Production and Characterization of Bifunctional Enzymes. Domain Swapping To Produce New Bifunctional Enzymes in the Aspartate Pathway†

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ABSTRACT: The bifunctional enzyme aspartokinase−homoserine dehydrogenase I from *Escherichia coli* catalyzes nonconsecutive reactions in the aspartate pathway of amino acid biosynthesis. Both catalytic activities are subject to allosteric regulation by the end product amino acid L-threonine. To examine the kinetics and regulation of the enzymes in this pathway, each of these catalytic domains were separately expressed and purified. The separated catalytic domains remain active, with each of their catalytic activities enhanced in comparison to the native enzyme. The allosteric regulation of the kinase activity is lost, and regulation of the dehydrogenase activity is dramatically decreased in these separate domains. To create a new bifunctional enzyme that can catalyze consecutive metabolic reactions, the aspartokinase I domain was fused to the enzyme that catalyzes the intervening reaction in the pathway, aspartate semialdehyde dehydrogenase. A hybrid bifunctional enzyme was also created between the native monofunctional aspartokinase III, an allosteric enzyme regulated by lysine, and the catalytic domain of homoserine dehydrogenase I with its regulatory interface domain still attached. In this hybrid the kinase activity remains sensitive to lysine, while the dehydrogenase activity is now regulated by both threonine and lysine. The dehydrogenase domain is less thermally stable than the kinase domain and becomes further destabilized upon removal of the regulatory domain. The more stable aspartokinase III is further stabilized against thermal denaturation in the hybrid bifunctional enzyme and was found to retain some catalytic activity even at temperatures approaching 100 °C.

The complex enzymes that are isolated and studied today are hypothesized to have evolved from more primitive precursors. At the metabolic level simple, monofunctional enzymes are seen as an early stage of evolutionary refinement. The clustering of isolated genes that encode for enzymes with related metabolic activities represents a significant advancement since the expression of related enzymes can now be controlled by common regulatory elements. At the protein level the association of individual enzymes into multienzyme complexes provides several distinct advantages. Catalytic events that take place on one enzyme can have a direct influence on the associated enzymes that are present in the complex. Single regulatory sites or regulatory subunits can control the coordinated activities of all of the enzymes in the complex. Also, the directed transfer of reactants from consecutive active sites can support more efficient metabolism. From this point of view the most highly evolved enzyme forms are seen to be multifunctional enzymes in which gene fusion has incorporated related catalytic activities into covalently coupled domains in a single polypeptide chain. Reactive intermediates could now be protected and channeled from one catalytic site to the next to minimize their degradation and to direct their metabolic flux. In addition, the binding of effectors at a single allosteric site can now regulate the activities at multiple catalytic sites.

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 (*1*). 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 (*2*), or phenylalanine when catalyzed by chorismate mutase−prephenate dehydratase (*3*). 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 (AK-HDH)[1] isoenzymes. In plants and microbial organisms there exist as many as three isofunctional aspartokinases. These isoenzymes are differentially regulated at both the

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[1] Abbreviations: AK, aspartokinase; AK-HDH, aspartokinase−homoserine dehydrogenase; ASADH, aspartate-β-semialdehyde dehydrogenase; HDH, homoserine dehydrogenase; HDH$^+$, homoserine dehydrogenase with the interface region attached; *thr*A, gene encoding for AK-HDH I; *thr*A_1, gene fragment encoding for the AK catalytic domain; *thr*A_2, gene fragment encoding for the HDH catalytic domain.

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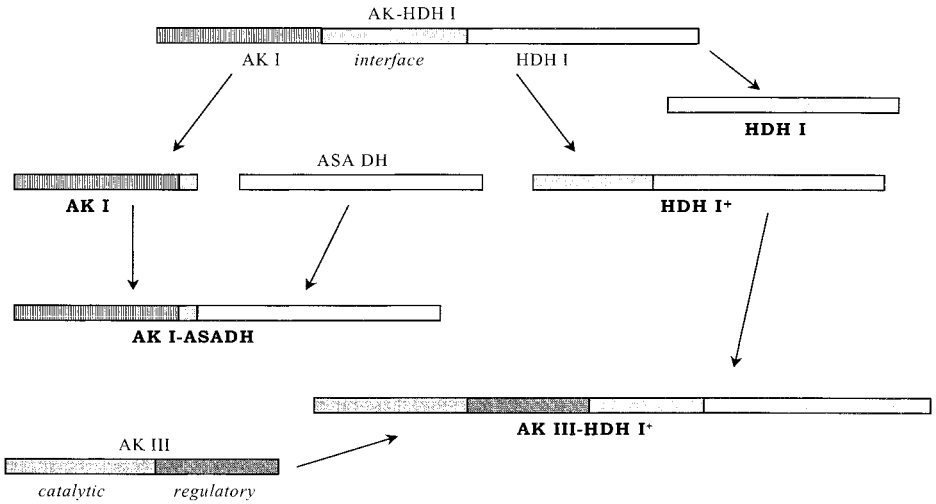
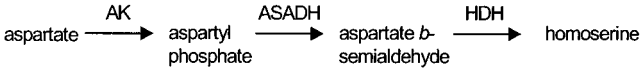


FIGURE 1: Construction of new monofunctional and bifunctional enzymes. The bars representing each domain are drawn approximately to scale, with the new enzyme constructs shown in bold. Key: AK-HDH I, native bifunctional aspartokinase−homoserine dehydrogenase I; AK I, aspartokinase I catalytic domain; interface, regulatory domain in aspartokinase−homoserine dehydrogenase I; HDH I, homoserine dehydrogenase I catalytic domain; HDH I^+, homoserine dehydrogenase I with attached regulatory domain; ASADH, aspartate semialdehyde dehydrogenase; AK I-ASADH, new aspartokinase I−aspartate semialdehyde dehydrogenase bifunctional enzyme; AK III, native aspartokinase III catalytic and regulatory domains; AK III-HDH I^+, new hybrid aspartokinase III−homoserine dehydrogenase I bifunctional enzyme.

protein level and the genetic level by the end product amino acids, threonine, isoleucine, lysine, and methionine. When these isoenzymes are present as bifunctional enzymes, they contain domains that catalyze the first reaction (the phosphorylation of aspartate, Scheme 1) and then the third reaction (the reduction of the aldehyde intermediate to produce homoserine) in the aspartate family of amino acid biosynthesis (*4*). A separate enzyme, aspartate-β-semialdehyde dehydrogenase (ASADH), catalyzed the intervening reaction, the reductive dephosphorylation of aspartyl phosphate (*5*).

Scheme 1: Central Reactions of the Aspartate Family of Amino Acid Biosynthesis



Since the intermediates in these reactions, an acylphosphate and a semialdehyde, are somewhat unstable, the potential advantage of fusing enzymes that can catalyze consecutive reactions to protect and channel these intermediates has not been realized with these bifunctional aspartokinases. Formation of a multienzyme complex between ASADH and the bifunctional AK-HDH would provide an avenue to solve this problem, but there has been no evidence to support the existence of a stable multienzyme complex of this type.

To examine the mechanisms of regulation in this pathway and to address the possibility of metabolite channeling, we report the creation and characterization of several new artificial bifunctional enzymes. The bifunctional AK-HDH I gene, *thr*A, from *Escherichia coli* has been divided into its separate and fully functional catalytic domains (Figure 1). Next, the new monofunctional aspartokinase I (AK I) domain was joined with ASADH to create an artificial bifunctional enzyme that can now catalyze consecutive reactions in this metabolic pathway. In addition, the naturally occurring monofunctional AK III isoenzyme has been combined with the homoserine dehydrogenase I (HDH I) catalytic domain to create a hybrid bifunctional enzyme. The kinetics, regulation, and stability of each of these new chimeras have been examined.

EXPERIMENTAL PROCEDURES

Materials. pGEM plasmids were from obtained Promega, and PTZ19 plasmid was from U. S. Biochemicals. Restriction enzymes, DNA linker sequences, T4 DNA ligase, buffer, and DNA markers were from New England Biolabs, plasmid DNA extraction kits and gel extraction kits were purchased from Quiagen, green A dye ligand resin was from Amigen, and *pfu* polymerase and the QuikChange mutagenesis kit were from Stratagene. All buffers, coupling enzymes, and remaining chemicals were from Sigma Chemical Co. A plasmid containing the *asd* gene and a plasmid containing the *thr*A and *thr*B genes were a gift from Dr. Georges Cohen of the Pasteur Institute (Paris). A bacterial strain of an *asd*-deficient G6MD3 cell line was a gift from Dr. Jack Priess. Aspartate β-semialdehyde was synthesized by the ozonolysis of L-allylglycine as previously described (*6*).

Transformation. TG1 cells were made competent by the addition of 100 mM $CaCl_2$. The ligation mixture was added to the competent cells, incubated for 1.5 h, heat shocked at 42 °C for 90 s, and then iced for 5 min. Cells were then spread onto ampicillin LB agar plates and incubated at 37 °C overnight. Colonies were inoculated into 5 mL of LB liquid media plus ampicillin and allowed to grow overnight at 37 °C with shaking. DNA was extracted from these cultures by use of the Quiagen plasmid extraction kit.

Formation of Monofunctional Enzymes. The AK I monofunctional enzyme was created by extracting the *thr*A gene section from the pIPII plasmid (*7*) using a restriction enzyme digest. To separate the kinase domain, an *Sml*I restriction site was selected after the end of the AK domain, about 110 bases into the interface region in the *thr*A gene. The resulting 860-base gene fragment, called *thr*A_1, was purified, ligated into an expression vector, and transformed into competent *E. coli* TG1 cells. High levels of AK were detected in the crude cell extract both by SDS−PAGE and by catalytic

Table 1: Kinetics and Regulation of the Aspartokinases

kinetic parameters	aspartokinase enzyme forms[a]				
	AK-HDH I	**AK I**	**AK I**-ASADH	**AK III**	**AK III**-HDH I$^+$
k_{cat} (s^{-1})	0.39 ± 0.02	6.3 ± 0.6	1.35 ± 0.03	98 ± 1.1	0.16 ± 0.02
K_m (aspartate)[b]	0.63 ± 0.08	0.47 ± 0.09	0.59 ± 0.02	0.51 ± 0.02	0.51 ± 0.01
k_{cat}/K_m ($M^{-1}\,s^{-1}$)	6.3×10^2	1.4×10^4	2.3×10^3	1.9×10^5	3.1×10^2
K_i (Thr)[b]	0.33 ± 0.04	ni[c]	ni	ni	ni
K_i (Lys)[b]	ni	ni	ni	0.50 ± 0.02	0.78 ± 0.09

[a] The aspartokinase activity of each enzyme was determined by a coupled assay as described in Experimental Procedures. [b] K_m and K_i values are in millimolar. [c] No inhibition observed.

activity. The homoserine dehydrogenase domain, consisting of approximately 350 amino acids, was removed from the *thr*A gene by a *Ban*I restriction digest which created a 2175-base fragment that includes nearly the entire interface region and continues through the C-terminus of the reading frame to another *Ban*I site that is 430 bases after the stop codon. Overexpression and purification of this domain, from this *thr*A_2^+ fragment, led to a highly active monofunctional HDH I$^+$ enzyme (where the + indicates the presence of the interface region). The *asd* gene was isolated from the pGEM7Zf plasmid using the restriction enzyme *Bam*HI, and both the gene and the remaining plasmid were purified by gel electrophoresis.

Formation of New Bifunctional Enzymes. AK was ligated to ASADH through a linker that was synthesized to couple the *Sml*I restriction site at the 5′-end of the *thr*A_1 gene that encodes for AK I to the *Bam*HI site at the 3′-end of the *asd* gene encoding for ASADH. ASADH was also ligated to HDH I through a pair of linkers. The *asd* gene encoding for ASADH was coupled to a 24 base pair linker duplex constructed with consecutive *Bam*HI, *Xmn*I, and *Eco*RI restriction sites. The *thr*A_2^+ fragment encoding for HDH gene section was ligated to a linker duplex composed of *Eco*RI, *Xmn*I, and *Sml*I restriction sites. The resulting product was isolated by gel electrophoresis and extracted from the gel.

Enzyme Assays. The catalytic activity of AK is monitored by coupled enzyme assays in which the rate of formation of either aspartyl phosphate (ASADH coupled assay) or ADP (pyruvate kinase−lactate dehydrogenase coupled assay) is monitored (*8*). The change in absorbance of the NADP/NADPH coenzyme is used to monitor the catalytic activity of both ASADH and HDH. For the AK-ASADH bifunctional enzyme the ADP coupled assay must be used to measure the isolated AK activity, and the increase in absorbance at 340 nm associated with the formation of NADPH is used to measure the isolated ASADH activity in the nonphysiological direction. For the determination of kinetic parameters the nonvaried substrate is fixed at saturating levels (at least 10K_m), and the other substrate is varied around its K_m value. Kinetic constants were determined by fitting the data using the kinetic programs of Cleland (*9*) that have been rewritten in Basic.

Enzyme Stability Studies. A solution of each enzyme form in 50 mM Hepes buffer, pH 8, was incubated at 25 °C for 5 min in an MJ Research Peltier-controller thermocycler. The incubation temperature was then increased in 5 deg increments with each temperature held for 5 min. A 10 μL aliquot was removed and assayed for catalytic activity, as described above, at the end of each incubation period. Activities were calculated as a percentage of the initial activity at 25 °C.

Table 2: Kinetics and Regulation of the Homoserine Dehydrogenases

kinetic parameters	homoserine dehydrogenase enzyme forms[a]			
	AK-**HDH I**	**HDH I**$^+$	**HDH I**	AK III-**HDH I**$^+$
k_{cat} (s^{-1})	0.24 ± 0.02	3.30 ± 0.02	0.51 ± 0.10	24 ± 7
K_m (Hse)[b]	1.2 ± 0.3	0.68 ± 0.02	17.2 ± 1.2	0.41 ± 0.09
k_{cat}/K_m ($M^{-1}\,s^{-1}$)	2.1×10^2	4.9×10^3	3.0×10^1	5.9×10^4
K_i (Thr)[b]	0.23 ± 0.07	0.04 ± 0.01	17 ± 5	0.15 ± 0.01
K_i (Lys)[b]	ni[c]	ni	ni	0.02 ± 0.003

[a] The homoserine dehydrogenase activity of each enzyme was determined by the assay described in Experimental Procedures. [b] K_m and K_i values are in millimolar. [c] No inhibition observed.

RESULTS

Separation of the Bifunctional AK-HDH I. The aspartokinase−homoserine dehydrogenases I and II in *E. coli* consist of two catalytic domains, a kinase domain and a dehydrogenase domain, joined by an interface region (*10*). The aspartokinase I domain encompasses approximately the first 250 amino acids on the N-terminal end of the bifunctional AK-HDH I enzyme (Figure 1). The gene fragment encoding this kinase domain, called *thr*A_1, was excised from the *thr*A gene and expressed separately. This purified monofunctional AK I domain retains catalytic activity and, in fact, has an enhanced k_{cat} compared to the intact bifunctional enzyme (Table 1). This high activity results in a greater than 20-fold increase in k_{cat}/K_m for the separated monofunctional AK I when compared to the same catalytic activity in the intact bifunctional enzyme.

The remaining portion of the *thr*A gene, called *thr*A_2^+, consists of the homoserine dehydrogenase catalytic domain along with the interface region. Expression of this gene fragment produces a monofunctional HDH I$^+$ that is also highly active. There is a greater than 10-fold increase in k_{cat} and a 2-fold decrease in K_m that, as with the AK I domain, also leads to a greater than 20-fold increase in k_{cat}/K_m for the separated monofunctional HDH I$^+$ compared to this activity in the native bifunctional enzyme (Table 2).

To examine the effect that the presence of the interface region has on the catalytic activity of the HDH domain, the interface was removed from this monofunctional HDH. This gene fragment, called *thr*A_2, encodes for only the 41 kDa HDH I domain (Figure 1). This separate catalytic domain has a lower k_{cat} once the interface region is removed and a K_m for homoserine that has increased by 25-fold. These changes result in a decrease of 2 orders of magnitude in k_{cat}/K_m upon deletion of the interface region. However, because of the enhanced activity of HDH I$^+$, this diminished catalysis

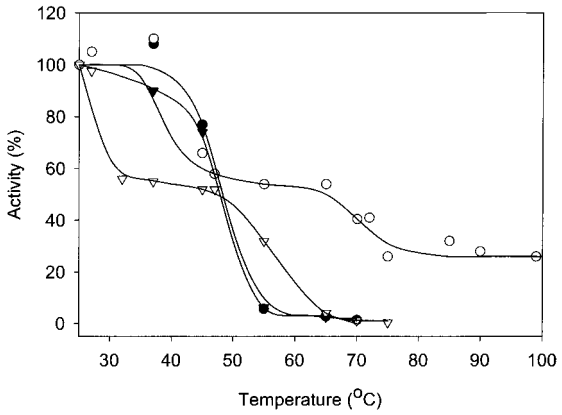


FIGURE 2: Stability of the aspartokinase activities of the various enzyme forms: (●) AK-HDH I; (▼) AK I; (▽) AK III; (○) AK III-HDH I$^+$. Each enzyme form was incubated at the prescribed temperature for 5 min in a Peltier-controlled thermocycler. Aliquots were removed after each incubation and assayed for catalytic activity as described in Experimental Procedures.

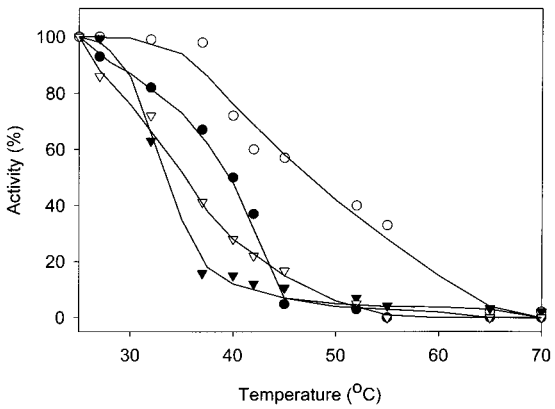


FIGURE 3: Stability of the homoserine dehydrogenase activities of the various enzyme forms: (●) AK-HDH I; (○) HDH I$^+$; (▼) HDH I; (▽) AK III-HDH I$^+$. The thermal stability studies were conducted as described in Figure 2.

is only 7-fold lower than that measured for the native bifunctional enzyme (Table 2).

Properties of the Monofunctional Enzymes. The quaternary structure of these separated catalytic domains was examined by native gel electrophoresis. HDH I$^+$ gives a single band on PAGE at the expected position for the tetramer. AK I appears to exist in an equilibrium, with nearly identical populations of the dimer and tetramer forms of the enzyme present.

The stability of each of the new monofunctional enzymes was determined and compared to that of the intact bifunctional enzyme. Each enzyme form was incubated at increasing temperatures, as described in Experimental Procedures, and the residual activity was measured. An examination of the activities of intact AK-HDH I as a function of temperature (Figure 2, filled circles) shows that the kinase activity of this enzyme is stable up to about 40 °C. Above this temperature the enzyme begins to lose activity, with complete inactivation of the kinase activity observed by 55 °C. The activity of monofunctional AK I follows the same thermal inactivation profile (Figure 2, filled triangles) as that of the intact bifunctional enzyme. The homoserine dehydrogenase activity of the native bifunctional enzyme is less stable, losing half of its catalytic power by 40 °C, and is completely inactivated at 45 °C (Figure 3, filled circles). Removal of both the kinase and interface domains leaves a monofunctional HDH I that is further destabilized, losing half of its activity at 30 °C (Figure 3, filled triangles). However, HDH I$^+$, with the interface region still attached, is more stable than either the separated catalytic domain or the intact bifunctional enzyme and does not become completely inactivated until temperatures above 60 °C (Figure 3, open circles).

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 (Table 1). The separated AK I domain has lost this allosteric regulatory capacity and is no longer inhibited by threonine even at concentrations as high as 200 mM. The separated HDH I domain with the interface region attached is still regulated and has become even more sensitive to threonine inhibition once the AK catalytic domain is removed (Table 2). However, further truncation, by removal of the interface region, causes a greater than 400-fold decrease in the sensitivity of monofunctional HDH I to feedback inhibition by threonine.

Construction of an AK-ASADH Bifunctional Enzyme. Now that stable and active monofunctional enzymes have been produced, these separate catalytic domains can be combined to generate new bifunctional enzymes. The separated *thr*A$_1$ gene fragment that encodes for AK I has been joined to the *asd* gene that encodes for ASADH to create a new bifunctional enzyme that, if fully active, should now be capable of catalyzing successive metabolic reactions. The genes were joined in-frame, as described in Experimental Procedures, with the 110-base segment from the interface region of AK-HDH I providing the interface to the *asd* gene (Figure 1). The new bifunctional construct was overexpressed in *E. coli* TG1 cells and purified using the procedure that was established for the purification of ASADH (*5*). Homogeneous enzyme was produced by this purification scheme despite the presence of the additional AK domain. Joining AK I to ASADH results in a 5−6-fold decrease both in k_{cat} and in k_{cat}/K_m when compared to the isolated monofunctional enzyme (Table 1). However, despite this decrease, these values are still 4-fold higher than those observed in the native bifunctional enzyme. In contrast, the k_{cat} of ASADH decreases by over 300-fold, but the K_m for ASA is unaffected by the fusion of the two catalytic domains (data not shown).

Construction of a Hybrid AK III-HDH I Bifunctional Enzyme. To examine the mechanism of feedback inhibition, a hybrid bifunctional enzyme was prepared by joining the *lys*C gene that encodes for the monofunctional AK III with the *thr*A$_2^+$ gene that encodes for HDH I including the interface domain (Figure 1). This new AK III-HDH I$^+$ bifunctional enzyme shows a 7-fold decrease in the kinase activity, as measured by both k_{cat} and k_{cat}/K_m, when compared to the native monofunctional AK III (Table 1). However, this hybrid enzyme has dramatically improved kinetic parameters for HDH activity. The k_{cat} is increased by 2 orders of magnitude, and the K_m for homoserine has decreased by a factor of 3 when compared to the parameters of the native bifunctional enzyme.

Properties of the New Bifunctional Enzymes. In addition to the capacity to catalyze consecutive reactions in the case of AK-ASADH, the newly created bifunctional enzymes have altered stabilities and regulatory properties when

compared to those of the native enzymes. Native, monofunctional AK III shows a biphasic thermal inactivation, losing about 40% of its activity after a 5 min incubation near 30 °C. The remaining catalytic activity is unaffected by increasing temperatures up to 50 °C and does not become completely inactivated until 65 °C (Figure 2, open triangles). The dehydrogenase activity of the hybrid bifunctional AK III-HDH I^+ enzyme is not stabilized by fusion to the more thermally stable AK III but, in fact, becomes somewhat less stable (Figure 3, open triangles) when compared to the stability of the native bifunctional enzyme. In contrast, the activity of AK III is further stabilized by fusion to HDH I. A biphasic inactivation is still observed for the fused AK III; however, the initial 40% loss of activity now occurs above 40 °C, with the final phase of denaturation taking place around 70 °C. Even more surprising is the retention of about 20% residual activity by this hybrid enzyme at temperatures approaching 100 °C (Figure 2, open circles).

Since the newly created monofunctional AK I has lost the regulation of its catalytic activity by L-threonine, neither of the catalytic activities of the bifunctional AK I-ASADH fusion enzyme are sensitive to threonine inhibition. 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 inhibition of the dehydrogenase activity by threonine this activity has now also become quite sensitive to inhibition by lysine (Table 2).

DISCUSSION

Generation of Monofunctional Enzymes. Bifunctional enzymes have been proposed to arise during evolution by the gene fusion of sequentially related monofunctional enzymes. A recent example of such a fusion event is the discovery of a yeast mutant that contains a new bifunctional enzyme in histidine metabolism (*11*). Here a frame-shift mutation causes a read-through of the normal stop codon at the end of the *his*D gene that encodes for histidinol dehydrogenase. With an additional insertion following the aborted stop codon the subsequent *his*C gene can be read in-frame, leading to a fusion of the dehydrogenase to the aminotransferase to form a new bifunctional enzyme. For more ancestral fusion events the subsequent alterations and mutations that could occur during evolution would be expected to lead to a highly integrated bifunctional enzyme that could prove difficult to deconvolute into its original separate catalytic components. However, earlier proteolytic cleavages of the bifunctional AK-HDH enzymes into fragments that retained some catalytic activity suggested that, in this case, reversal of this ancestral gene fusion event may be possible.

Treatment of AK-HDH I with trypsin led to the isolation of a separate protein fragment that still possessed some residual aspartokinase activity (*12*). Similar proteolysis of AK-HDH II with subtilisin resulted in the formation of a monofunctional homoserine dehydrogenase fragment (*13*). Encouraged by these earlier results, we undertook the separation of these catalytic activities at the genetic level. Each of these catalytic activities in AK-HDH I has now been expressed as highly active and stable monofunctional enzymes. In contrast to the 70−95% loss of activity that was observed on proteolytic cleavage, expression of the respective gene fragments led to purified catalytic domains with enhanced activity, with the k_{cat}/K_m for both the AK and the HDH activities increasing by over 20-fold.

The homoserine dehydrogenase domain with the attached interface region (Figure 1, HDH I^+) is found as a fully functional tetramer, suggesting that a major portion of the subunit contacts are retained in this domain. In contrast, the AK I catalytic domains were observed to be partially dissociated into dimers. The aspartokinase activity of native AK-HDH I is more stable than the homoserine dehydrogenase activity, with negligible activity loss in AK activity until the incubation temperatures exceed 40 °C. However, over 70% of the HDH activity in the native, bifunctional enzyme is lost when incubated at the same temperature. The thermal stability of AK I is unaffected by its separation from the native bifunctional enzyme. The separated monofunctional HDH I^+ is stabilized while it remains covalently attached to the interface domain. In fact, the stability profile of HDH I^+ resembles that of the more thermally stable AK I activity. However, when this HDH catalytic domain is expressed in the absence of the interface, this domain is dramatically destabilized, with negligible residual activity at temperatures above 40 °C.

Formation of New Bifunctional Enzymes. Combining the gene fragment that encodes for the separate AK domain with the gene for the intervening enzyme in this pathway leads to a new bifunctional enzyme. While not unprecedented, there have been relatively few examples of artificial bifunctional enzymes created by gene fusion. Dihydrofolate reductase was fused to the N-terminus of thymidylate synthase by a five amino acid linker (*14*) to mimic a naturally occurring bifunctional enzyme that is found in protozoa and plants (*15*). A carboxy-terminal truncated aminocyclopropane-1-carboxylate (ACC) synthase has been fused with an amino-terminal truncated ACC oxidase to create an active bifunctional enzyme that can cleave *S*-adenosylmethionine to directly produce ethylene (*16*). In some cases attempts at gene fusion have not succeeded because of the instability of one of the enzymes or because of the inability of one of the enzymes to correctly fold after fusion. The inclusion of a variable linker between the respective genes has been examined as a means to provide a flexible tether to join two enzyme domains. Citrate synthase has been fused to malate dehydrogenase by a flexible three amino acid, Gly-Ser-Gly, linker (*17*). There is little change in the kinetics of these activities upon fusion (*18*), and this new construct has been shown to restore the growth ability of yeast that is deficient in both catalytic activities. β-Galactosidase has been fused to several different enzymes that are involved in galactose metabolism (*19*). The fusion with galactose dehydrogenase leads to a bifunctional enzyme in which the β-galactosidase activity is essentially unchanged; however, the dehydrogenase activity is somewhat diminished. Both the stability and the catalytic activity of galactose dehydrogenase can be enhanced when fused with a linker of increasing chain length (*20*). Several linkers, ranging from 3 to 13 amino acids, were examined. The shortest linker leads to fused enzymes with lower stabilities and an elevated K_m for galactose compared to the native monofunctional enzymes, while optimal activity was achieved with a 9 amino acid linker.

With the fusion of AK to ASADH the aspartokinase enzymes already contain a nearly 40 amino acid interface fragment (in the case of AK I), or a 188 amino acid regulatory domain (in the case of AK III), that should serve as a flexible linker to allow proper folding (Figure 1). In addition, the high-resolution structure of ASADH indicated that the carboxy terminus is solvent exposed (*21*) and is therefore accessible for covalent linkage. In each case that was attempted between these respective genes, fusion has led to properly folded catalytic domains. Each of these new bifunctional enzymes is fully active, with kinetic parameters that are, in most cases, comparable to or even exceeding those of the native enzymes.

Enzyme Regulation. Previous studies (*22*) of AK-HDH I that had been denatured by treatment with guanidinium chloride showed that protein refolding and recovery of catalytic activity occur in three distinct stages. Initial refolding of the monomer leads to the recovery of AK activity that is not sensitive to threonine inhibition. Next, dimerization allows recovery of HDH activity and, simultaneously, the recovery of threonine regulation of both catalytic activities. Finally, the dimers associate to form stable tetramers. Since the restoration of AK activity in the native bifunctional enzyme takes place independently of allosteric regulation, it is not surprising that monofunctional AK is not sensitive to threonine inhibition. Similar results had been observed with a proteolytic fragment of AK-HDH I that retained a very low level of AK activity (*12*).

The HDH I domain retains its sensitivity to threonine regulation in the presence of the interface region. In fact, this monofunctional enzyme is now exquisitely sensitive to threonine, with a K_i value that is 6-fold lower than that of the native bifunctional enzyme. Removal of this interface region leads to a dramatic diminution, but not the elimination, of this threonine sensitivity. Even in the absence of the interface region, which is the presumed site of threonine binding (*10*), the HDH activity is still weakly inhibited by L-threonine. This inhibition is stereospecific, since D-threonine is not an inhibitor of the HDH domain when examined at concentrations up to 100 mM. These results suggest that the allosteric site of AK-HDH I is located at or near the domain interface or at least that structural elements within the HDH catalytic domain contribute to the formation of the allosteric site. This observation is consistent with the strong coupling between the recovery of HDH activity upon dimerization and the return of allosteric regulation (*22*).

The structure of a monofunctional form of HDH from yeast has recently been determined (*23*). This enzyme is not subject to feedback inhibition by threonine (*24*) despite having a high sequence homology to the HDH catalytic domain from *E. coli*. However, the start of the yeast HDH does not include the first 30 or so amino acids that are present in the monofunctional *E. coli* HDH. This missing region in yeast corresponds to the sequence in *E. coli* that is proposed to be involved in threonine binding.

The retention of lysine regulation of AK III, and threonine regulation of HDH I, in the hybrid bifunctional enzyme provides further support for the intact structure of these catalytic domains in this chimeric construct. The AK III activity is oblivious to the binding of threonine at its regulatory site in the HDH I domain. However, the binding of lysine at the AK III regulatory site leads to structural changes that are felt both at the AK III active site within the catalytic domain and at the HDH I active site in the adjacent domain. Whether this new site−site communication takes place within the hybrid bifunctional subunit or across subunit boundaries remains to be established.

Now that active bifunctional enzymes have been created that catalyze consecutive reactions in the aspartate biosynthetic pathway, we are in a position to assess whether these artificial bifunctional enzymes can convey distinct advantages to the organism in terms of enhanced catalytic activities, improved potential for the channeling of metabolic intermediates, or altered regulation of this branched pathway.

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