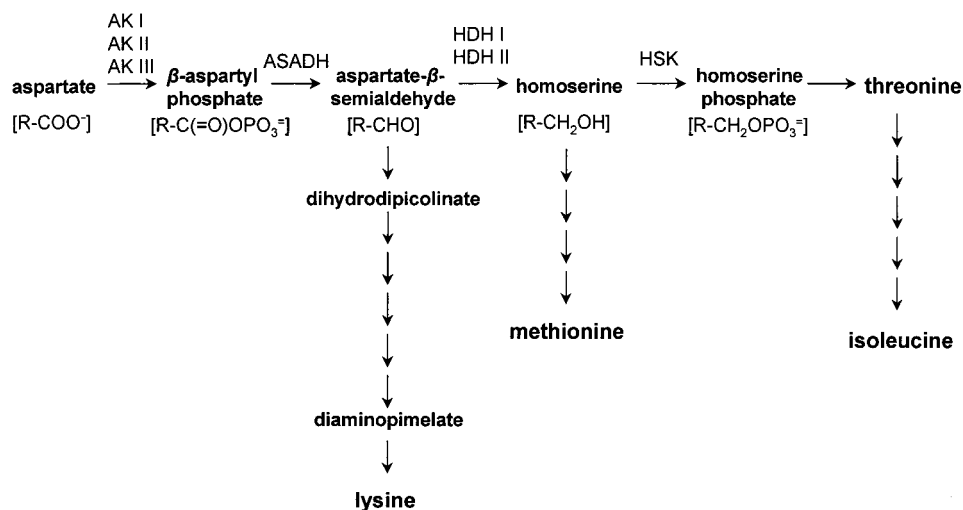


FIGURE 1. Aspartate pathway of amino acid biosynthesis. Each arrow represents an enzyme-catalyzed conversion, and the central enzymes that catalyze the core reactions of the pathway are listed. AK, aspartokinase isozymes; ASADH, aspartate- β -semialdehyde dehydrogenase; HDH, homoserine dehydrogenase isozymes; HSK, homoserine kinase.

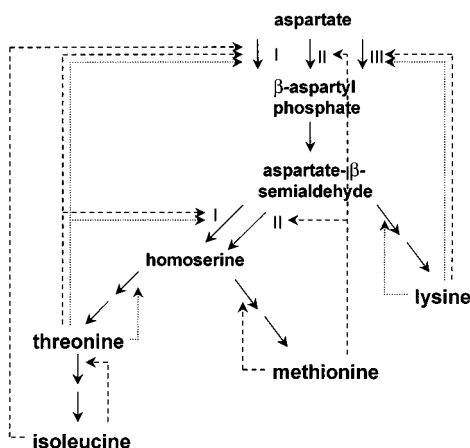


FIGURE 2. Regulation of the aspartate pathway in *Escherichia coli*. The flux through the branches of this pathway is regulated at two levels, by feedback inhibition from the end product amino acids (····) and by repression of the synthesis of key regulatory enzymes (- - -).

AK-HDH II) catalyze a phosphorylation and then, after an intervening reduction catalyzed by a separate enzyme, a second reduction to produce the intermediate homoserine. A third monofunctional enzyme, aspartokinase III, also catalyzes the phosphorylation of aspartic acid that is the commitment step in this biosynthetic pathway. AK-HDH I (EC 2.7.2.4) in this bacterium is a tetramer composed of identical subunits of molecular weight 86 000, with a known amino acid sequence.^{5,6} Each polypeptide chain contains both catalytic activities; however, these activities reside on separate structural domains. The amino-terminal moiety carries the kinase activity and the carboxy-terminal region contains the dehydrogenase activity, with the central interface domain reported to be the site of allosteric regulation.⁷ An improved purification scheme, utilizing ternary elution from a dye-ligand column,⁸ has led to the production of highly purified AK-HDH I with excellent overall yield. A similar improved

Table 1. Alternative Substrates for the Aspartokinase Isoenzymes^a

substrate	aspartokinase I		aspartokinase III	
	k_{cat} (%)	K_{m} (mM)	k_{cat} (%)	K_{m} (mM)
L-aspartic acid	100	1	100	0.6
L-aspartate α -amide	15	50	53	184
L-aspartate α -benzyl ester	17	4	84	5.3
<i>N</i> -formyl-L-aspartate			57	41
<i>N</i> -acetyl-L-aspartate			30	68
L-asparagine ^b	15	4	45	16
L-aspartate β -benzyl ester ^b	11	3	86	2.5
L-aspartate β -methyl ester ^b	65	20	88	4.9

^a Reaction conditions: activity was determined with a coupled pyruvate kinase/lactate dehydrogenase assay containing 100 mM Hepes (pH 8.0), 100 mM KCl, 2 mM MgATP, 0.7 mM PEP, 0.1 mM NADH, varying concentrations of the alternative substrates, and 8–15 μg of AK. ^b Phosphorylation at the α -carboxyl group.

purification, with hydrophobic chromatography as the final step, led to the production of pure AK III.⁹

Substrate Specificity. Aspartokinase I has a somewhat relaxed specificity toward its amino acid substrate (Table 1). While L-aspartic acid is the amino acid substrate of choice, a free α -carboxyl group is not essential for substrate recognition. The α -amide and several different α -esters of aspartate were found to be competent alternative substrates.¹⁰ However, aspartate analogues in which the α -amino group is either derivatized or replaced are neither substrates nor inhibitors for AK I, indicating the importance of the α -amino group as a binding determinant. In contrast, *N*-derivatized aspartate analogues such as *N*-formyl and *N*-acetyl aspartate are substrates for AK III with only slightly reduced k_{cat} values (Table 1), but with significantly elevated K_{m} values.⁹ These specificity differences can be exploited to develop differential inhibition of these highly homologous enzymes and to dissect the substrate recognition elements at the active site. The requirements for a phosphoryl donor were also investigated using structural analogues of ATP. With the excep-

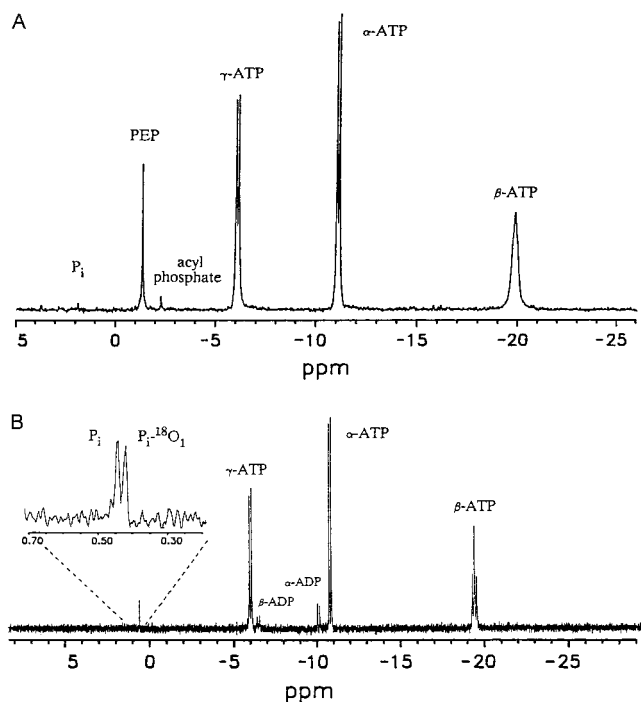


FIGURE 3. ^{31}P NMR spectrum for the reaction of L-aspartate- β -methyl ester with aspartokinase I. (A) Reaction run in ^{16}O water. The peak at -2.3 ppm is the acyl phosphate product of the enzyme-catalyzed reaction. (B) Reaction run in 50% ^{18}O water. This reaction was run until the PEP had been depleted. The expanded inset shows the unlabeled and the mono- ^{18}O -labeled phosphate peaks.

tion of 2'-deoxy ATP,¹¹ all of the triphosphates tested, including CTP, GTP, UTP, and several ATP derivatives, were found to be unreactive toward AK I.

Reversal of Regiospecificity. Aspartate analogues with derivatized β -carboxyl groups were examined as potential competitive inhibitors of the aspartokinases. Unexpectedly, these analogues, including compounds such as asparagine, aspartate- β -hydroxamate, and both the β -methyl and β -benzyl esters of aspartate, appear to be alternative substrates and not inhibitors.¹⁰ This is surprising because the β -carboxyl group of aspartate is the acceptor site for phosphorylation, and blocking this group by derivatization should prevent phosphoryl transfer. It is possible either that the enzyme could hydrolyze these derivatives before phosphorylation, or that binding of these substrate analogues to the enzyme allows water to act as the attacking nucleophile to hydrolyze ATP (an induced adenosine triphosphatase reaction). To determine whether phosphorylation of these β -derivatized aspartates had actually occurred, the putative acyl phosphate products that would be obtained from these alternative substrates were searched for and then characterized by coupled enzyme assays and also by NMR spectroscopy. These studies confirmed the production of a phosphorylated product from each of the β -derivatized aspartates, products that can be directly observed by ^{31}P NMR (Figure 3A). In addition, ^{18}O labeling studies have verified not only the production of these acyl phosphate products, but also their mechanism of nonenzymatic hydrolysis after the products are released from the enzyme. At neutral pH this hydrolysis involves attack of water on the phosphate of

the product with cleavage of the ester linkage. When the enzymatic production of these phosphorylated products is conducted in 50% ^{18}O water, a single labeled oxygen is found in half of the phosphate that is released upon hydrolysis (Figure 3B).¹⁰ At higher pH hydroxide becomes the attacking species and the mechanism switches to attack at the carbonyl carbon. This leads to incorporation of the labeled oxygen into the aspartic acid analogue and the release of unlabeled phosphate (data not shown).

Clearly, the only viable explanation for the products obtained from these enzyme-catalyzed reactions is that β -derivatized analogues of aspartate are capable of productive binding to aspartokinase through a reversal of regiospecificity to make the α -carboxyl group available as a phosphoryl acceptor. In support of this hypothesis is the observation that analogues in which both the α - and the β -carboxyl groups have been derivatized are no longer substrates for AK I. Many, but not all, of these α -acyl phosphates have also been shown, by coupled enzyme assays, to be viable substrates for the next two enzyme-catalyzed steps in this metabolic pathway.¹⁰ This scenario raises the possibility of producing enzyme-generated alternative substrates that can potentially serve as anti-metabolites for the downstream reactions in this biosynthetic pathway.

Identification of Functionally Important Amino Acids.

Earlier chemical modification studies^{12,13} of AK had indicated the potential involvement of sulfhydryl groups; however, those cysteines appeared to play more of a structural rather than a catalytic role. An examination of the pH profiles of the kinase activity of AK-HDH I shows a loss of activity near neutral pH upon protonation of a single cationic acid group.¹⁴ We turned to chemical modification studies with group-specific reagents to aid in the identification of this functional group. Inactivation of the enzyme with diethylpyrocarbonate, and reversal of this inactivation by hydroxylamine, supports the identification of this essential amino acid as a histidyl residue. This enzyme also loses activity at high pH as a consequence of the ionization of neutral acid groups with pK values near 10. Chemical modification studies with *N*-acetylimidazole (NAI) and with tetranitromethane (TNM) supported the assignment of these groups as tyrosyl residues, although the observed modification of multiple groups by this reagent also suggests a structural rather than a catalytic role for these functional groups in AK-HDH I.¹⁴

Inactivation studies with AK III, and subsequent substrate protection studies, also confirmed the presence of an essential reactive histidine in this aspartokinase. However, a careful analysis of the inactivation of AK III by the purported tyrosine-specific reagents NAI and TNM exposed the possibility of some cross-reactivity with enzymatic sulfhydryl groups.⁹ Protection studies in the presence of MgATP suggest that this cysteine may be located at or near the nucleotide binding site. The conclusion from these modification studies is a lesson that has been learned before. Group-specific reagents can provide support for the identification of an essential

amino acid functional group in an enzyme. However, even with many of the newer and more selective modification reagents, it is important to run controls to verify the nature of the modification and not to rely on chemical modification studies as the sole criteria for functional group identification.

Aspartate β -Semialdehyde Dehydrogenase

L-Aspartate- β -semialdehyde dehydrogenase (ASADH, EC 1.2.1.11), the second enzyme in the central pathway, is a dimer composed of identical subunits. The DNA sequence encodes for a subunit composed of 367 amino acids with an overall molecular weight of 39 950.¹⁵ In the biosynthetic direction ASADH catalyzes the formation of L-aspartate- β -semialdehyde (ASA) by the reductive dephosphorylation of L- β -aspartyl phosphate (BAP), utilizing the reducing power of NADPH. The ASADH reaction is related to the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase, and similar chemical mechanisms have been proposed for each enzyme.^{16–18} This mechanism involves the formation of a thioester intermediate resulting from attack of an active site cysteine thiolate on the carbonyl group of BAP, followed by expulsion of phosphate. Hydride transfer from NADPH and collapse of the resulting tetrahedral intermediate leads to release of the ASA product.

Identification of Functionally Important Amino Acids.

Chemical modification studies with 2,2'-dithiobis(5-nitrobenzoate) (DTNB)¹⁶ and *N*-ethylmaleimide (NEM)¹⁸ have indicated an essential role for a cysteine thiolate in the ASADH reaction. Substrate protection studies against NEM inactivation, conducted in the presence of ASA, have suggested that this essential cysteine is located in or near the enzyme active site. In addition, pH profile studies have indicated a role for a neutral acid group, with a p*K* value similar to that observed during NEM modification, that must be ionized for enzymatic activity.¹⁸ L-2-Amino-4-oxo-5-chloropentanoic acid, an alkylating substrate analogue of ASA, irreversibly inactivates ASADH. An isolated peptide containing the modified amino acid, tentatively identified as a histidine, was reported to have the amino acid sequence Phe-Val-Gly-Gly-Asp-**His**-Thr-Val-Ser.¹⁹ Haziza et al. subsequently determined the nucleotide sequence of the entire *asd* gene of *E. coli* that codes for ASADH¹⁵ and found a corresponding sequence encoding for amino acid residues 130–138 of their deduced sequence. However, they found a substitution of asparagine for aspartic acid and cysteine for histidine at residues 134 and 135, respectively. These results are consistent with the cysteine located at position 135 of the amino acid sequence as the most likely candidate for the active site cysteine nucleophile.

Mutagenesis Studies. Mutants of ASADH were constructed by oligonucleotide-directed mutagenesis to examine the role of this putative active site cysteine, and to identify additional amino acids functional groups that may play a role in the mechanism of the enzyme-catalyzed reaction. Kinetic parameters were determined in the

Table 2. Kinetics of Mutated Aspartate Semialdehyde Dehydrogenases^a

enzyme	k_{cat} (s ⁻¹)	k_{cat} (%)	L-ASA ^b		phosphate	
			K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
wild type	610	100	0.17	3.6×10^6	4.8	1.3×10^5
C135A	<0.005	<0.001				
C135S	2.0	0.3	0.15	1.3×10^4	1.2	1.5×10^3
Q162N	47	8	0.15	3.1×10^5	4.6	1.0×10^4
Q162H	177	29	0.25	7.1×10^5	4.7	3.8×10^4
Q39D	290	48	0.13	2.3×10^6	4.7	6.2×10^4
R267L	58	10	5.3	1.1×10^4	19	3.0×10^3

^a The reaction was monitored by following the oxidative phosphorylation of L-ASA. Reaction conditions: 200 mM Ches buffer (pH 8.6), 25 °C, saturating (1 mM) NADP, varying concentration of ASA and phosphate, and 0.055–3.8 $\mu\text{g}/\text{mL}$ enzyme. ^b L-ASA, L-aspartate β -semialdehyde.

nonphysiological direction, the oxidative phosphorylation of ASA, for both the native enzyme and mutants in which Cys-135 was replaced with either an alanine or a serine. A complete loss of enzyme activity is observed upon the substitution of alanine at this position in ASADH (Table 2). While the alanine mutant is inactive, the corresponding serine mutant (C135S) does show some enzymatic activity, with the maximum velocity reduced by about 500-fold compared to that of the wild-type enzyme.²⁰ In this mutant the K_{m} for ASA is unchanged from that in the native enzyme, while the K_{m} for phosphate is actually 4-fold lower in the Ser-135 mutant. These results suggest that there are no major conformational changes resulting from the serine substitution that would adversely affect the ability of the enzyme to bind its substrates. Taken together with the earlier modification studies, these results present a compelling case for the essential catalytic role of Cys-135.

A sequence homology search among the ASADHs that have been isolated from other species, and comparisons with the structures of functionally similar D-glyceraldehyde-3-phosphate dehydrogenases (GAPDH) that have been solved from several species, have been utilized to select appropriate targets for mutagenesis (Figure 4). A highly conserved active site histidine in the GAPDH family (His-176) has been suggested to enhance catalysis by deprotonating the active site cysteine (Cys-149) to generate the thiolate nucleophile.^{21,22} However, a fully conserved glutamine is found in the ASADH family at the position corresponding to this histidine. While it is not chemically feasible for an amide to substitute for the acid–base catalytic role of histidine, this glutamine must be playing some essential role. A conservative substitution of an asparagine for the glutamine at this position (Q162N) results in a decrease in k_{cat} to less than 10% that of the native activity.²³ Substitution of a histidine at this position in ASADH, to mimic the amino acid found at this position in GAPDH, gives a 4-fold activity enhancement over the Q162N mutant (Table 2). However, the k_{cat} of this mutant is still less than 30% that of the native activity, supporting an essential but at this point undetermined role for this glutamyl residue.

A conserved arginine (Arg-267) has been found to align with the Arg-231 in GAPDH (Figure 4) that has been

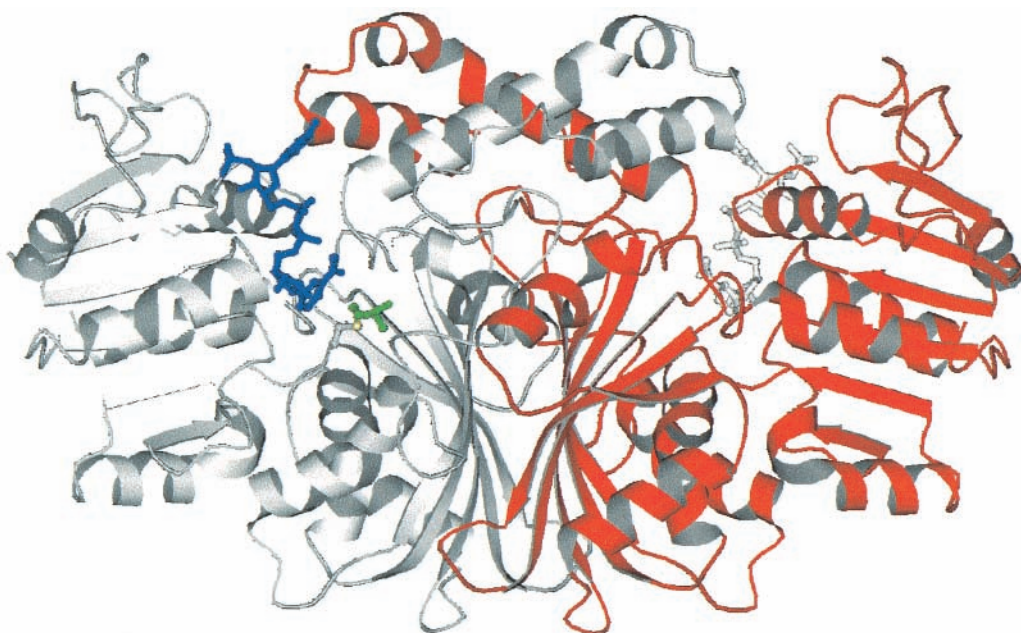


FIGURE 5. Ribbon drawing of the structure of aspartate- β -semialdehyde dehydrogenase from *Escherichia coli*.²⁴ The catalytically active dimer is shown, with one of the subunits shaded in red. The position of bound NADP is shown (in blue) in the unshaded subunit as well as the active site cysteine-135 modified with *S*-methylcysteine sulfoxide (in green).

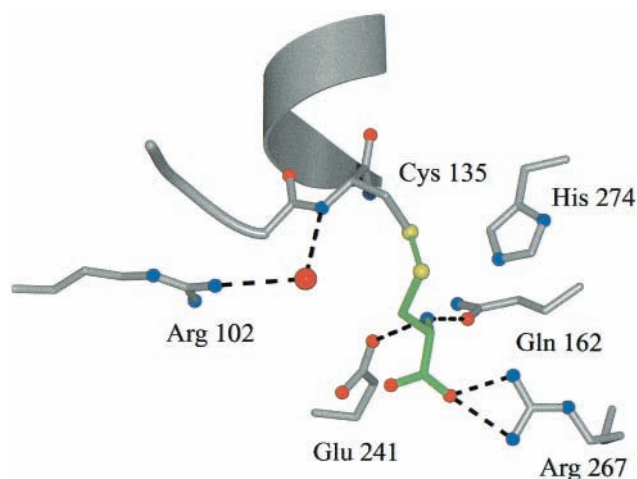


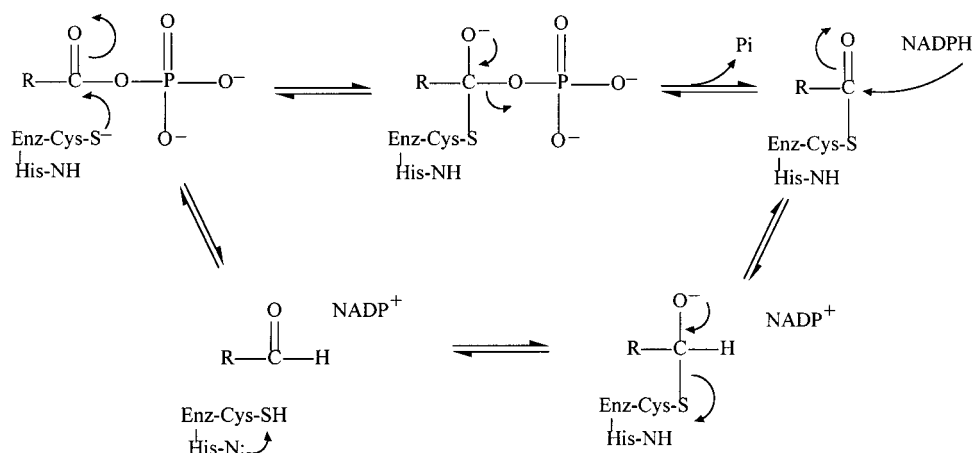
FIGURE 6. Active site structure of aspartate- β -semialdehyde dehydrogenase showing the nucleophile (Cys-135) covalently inactivated by *S*-methylcysteine sulfoxide (shown in green), the catalytic base (His-274), the orienting group for the catalytic base (Gln-162), and the substrate binding groups (Glu-241 and Arg-267).⁴⁰

entially to the E-NADPH complex, and there is ordered release of the products ASA and NADP. The chemical mechanism proposed for wild-type ASADH follows a four-step sequence (Scheme 1). The active site nucleophile is generated by deprotonation of Cys-135 by the adjacent His-274. His-274 is positioned for this role by a hydrogen bond to Gln-162. The binding of aspartyl phosphate is assisted by an electrostatic interaction of the substrate carboxyl group with the guanido group of Arg-267. Attack of the active site thiolate at the carbonyl carbon of aspartyl phosphate, followed by collapse of the tetrahedral intermediate with expulsion of phosphate, leads to hydride transfer to this intermediate. Expulsion of thiol (assisted

by protonation from His-274) generates ASA. Deprotonation of Cys-135 is required to initiate another catalytic cycle.

Oxyanion Specificity. The structure of an enzyme–NADP–substrate analogue (*S*-methylcysteine sulfoxide) complex has been determined; however, an even more informative structure would be that of the acyl-enzyme intermediate. This acyl-enzyme form is reasonably stable, to the point where it can be isolated by gel filtration chromatography in the absence of phosphate.¹⁶ Further stabilization of this intermediate may allow the crystallization of this complex. To this end a variety of oxyanions have been tested as possible alternative substrates or competitive inhibitors against phosphate for the reverse (oxidative) reaction. Arsenate and vanadate were found to be good alternative substrates (Table 3), with V_{\max} values which are 50–70% that with phosphate and a K_m for vanadate that is 20-fold lower than that for phosphate.²⁶ Periodate, tellurate, phosphonate, tungstate, and perrhenate were all found to be competitive inhibitors of ASADH, with K_i values ranging from 0.2 mM for periodate to 140 mM for perrhenate. These results have been analyzed to determine how molecular geometry, anion size, and charge density contribute to the enzyme's ability to discriminate among possible substrates or inhibitors. Tetrahedral geometry is required, and a high negative charge density on the peripheral oxygens is an important component for substrate recognition by ASADH.²⁶

The binding of competitive inhibitors in the phosphate binding site was found to stabilize the acyl-enzyme intermediate. The slow hydrolysis of this intermediate is further decreased by at least 20-fold upon changing from pH 8 to pH 7 in the presence of saturating levels of periodate. Crystallization of this intermediate complex will

Scheme 1. Chemical Mechanism of Aspartate- β -Semialdehyde Dehydrogenase**Table 3. Kinetics for Oxyanions Interacting with Aspartate Semialdehyde Dehydrogenase^a**

oxyanion	V_{\max} (min^{-1})	K_m (mM)	V_{\max}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
phosphate	710	2.9	4.1×10^3
arsenate	510	1.6	5.3×10^3
vanadate	330	0.14	3.9×10^4

^a Reaction conditions: 200 mM Ches buffer (pH 8.6), 25 °C, saturating (1 mM) NADP, saturating (0.5 mM) ASA, and varying concentrations of the oxyanions.

provide a snapshot of an important step in the catalytic cycle of ASADH.

Homoserine Dehydrogenase

Homoserine dehydrogenase (HDH) catalyzes the NADPH-dependent reduction of ASA to produce homoserine. This reaction occurs at a key branch point in the pathway, with the substrate serving as the precursor for the biosynthesis of lysine and the product leading to methionine, threonine, and isoleucine (Figure 1). Regulation of the reaction catalyzed by HDH is crucial for controlling the flux through this branched pathway. In Gram-negative bacteria such as *E. coli*, HDH is found as a catalytic domain in a bifunctional enzyme with aspartokinase, and allosteric regulation of both activities is mediated through the aspartokinase domain.²⁷ In contrast, HDH in Gram-positive bacteria is a separate enzyme and a different mechanism of regulation has evolved. The regulation by threonine of the homoserine dehydrogenase from *Corynebacterium glutamicum* is abolished by mutations near the C-terminus.^{28,29} The monofunctional HDH from *Bacillus subtilis* is highly homologous to that from *C. glutamicum*. An examination of the sequences of these enzymes shows that they contain an additional C-terminal domain of about 100 amino acids that is not found in the *E. coli* enzyme.³⁰ On the basis of sequence alignments, hybridization, and deletion studies, this domain appears to be the site of allosteric regulation by threonine. The enzyme that has been isolated from yeast is the smallest known homoserine dehydrogenase. This monofunctional enzyme is missing the C-terminal extension that is found in Gram-positive bacteria and consequently is not regulated by threonine.³¹

The structure of this yeast HDH has recently been solved.³² The enzyme is a dimer, with each monomer composed of a nucleotide binding region, a catalytic region, and a subunit interface region. The active site structure of HDH identifies two binding groups, Asp-214 and Glu-208, that help to orient the substrate ASA. The proposed mechanism, based on both structural and mutagenic studies, is unusual for a pyridine-linked dehydrogenase. Instead of the typical proton donors found in these enzymes, an active site lysine (Lys-223), positioned through an electrostatic interaction with Asp-219, is proposed to donate a proton to the alkoxide of ASA that forms during hydride transfer from NADPH. This role is supported by a mutation of this lysine that results in a completely inactive enzyme.³²

At this point in the pathway homoserine can be directed, by condensation with succinyl-coenzyme A, to the production of methionine. Alternatively, homoserine can be phosphorylated by homoserine kinase, leading to threonine and subsequently to isoleucine (Figure 1).

Homoserine Kinase

Homoserine kinase (HSK, EC 2.7.1.39), the fourth enzyme in the aspartate pathway of amino acid biosynthesis, catalyzes the phosphorylation of L-homoserine to L-homoserine phosphate. This activated intermediate is the precursor for the synthesis of threonine, catalyzed by threonine synthase.

Substrate Specificity. As is true for all kinases, a divalent metal ion, normally magnesium, is essential for catalytic activity. But, in addition, divalent metal ions have also been reported to increase the affinity of the enzyme for L-homoserine.³³ A wide range of structural analogues of L-homoserine have been examined as possible alternative substrates for the reaction catalyzed by HSK. Both kinetic and ³¹P NMR studies have shown that the enzyme is able to phosphorylate a hydroxyl group at either carbon 4 or 5 of L-homoserine analogues, but neither L-threonine nor L-serine is a substrate of the enzyme. For four-carbon L-homoserine analogues, the carboxyl group at the α -position can be a carboxyl, or could be substituted with an ester or even a hydroxymethyl group. The enzyme can

Table 4. Kinetics of Homoserine Kinase Mutants^a

enzyme	k_{cat} (s ⁻¹)		kinase/ATPase ratio
	kinase	ATPase	
wild type	18.3	0.016	1140
R234L	0.20	0.049	4.1
R234C	0.02 ^b	0.28	0.08
R234H	0.073	0.37	0.19
H202L	9.06	0.071	128
H139L	0.5 ^b	2.51	0.21
H205Q	0.005 ^b	0.036	0.14

^a Reaction conditions: activity was monitored by a coupled pyruvate kinase/lactate dehydrogenase assay containing 100 mM Hepes/Tris (pH 8.0), 100 mM KCl, 2 mM MgATP, 0.7 mM PEP, and 0.1 mM NADH. ^b Estimated from the partition ratio of kinase to ATPase activity determined by phosphorus-31 NMR spectroscopy

also accommodate large, hydrophobic groups at the carboxyl end of the substrate. L-Homoserine esters with ester groups up to four carbons long are processed without a substantial sacrifice in catalytic efficiency, although the selectivity ($k_{\text{cat}}/K_{\text{m}}$) for homoserine is 20- to 80-fold higher than for these homoserine esters.³⁴ These results suggest the presence of a hydrophobic pocket adjacent to the binding site of the carboxyl group of the substrate on the active site, with hydrophobic interactions between the alkyl groups and the surrounding of the pocket at least partially compensating for the loss of an ionic interaction to aid in the productive orientation of these alternative substrates.

Identification of Functionally Important Amino Acids.

An examination of the pH profile shows that this enzyme loses activity upon protonation of a single functional group and upon deprotonation of a second functional group, with both groups appearing to be of the cationic acid type.³⁵ Incubation of the enzyme with DEP leads to the complete loss of enzyme activity. Spectral and chemical characterization of the derivatized enzyme has shown that this activity loss is caused by the modification of a histidyl residue, and the presence of substrates protects a single histidine from modification. Treatment of the enzyme with pyridoxal-5'-phosphate also results in enzyme inactivation. The spectral evidence for the formation of a Schiff base supports the presence of a reactive lysine. The complete protection against modification afforded by substrates and inhibitors also indicates that homoserine kinase contains a lysine that is essential for catalytic activity.³⁵

Mutagenesis Studies. The choice of the appropriate amino acid targets for site-directed mutagenesis was based on the earlier pH and chemical modification studies, and also on sequence comparisons among related enzymes. Arg-234, the only conserved arginine, was first replaced with the neutral amino acid leucine. The K_{m} of R234L for L-homoserine increases by nearly 300-fold and the k_{cat} decreases by 90-fold compared to those of the wild-type enzyme (Table 4). However, there is less than a 2-fold change in the K_{m} for ATP, and the inherent ATPase activity of HSK (<0.1% of the kinase activity in the native enzyme) actually increases by 3-fold in the R234L mutant.³⁴ Arg-234 was also replaced with cysteine and histidine.

The R234C mutant has no observable homoserine kinase activity, measured by using a coupled enzyme assay; however, it now has an ATPase activity that is nearly 20 times that of the wild-type enzyme. The R234H mutant also has diminished kinase activity (0.4% of wild type) and an enhanced ATPase activity.

There are three histidines that are partially conserved in HSK. Mutation of the most conserved histidine (His-139) to leucine leads to an enzyme with dramatically enhanced ATPase activity, about 150-fold greater than that of the wild-type enzyme, and with diminished kinase activity (Table 4). When calculated from the ratio of kinase to ATPase products by using ³¹P NMR spectroscopy, the kinase activity of H139L is less than 3% that of the wild-type enzyme. Replacing histidine-202 with leucine leads to only a 50% decrease in k_{cat} , while substitution of histidine-205 with glutamine leads to an enzyme with an unaltered K_{m} for ATP and an ATPase activity that is within a factor of 2 of the wild-type enzyme.³⁴ However, the kinase activity of H205Q, as determined from ³¹P NMR, is less than 0.03% that of wild-type enzyme (Table 4).

These site-directed mutagenesis studies have confirmed a role for arginine-234 in the binding of the carboxyl group of L-homoserine, and the involvement of two histidines (His-139 and His-205) at the homoserine site. Mutations at these sites have led to the decoupling of the kinase activity from an inherent ATPase activity in the enzyme. These results suggest the presence of independent domains for the binding of ATP and homoserine in HSK. Alterations that interfere with homoserine binding will actually enhance the ability of homoserine kinase to hydrolyze ATP.

Creation of New Bifunctional Enzymes

Existing Bifunctional Enzymes. There are many examples of well-characterized bifunctional enzymes, particularly in amino acid metabolism. For example, in aromatic amino acid biosynthesis the α -subunit of tryptophan synthase catalyzes the production of indole, which is then channeled to the β -subunit to condense with serine for the synthesis of tryptophan.³⁶ Two different bifunctional enzymes catalyze the conversion of chorismate, directing the intermediate prephenate to the synthesis of either tyrosine, in the case of chorismate mutase-prephenate dehydrogenase,³⁷ or phenylalanine when catalyzed by chorismate mutase-prephenate dehydratase.³⁸ In these, and in virtually all other examples, bifunctional enzymes catalyze consecutive reactions either to channel a metabolic intermediate to the next catalytic site, or to direct the metabolic flux down one branch of a pathway. A notable exception to the utility of consecutive reaction catalysis is the aspartokinase-homoserine dehydrogenase isoenzymes that catalyze nonconsecutive reactions.

To examine the mechanisms of regulation in this pathway, and to potentially address the question of metabolite channeling, we have cleaved the gene that encodes for the bifunctional AK-HDH I and have separately expressed each of the catalytic domains. To study

Table 5. Kinetics of the Monofunctional Aspartokinases and Homoserine Dehydrogenases

kinetic parameters	aspartokinase activity ^a			homoserine dehydrogenase activity ^a		
	AK-HDH I ^b	AK I ^b	AK III ^b	AK-HDH I ^b	HDH I' ^b	HDH I ^b
k_{cat} (s ⁻¹)	0.39	6.3	98	0.24	3.30	0.51
K_{m} (aspartate or homoserine) (mM)	0.63	0.47	0.51	1.2	0.68	17.2
$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)	6.3×10^2	1.4×10^4	1.9×10^5	2.1×10^2	4.9×10^3	3.0×10^1

^a Aspartokinase activity was monitored by a coupled pyruvate kinase/lactate dehydrogenase assay while homoserine dehydrogenase activity was monitored directly by the reduction of NADP. ^b AK-HDH I, native bifunctional aspartokinase-homoserine dehydrogenase I; AK I, aspartokinase I catalytic domain; AK III, native aspartokinase III; HDH I', homoserine dehydrogenase catalytic domain with attached interface region; HDH I, homoserine dehydrogenase catalytic domain.

Table 6. Kinetics of the New Bifunctional Enzymes

kinetic parameters	aspartokinase activity ^a		homoserine dehydrogenase activity ^a	
	AK I-ASA DH ^b	AK III-HDH I' ^b	AK-HDH I ^b	AK III-HDH I' ^b
k_{cat} (s ⁻¹)	1.35	0.16	0.24	24
K_{m} (aspartate or homoserine) (mM)	0.59	0.51	1.2	0.41
$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)	2.3×10^3	3.1×10^2	2.1×10^2	5.9

^a Aspartokinase activity was monitored by a coupled pyruvate kinase/lactate dehydrogenase assay while homoserine dehydrogenase activity was monitored directly by the reduction of NADP. ^b AK I-ASA DH, fusion of aspartokinase I catalytic domain with aspartate semialdehyde dehydrogenase; AK III-HDH I', hybrid fusion of native aspartokinase III with the catalytic domain and interface region of homoserine dehydrogenase I; AK-HDH I, native aspartokinase-homoserine dehydrogenase I.

the flux and regulation of metabolites through this pathway we have combined different catalytic domains to create several new artificial bifunctional enzymes.

Expression of New Monofunctional Enzymes. The bifunctional AK-HDH I gene, *thrA*, from *E. coli* has been divided into its separate and fully functional catalytic domains.³⁹ The aspartokinase I domain encompasses approximately the first 250 amino acids on the *N*-terminus of the bifunctional AK-HDH I enzyme. The gene fragment encoding this domain, called *thrA*₁, was excised from the *thrA* gene and expressed separately. This purified monofunctional AK I domain has high catalytic activity, with an enhanced k_{cat} compared to that of the intact bifunctional enzyme (Table 5). The remaining portion of the *thrA* gene, called *thrA*₂', consists of the interface region and the homoserine dehydrogenase catalytic domain. Expression of this gene fragment produces a monofunctional HDH I' that is also highly active, with over a 10-fold increase in k_{cat} and a 2-fold decrease in K_{m} compared to this activity in the native bifunctional enzyme (Table 5).

To examine the effect that the presence of the interface region might have on the catalytic activity of the HDH domain, this region was removed from the monofunctional HDH. This gene fragment, called *thrA*₂, encodes for only the 41 kDa HDH domain. The separate catalytic domain has a lower k_{cat} without the interface region present, and a K_{m} for homoserine that has increased by 40-fold. However, this diminished catalysis is only 7-fold lower than that measured for the native bifunctional enzyme (Table 5).

Regulation of the Monofunctional Enzymes. Native AK-HDH I is subject to feedback inhibition by the end product amino acid L-threonine, with an inhibition constant of about 0.3 mM. The separated AK I domain has lost this allosteric regulatory capacity, and is no longer inhibited by threonine at concentrations up to 200 mM. The separate HDH I domain is still regulated, and becomes even more sensitive to threonine inhibition when

the AK catalytic domain is removed. These results are in contrast to the previous reports that the regulation of HDH by threonine is mediated through the aspartokinase domain.²⁷ However, further truncation, by removal of the interface region, causes a greater than 400-fold decrease in the sensitivity of HDH to feedback inhibition by threonine.³⁹

Fusion To Form Bifunctional Enzymes. Now that each catalytic domain has been shown to be fully active when expressed separately, the next question to be addressed is whether these genes can be fused to form new bifunctional enzymes that can catalyze consecutive reactions. The separated *thrA*₁ gene fragment that encodes for AK I was joined in frame to the *asd* gene that encodes for ASADH to create a new bifunctional enzyme that could now potentially catalyze the coupled conversion of aspartate to aspartate- β -semialdehyde. Joining AK I to ASADH results in a 5- to 6-fold decrease both in k_{cat} and in $k_{\text{cat}}/K_{\text{m}}$ for AK I when compared to the values for the isolated monofunctional enzyme (Table 6). However, these values are still 4-fold higher than those observed in the native bifunctional enzyme.

To examine the mechanism of end product inhibition, a hybrid bifunctional enzyme was prepared by joining the *lysC* gene that encodes for the monofunctional AK III with the *thrA*₂' gene that encodes for HDH I' with the interface domain. Native AK III is subject to feedback inhibition by the end product amino acid lysine. This inhibition is retained in the hybrid bifunctional enzyme with HDH I, while the HDH activity in this enzyme remains sensitive to threonine inhibition. However, in addition to the inhibition by threonine, the dehydrogenase activity has now become quite sensitive to inhibition by lysine.³⁹ Thus, fusion of these two catalytic domains has allowed the binding of an allosteric inhibitor (lysine) near one catalytic domain (AK III) to be communicated across the fusion interface to influence the activity of the other catalytic domain (HDH).

Conclusions and Future Directions

Enzymes, with few exceptions, are noteworthy for their ability to catalyze stereoselective and regioselective transformations. These specificities are certainly present in the phosphorylation of aspartic acid that is catalyzed by the aspartokinases. Only the L-isomer of aspartic acid is a substrate and only the β -carboxyl group of the substrate is phosphorylated. However, these enzymes have been found to bind β -derivatized analogues of aspartic acid, compounds that would not normally be encountered in vivo. In doing so the regioselectivity is reversed and the α -carboxyl group becomes the phosphoryl acceptor. Several of these α -acyl phosphates have also been shown to be alternative substrates for subsequent enzyme-catalyzed steps in this metabolic pathway. By allowing these enzymes to carry out the synthesis, the products of these alternative reactions become potential candidates for active-site-directed inhibitors or inactivators of the downstream enzymes in the pathway.

The high-resolution structure of aspartate semialdehyde dehydrogenase confirmed the active site structural assignments that were made on the basis of chemical modification and mutagenesis studies. A previously unrecognized histidine was discovered in this structure that plays the role of an active site base to deprotonate the catalytic cysteine and generate the thiolate nucleophile. The ASADHs from a range of infectious microorganisms have the same set of functionally important amino acids that have been identified in the *E. coli* enzyme; however, their overall sequence identity varies from over 80% to less than 25%. The purification, mechanisms, and structures of these enzymes are being investigated with the goal of identifying differences between these enzymes that can be used for targeted inhibition.

It is not clear why Nature chose to fuse the genes encoding for aspartokinase and homoserine dehydrogenase to make a bifunctional enzyme that catalyzes non-consecutive reactions. Separating these genes leads to two independent fully functional enzymes. Joining the newly produced monofunctional aspartokinase I to aspartate semialdehyde dehydrogenase creates an artificial bifunctional enzyme that can now catalyze consecutive reactions. Preliminary experiments have demonstrated a direct channeling of the unstable acyl phosphate intermediate between the active sites in this newly constructed fusion enzyme. Future studies will focus on the production of additional bifunctional constructs, a trifunctional enzyme that can catalyze the direct conversion of aspartic acid to homoserine, and an examination of intermediate channeling and regulation in this pathway. Completion of these studies will provide the basis for the development of inhibitors and activators of this essential pathway.

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