Production and Characterization of Bifunctional Enzymes. Substrate Channeling in the Aspartate Pathway†

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Received October 31, 2001; Revised Manuscript Received January 23, 2002

ABSTRACT: The direct channeling of an intermediate between enzymes that catalyze consecutive reactions in a pathway offers the possibility of an efficient, exclusive, and protected means of metabolite delivery. Aspartokinase−homoserine dehydrogenase I (AK-HDH I) from *Escherichia coli* is an unusual bifunctional enzyme in that it does not catalyze consecutive reactions. The potential channeling of the intermediate β-aspartyl phosphate between the aspartokinase of this bifunctional enzyme and aspartate semialdehyde dehydrogenase (ASADH), the enzyme that catalyzes the intervening reaction, has been examined. The introduction of increasing levels of inactivated ASADH has been shown to compete against enzyme−enzyme interactions and direct intermediate channeling, leading to a decrease in the overall reaction flux through these consecutive enzymes. These same results are obtained whether these experiments are conducted with aspartokinase III, a naturally occurring monofunctional isozyme, with an artifically produced monofunctional aspartokinase I, or with a fusion construct of AK I-ASADH. These results provide definitive evidence for the channeling of β-aspartyl phosphate between aspartokinase and aspartate semialdehyde dehydrogenase in *E. coli* and suggest that ASADH may provide a bridge to channel the intermediates between the nonconsecutive reactions of AK-HDH I.

The channeling of intermediates between consecutive enzymes in a metabolic pathway can offer significant advantages to an organism. Unstable, reactive intermediates can be protected from solvent and directly transferred from one active site to the next to minimize their degradation. Channeling can also be used to direct the flux of a pathway by eliminating the competition from other enzymes for common metabolic intermediates. Substrate channeling would also seem to present some distinct kinetic advantages in substrate delivery as compared to the free diffusion of these intermediates.

There are a number of well-characterized examples of substrate channeling within multifunctional enzymes. 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 (*2*) or phenylalanine (*3*). Carbamoyl phosphate synthase protects three reactive intermediates, ammonia, carbamate, and carboxyphosphate, channeling each of them through a portion of a contiguous 96 Å tunnel from their respective sites of production leading to the final formation of carbamoyl phosphate (*4*). The bifunctional enzyme glutamate formimidoyl transferase−cyclodeaminase has been proposed to channel N^5-formiminotetrahydrofolate in consecutive reactions in the histidine degradative pathway. A recent structure of the transferase domain shows the presence of an electrostatic tunnel through the domain that is postulated to be responsible for channeling the folate intermediate (*5*). Folate is also a channeled intermediate in the bifunctional enzyme dihydrofolate reductase−thymidylate synthase that is found in the *de novo* biosynthetic pathway for 2′-deoxythymidylate in protozoa and certain plants. Here the channeling is postulated to occur not through a tunnel that is located in the interior of the protein but along an "electrostatic highway" on the protein surface (*6*, *7*).

The proposed channeling of intermediates between separate, soluble enzymes has been a more contentious issue. Several papers have reported the channeling of reducing equivalents, in the form of reduced pyridine nucleotide, between stereospecifically related enzyme pairs such as between glycerol-3-phosphate dehydrogenase and lactate dehydrogenase (*8*, *9*). Evidence for the channeling of oxaloacetate (OAA)[1] between aspartate aminotransferase and malate dehydrogenase has also been reported; however, the conclusions that were drawn from these studies have been challenged (*10*, *11*). Evidence has also been presented to support the channeling of OAA between malate dehydrogenase and citrate synthase both in a poly(ethylene glycol)

† This work was supported by a grant from the National Science Foundation (MCB0196107).

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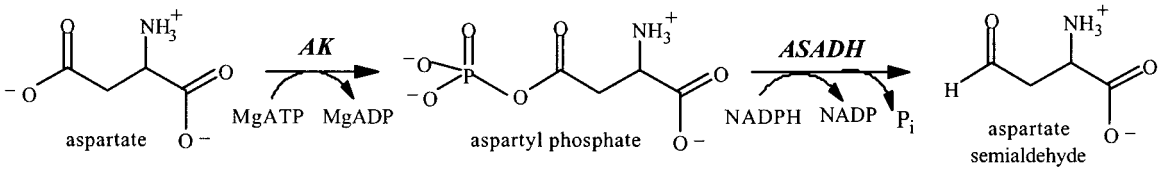
[1] Abbreviations: AK, aspartokinase; AK III, monofunctional aspartokinase, isozyme III; AK-HDH I, aspartokinase−homoserine dehydrogenase I; AMP-PNP, 5′-adenylyl imidodiphosphate; ASADH, aspartate-β-semialdehyde dehydrogenase; BSA, bovine serum albumin; DEPC, diethyl pyrocarbonate; DTNB, 2,2′-dithiobis(5-nitrobenzoate); HDH, homoserine dehydrogenase; OAA, oxaloacetate; SMCS, *S*-methylcysteine sulfoxide.

10.1021/bi0159074 CCC: $22.00
Published on Web 02/16/2002

Scheme 1: Enzyme-Catalyzed Conversion of Aspartate to Aspartate Semialdehyde



precipitate of the free enzymes (*12*) and in a fusion protein between these two enzymes (*13*). The evidence in support of substrate channeling in several of these enzyme systems has recently been reviewed (*14*).

In these studies of substrate channeling between separate enzymes the supporting evidence has been obtained through kinetic studies, by the trapping of intermediates, or by isotope dilution studies. The kinetic approach examines the effect of the addition of the second enzyme in the putative channeling pair on either the rate of buildup or the rate of depletion of the intermediate (*15*). Trapping studies use the presence of an additional enzyme to divert any intermediate that is released (*12*), and dilution studies examine the washout of radiolabeled intermediate in the presence of an excess of unlabeled intermediate once it is produced from the labeled substrate (*16*). Each of these approaches requires extensive controls to be run and, unfortunately, does not always provide a definitive answer. Recently, a new approach has been developed in which the effects of adding excess levels of an inactivated form of the second enzyme are examined in a putative channeling pathway (*17*). Intermediate channeling requires at least some transient contact between the enzyme pair, and the presence of high levels of an inactive enzyme form should compete with, and can potentially eliminate, any contact and therefore any channeling. We have applied this approach to examine the possibility of channeling of the reactive intermediate β-aspartyl phosphate between several forms of aspartokinase that produce this intermediate and aspartate β-semialdehyde dehydrogenase, the next enzyme in the pathway that converts this intermediate to the aldehyde (Scheme 1). The aldehyde product is then partitioned down several branches of this biosynthetic pathway, leading to the end product amino acids lysine, threonine, isoleucine, and methionine.

EXPERIMENTAL PROCEDURES

Materials. Substrates, buffers, ATP, 2,2′-dithiobis(5-nitrobenzoate) (DTNB), guanidine hydrochloride, and other reagents were obtained from Sigma. *S*-Methyl-L-cysteine sulfoxide (SMCS) was obtained from Karlan Research Products, and Centricon spin concentrators were purchased from Amicon.

Enzyme Preparation. Aspartokinase (AK) (*18*) and aspartate semialdehyde dehydrogenase (ASADH) (*19*) were prepared as previously described. The inactive C135A and low activity C135S mutants of ASADH were prepared by recombinant circle PCR as described earlier (*20*). The AK-ASADH bifunctional enzyme was created by fusion of the respective genes after the AK I domain was separately expressed (*21*). A mutant of ASADH was created in which a portion of the loop consisting of amino acids 171−205 was removed. The *asd* gene was treated with the restriction enzyme *Msl*I, purified by gel electrophoresis, and then blunt end religated in-frame to eliminate the codons for the 12 amino acids from His-171 to Leu-182. Competent TG1 *Escherichia coli* cells were transformed with the pGEM7Zf vector containing the truncated gene insert. Cells were grown, and the mutant enzyme was purified as described previously for the wild-type enzyme.

Enzyme Assays. Enzyme activities were measured on a Perkin-Elmer Lambda-1 spectrophotometer equipped with a thermostated cell holder connected to a circulating water bath. AK activity was assayed either by following the production of ADP using a pyruvate kinase/lactate dehydrogenase coupled assay or by following the production of β-aspartyl phosphate using ASADH as the coupling enzyme. ASADH activity was assayed by following either the appearance or the disappearance of NADPH at 340 nm. Assays were conducted at 25 °C with 120 mM Hepes−Tris buffer, pH 8.6, 200 mM KCl, and saturating levels of substrates in a total volume of 1 mL. Protein concentrations were determined by the method of Bradford (*22*).

Enzyme Inactivation. ASADH was inactivated by treatment of 2 mg of enzyme with a 100 μM solution of DTNB for 60 s to modify the essential active site cysteine nucleophile (*23*), followed by centrifugation using a Centricon-10 spin filter concentrator to remove the excess DTNB. Alternatively, 100 μg of ASADH was incubated at room temperature for 20 min with 190 mM NADP and 200 mM SMCS to form a ternary complex in which the active site cysteine is selectively modified (*24*). The inactivated enzyme mixture was then washed with 100 mM Tris-HCl (pH 7.0) and concentrated in a Centricon-10 spin concentrator to remove excess reagent. AK III was inactivated by treatment with 4 mM diethyl pyrocarbonate (DEPC) for 1 h, followed by concentration in an Amicon-30 concentrator and washing to remove any unreacted reagent.

Substrate Channeling Assay. The conversion of aspartate to ASA was measured by the coupled AK/ASADH assay at from 5 to 50 nM enzyme concentrations, with the relative amount of each enzyme adjusted to give comparable catalytic activities under the assay conditions. Assays were conducted in 100 mM Tris buffer, pH 8.6, with 200 mM KCl, 4 mM MgATP, 15 mM L-aspartic acid, and 100 μM NADPH. To test for channeling, increasing amounts of an inactivated form of ASADH (DTNB inactivated, SMCS inactivated, or C135 mutants) were added, and the rates of ASA production were measured. Controls, including the addition of a noncatalytic protein (bovine serum albumin) or active ASADH, were run to establish the specificity of any suppression of ASA production that is observed.

RESULTS

Suppression of Substrate Channeling from Native AK by Inactive ASADH. The rate of production of ASA from aspartate was determined with fixed, equimolar concentra-

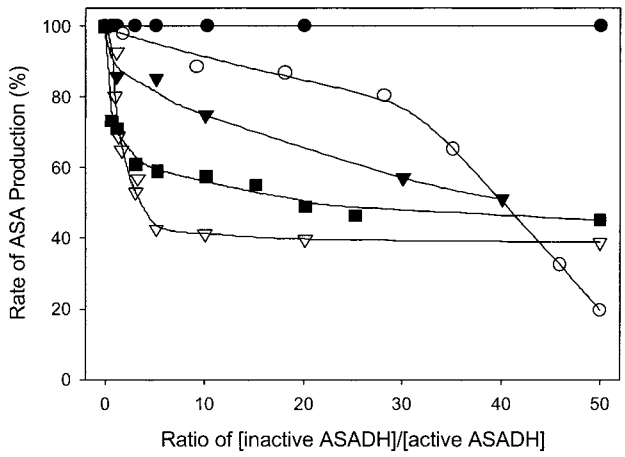


FIGURE 1: Effect of increasing levels of inactivated ASADH on the overall rate of production of ASA. Various inactivated forms of ASADH were titrated into a reaction mixture containing equimolar amounts of AK III and ASADH. The rate of production of ASA from aspartic acid was measured by the increase in absorbance at 340 nm. Symbols: (●) BSA control; (■) C135A; (▽) C135S; (▼) SMCS-inactivated ASADH; (○) DTNB-inactivated ASADH.

tions of AK and ASADH. Once this base rate had been established, an inactivated form of ASADH was titrated into this reaction mixture to determine if there was an effect on the overall rate of conversion of aspartic acid through β-aspartyl phosphate to ASA by these two enzymes. The addition of C135A ASADH, an inactive mutant in which the active site cysteine nucleophile has been replaced by alanine (*20*), caused a decrease in the rate of production of ASA. At a 1:1 ratio of inactive (C135A) to active ASADH the production of ASA had decreased to about 70% of the base rate in the absence of the inactive enzyme. Further titration with C135A ASADH resulted in an additional decrease in the rate that eventually plateaued at about 40% of the original rate at a 5:1 ratio of inactive to active ASADH (Figure 1). Titration with C135S, an active site mutant with very low catalytic activity, has similar effects on the overall rate, as did C135A. Titration with active ASADH instead of the inactive mutant resulted in a slight increase in the overall rate of conversion (data not shown) while titration with a noncatalytic protein (BSA) had no effect on the conversion rate even at a 50:1 molar excess over active ASADH (Figure 1).

The maximal effect on the overall rate of ASA production is seen at a severalfold molar excess of inactivated ASADH. The mutant forms of ASADH (C135S and C135A) possess little or no catalytic activity but are still capable of binding substrates and products. It is possible that the suppression that is observed in ASA production is simply a consequence of an increasing fraction of the β-aspartyl phosphate intermediate being sequestered by the increasing amount of inactive enzyme, thus lowering the effective concentration that is available as a substrate for the active ASADH. The highest concentrations of inactivated ASADH never exceeded about 1 μM, which is the maximum amount of aspartyl phosphate ($K_i = 2$ μM) (*19*) that could be bound. However, if sequestering this intermediate was the primary mode of suppression of ASA production, then the time course for these reactions would show an initial lag until aspartyl phosphate accumulates to a sufficient level to support the steady-state rate of ASADH. The time courses for these coupled assays were linear throughout the measured period, suggesting that any binding of this intermediate by these ASADH mutants must have a negligible effect on the overall rate. To further test the possible effects of intermediate binding, these experiments were repeated using wild-type ASADH that had been covalently inactivated by treatment with either the active site directed inactivator SMCS or the sulfhydryl reagent DTNB. These modifications of the active site cysteine prevent substrate binding. Titration with SMCS-modified ASADH also leads to a decrease in the rate of production of ASA in the coupled AK-ASADH reaction (Figure 1). This decrease requires a higher concentration of the inactivated enzyme but does eventually plateau at a rate similar to that observed with the mutant ASADHs. Titration with DTNB-inactivated ASADH has only a modest effect on the overall rate at low molar ratios but at higher concentrations leads to an even more dramatic inhibition of the rate of ASA production.

ASADH is a homodimer with an N-terminal nucleotide binding domain and a dimerization domain containing a 40-residue loop that is located between the two active sites (*25*). A mutant of ASADH was created in which a 12 amino acid portion of this loop was removed to test the impact that this may have on the interactions between AK and ASADH. This mutant retains the ability to dimerize and, despite a 60-fold decrease in k_{cat}/K_m, retains K_m values for ASA and NADP that are comparable to those of the native enzyme (James and Viola, unpublished results). These results demonstrate that the overall structure of this truncated mutant has not been dramatically altered by the excision of this loop. This mutant was then inactivated, as described above, by treatment with SMCS in the presence of NADP. In contrast to what was previously observed, titration with this SMCS-inactivated mutant was found to have no effect on the rate of ASA production in the coupled enzyme reaction, even when added at 50-fold excess over the active ASADH levels.

Reversal of Channeling Suppression. If the observed decrease in the rate of production of ASA is a result of the competition of inactivated ASADH binding to AK, then increasing the amount of active ASADH should overcome this inhibition. A 10-fold excess of either C135S or C135A ASADH was added to an equimolar mixture of active AK and ASADH. As before, the overall rate decreased to less than half of the uninhibited rate. Each of these mixtures was then titrated with increasing amounts of active ASADH to lower the molar excess of inactive ASADH. In each case, as the ratio of inactive to active ASADH was decreased, the rate of production of ASA increased, approaching the original uninhibited rate (Figure 2), thus demonstrating that this suppression is completely reversible.

Suppression of Substrate Channeling from Artificial AK Constructs. The channeling of substrates between AK and ASADH was examined from two artificial constructs, the AK I catalytic domain that had been excised from the AK-HDH I bifunctional enzyme and a new bifunctional enzyme created by joining the AK I catalytic domain to ASADH (*21*). Adding increasing amounts of the inactive C135A ASADH mutant to an equimolar mixture of the AK I catalytic domain and active ASADH leads to a similar decrease in the overall rate of production as was observed with AK III (Figure 3). For the artificial bifunctional enzyme

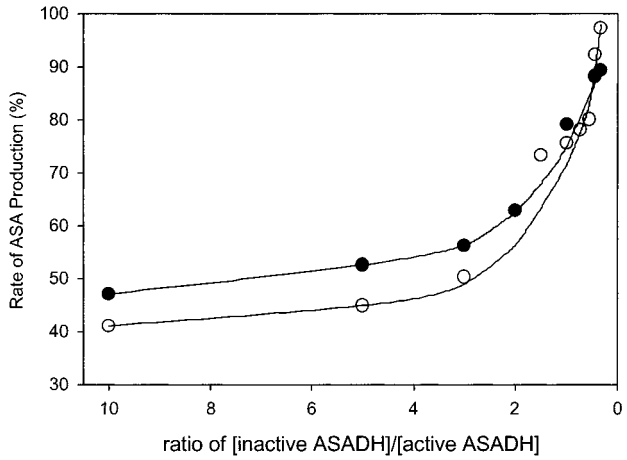


FIGURE 2: Addition of active ASADH to restore the overall rate of production of ASA. A 10-fold molar excess of either C135S ASADH (●) or C135A ASADH (○) was added to an equimolar mixture of AK III and ASADH. These mixtures were then titrated with active ASADH, and the rate of production of ASA from aspartic acid was measured.

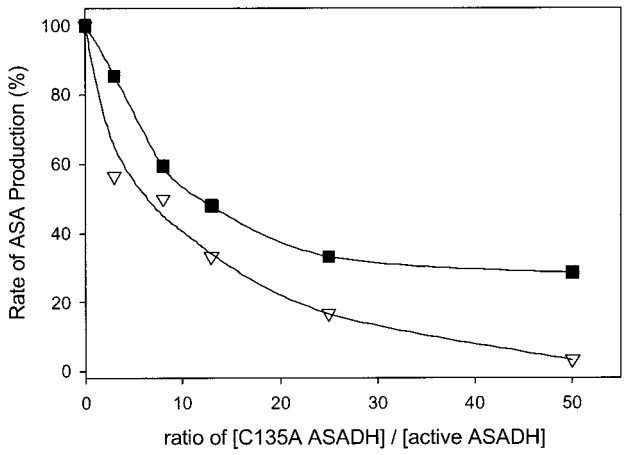


FIGURE 3: Effect of the addition of inactive C135A ASADH on the rate of production of ASA. The reaction mixture consists of an equimolar mixture of the separated AK I catalytic domain and ASADH (■) or a fixed concentration of the AK I-ASADH bifunctional enzyme (▽).

both catalytic activities now reside on the same polypeptide chain. However, even in this case the addition of inactive ASADH still results in the suppression of the rate of production of ASA. Here the suppression of ASA production is even more dramatic, with the rate at excess levels of C135A ASADH less than 5% that of the uninhibited rate.

Suppression of Substrate Channeling by Inactive AK. If substrate channeling is occurring between two enzymes, as has now been demonstrated between AK and ASADH, then the addition of an inactive form of either enzyme should compete for the interaction between the active enzyme pair and would suppress the rate of product formation. To test this hypothesis, AK III was inhibited by forming an abortive ternary complex with the ATP analogue 5′-adenylyl imidodiphosphate (AMP-PNP) and the competitive inhibitor α-aminobutyrate. This inhibited form of AK III was then titrated into an equimolar mixture of uninhibited AK III and ASADH. Increasing concentrations of inhibited AK III cause a substantial suppression in the overall rate of ASA production. At a 25:1 ratio of inhibited to uninhibited AK III, that ASA production rate is less than 10% of the original rate

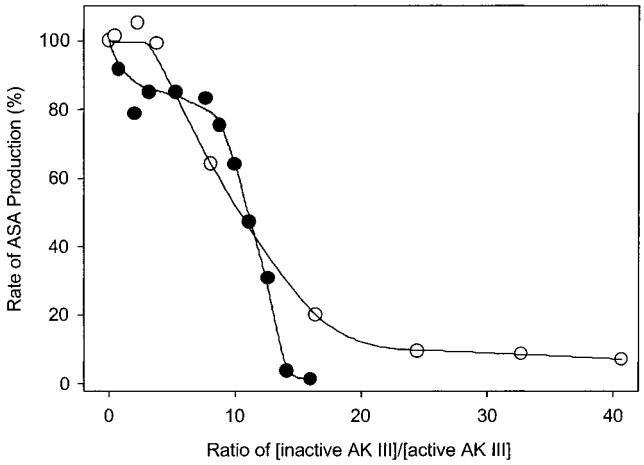


FIGURE 4: Effect of increasing levels of inactivated AK III on the overall rate of production of ASA: (○) AK III/AMP-PNP/α-aminobutyrate ternary complex; (●) DEPC-inactivated AK III.

(Figure 4). While these inhibitors form a tight-binding ternary complex with AK III, upon dilution into the reaction mixture any inhibitor that dissociates will bind to active AK III, and this could explain the overall suppression of ASA production that is observed. To eliminate this possibility, a sample of AK III was covalently inactivated with diethyl pyrocarbonate (DEPC). We had previously shown that AK III is sensitive to DEPC inactivation through the modification of several tyrosyl residues (*26*). Titration of the active AK III-ASADH mixture with DEPC-inactivated AK III also results in suppression of ASA production, and at a 15-fold excess of inactive AK III, the production of ASA is almost completely shut down (Figure 4).

DISCUSSION

Mechanisms of Substrate Transfer. For a coupled reaction between consecutive enzymes in a metabolic pathway there are several mechanisms by which the product of the first enzyme-catalyzed reaction can find its way to the active site of the second enzyme. Release of this product from the first enzyme will lead to an accumulation in bulk solution that can then freely diffuse to the second enzyme. Facilitated diffusion, either by a carrier molecule or by some constrained pathway, can potentially increase the transfer efficiency. Finally, substrate channeling in which this product is directly transferred from the first to the second enzyme without exposure to bulk solution would offer maximal transfer efficiency. Determination of the overall rate of a coupled enzyme reaction offers no insight into the mechanism of product transfer since each or all of the above mechanisms could be operating to some extent under the reaction conditions.

Suppression of Aspartyl Phosphate Channeling. If the overall rate of production of ASA is controlled by the free diffusion of the aspartyl phosphate product of AK to the active site of ASADH, then the addition of inactive enzyme into this reaction mixture should have no effect on this rate. However, the addition of increasing amounts of inactive ASADH does inhibit the rate of ASA production. This observation is taken as supporting evidence that the direct channeling of aspartyl phosphate between AK and ASADH must play at least some role in the *in vitro* mechanism of

intermediate transfer in this coupled reaction. To have direct channeling of a metabolic intermediate between consecutive enzymes in a pathway, there must be at least transient contact between these enzymes, even if a stable complex cannot be isolated. Given the large number of proteins that could potentially encounter each other in a cell, a specific interaction between a pair of enzymes that is designed to channel a metabolic intermediate would be expected to involve some surface complementarily that would lead to some selective recognition. The introduction of an inactivated form of one enzyme in this pair would interact with its active partner in a similar manner as long as the method of inactivation does not alter the complementary surface.

The maximal suppression of ASA production requires the addition of excess levels of inactive ASADH over active ASADH. Furthermore, this rate does not approach zero but reaches a limiting value that is unchanged even at a 50-fold excess of inactive enzyme. These results are consistent with both free substrate diffusion and direct substrate channeling operating in the transfer of the unstable metabolic intermediate aspartyl phosphate from AK to ASADH. On the basis of these results more than half of the flux between AK and ASADH must proceed *via* the direct substrate channeling mechanism.

For maximal channeling efficiency two enzymes should interact so as to align their respective active sites. This will serve to minimize the distances that must be traversed during the facilitated diffusion. The binding of inhibitors can alter the active site geometry of an enzyme. The more substantial this alteration, the more likely that the ability to interact with the subsequent enzyme in the pathway would be impaired. It is clear, from the different concentrations required among the inactived forms of ASADH to achieve suppression, that the method of inactivation can affect the ability of inactive ASADH to compete with active ASADH for binding to AK. The active site mutants of ASADH, C135A and C135S, are expected to have minimal overall structural perturbations. These enzyme forms cause suppression of β-aspartyl phosphate transfer at the lowest added concentrations. These mutants appear to have comparable affinity to that of active ASADH for binding to AK III, since the rate of ASA production decreases to about half of the maximal suppression at a 1:1 ratio of mutant to active ASADH. Inactivating ASADH with the active site directed inactivator SMCS in the presence of NADP alters the structure of ASADH by causing a domain rotation that leads to a closure of the active site (*24*). This domain movement must affect the binding of ASADH to AK, since a higher level of SMCS-inactivated ASADH is required to achieve comparable suppression of ASA production. Inactivation with DTNB, a bulkier reagent without any of the complementary functional groups of SMCS, would be expected to cause a greater structural perturbation, and the DTNB-inactivated enzyme requires the highest levels before affecting the rate of substrate transfer. In each case the competition between the various inactivated forms of ASADH for AK binding is noncovalent and can be readily reversed simply by adding higher levels of active ASADH.

The AK catalytic domain was fused in-frame to ASADH to create a bifunctional enzyme that is capable of catalyzing consecutive reactions (*21*). Since these two activities are now fused, it is possible that this artificial bifunctional enzyme can directly channel the intermediate aspartyl phosphate within the confines of the polypeptide chain. However, to achieve this channeling, the two active sites must be in close proximity and in the proper orientation. If these conditions were met, then the addition of inactivated ASADH would be expected to have no effect on the rate of internal channeling within this newly created bifunctional enzyme. The dramatic suppression of the rate of production of ASA from aspartate observed upon addition of the inactive C135A mutant (Figure 3) clearly establishes that substrate channeling does not take place within this bifunctional enzyme but must occur between different molecules of this fusion enzyme.

The high-resolution structure of AK has not been determined, so it is not yet possible to search for complementary surfaces on AK and ASADH that would suggest possible contact points for binding. An examination of the ASADH structure indicates the presence of several surface regions that could be involved in protein−protein interactions. In particular, there is a 40 amino acid loop located near the active site that provides part of the subunit contacts in the ASADH dimer (*25*). This loop is not present in the ASADHs from some species, although all of these enzymes are functional dimers. Removal of a 12 amino acid portion of this loop from the *E. coli* enzyme does not have a dramatic effect on the K_m values for each substrate. However, this mutant enzyme has now lost the ability to suppress the production of ASA in the coupled assay. This suggests that this loop provides at least part of the contact surface that interacts with AK to allow substrate channeling.

The suppression of ASA production in a coupled AK-ASADH enzyme reaction provides definitive evidence of some degree of substrate channeling between these enzymes in dilute aqueous solution. Channeling of aspartyl phosphate will minimize possible losses from hydrolysis in solution, thus resulting in more efficient metabolism. In the significantly more crowded environment within cells enzyme concentrations are much higher. The transient contacts that take place between these enzymes would be expected to be more frequent and more persistent under these conditions, leading to enhanced flux through this direct transfer mechanism. The presence of any additional proteins, as yet unidentified, that could interact with and stabilize these contacts would further enhance substrate channeling. The presence of a stabilized complex between AK-HDH and ASADH in the cell would allow ASADH to act as a bridge to channel and reduce the β-aspartyl phosphate product of the aspartokinase reaction and deliver aspartate β-semialdehyde to the homoserine dehydrogenase catalytic domain.

In contrast to the partial suppression of ASA production caused by inactivated ASADHs, the addition of inactivated AK results in nearly complete suppression; however, a greater excess of inactivated AK is required to achieve maximal suppression than was needed for the partial suppression by inactive ASADH. These results indicate that these modified forms of AK have structural alterations that lead to a lower affinity for ASADH than that of the native AK. The activity in the presence of covalently inactivated AK is less than 5% that of the uninhibited rate, suggesting that under the appropriate conditions substrate channeling between AK and ASADH can be responsible for the majority of the flux through this pathway. Since there are three AK isozymes in *E. coli*, their relative binding affinities to

ASADH, and how the binding of allosteric regulators to each isozyme alters that affinity, may provide an additional mode of regulation. Different protein−protein affinities will alter the flux through the different branches of the aspartate pathway and will therefore affect the relative levels of the end product amino acids.

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BI0159074