A Novel, Definitive Test for Substrate Channeling Illustrated with the Aspartate Aminotransferase/Malate Dehydrogenase System†

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ABSTRACT: A novel method is presented that establishes definitively the existence or nonexistence of direct metabolite transfer between consecutive enzymes in a metabolic sequence. The procedure is developed with the specific example of channeling of oxaloacetate between *Escherichia coli* aspartate aminotransferase (AATase) and malate dehydrogenase (MDH). The assay is carried out in the presence of a large excess of inactive variants of AATase. These mutants would outcompete the much smaller quantities of wild-type AATase for any docking sites on MDH and thus decrease the rate of the coupled L-aspartate to oxaloacetate to malate sequence only if the direct metabolite transfer mechanism is operative. The results show that oxaloacetate is not transferred directly from AATase to MDH because no decrease in rate was observed in the presence of ∼100 μM inactive mutants. This concentration is 10 times the physiological AATase concentration, which was determined in this work. The methodology can be applied generally.

Substrate channeling is defined by the transfer of the reaction product of one enzyme to the next in a metabolic sequence without equilibrating with the bulk fluid of the cell (see ref *1* for a review). Direct metabolite transfer should be advantageous where the intermediate (i) is chemically labile, (ii) can react with more than one enzyme in the cellular compartment, or (iii) reacts with the second enzyme with a diffusion-controlled rate constant. Although this process has frequently been invoked, definitive proof is lacking in all but a few cases. Thus, the goal of this research was to develop a diagnostic test for substrate channeling where both the positive and the negative result would be conclusive. This method was developed initially with aspartate aminotransferase (AATase)[1] and malate dehydrogenase (MDH) from *E. coli*.

AATase and MDH catalyze the following sequence of reactions:

L-aspartate + α-ketoglutarate ⟶ oxaloacetate + L-glutamate

oxaloacetate + NADH ⟶ malate + NAD^+

net: L-aspartate + α-ketoglutarate + NADH ⟶ malate + L-glutamate + NAD^+

Channeling of oxaloacetate (OAA) between the aminotransferase and dehydrogenase is teleologically reasonable because (i) OAA is chemically labile in that it can decarboxylate to pyruvate through a nonenzymatic process; (ii) OAA is a substrate for not only MDH but also for citrate synthase (CS); and (iii) the reaction catalyzed by AATase is partially diffusion limited (*2*).

EXPERIMENTAL PROCEDURES

Materials. Reagents were obtained from the following suppliers: L-aspartate and L-cysteine sulfinate, Aldrich; NADH and PLP, Sigma; α-ketoglutarate, Fluka; and buffers, Fisher and Research Organics. PPL−Asp was a gift from Michael Toney.

Preparation of WT and Mutant AATases and MDH. The R292D and $K258E_Q$ mutations in AATase have been previously described (*3, 4*). Both the WT and mutant forms of AATase were overexpressed using the plasmid pJO2 in an AATase-deficient strain of *E. coli*, MG204 (*his*-23(Am), *proB*, *trpA*-605(Am), *lac*I3, *lacZ*118(Oc), *gyrA*, *rpsL*, Δ*aspC*, Kan^r, *tyrB*, *RecA*::Tn10, *ilvE*), and purified (*4*). All preparations were at least 90% pure, as judged by SDS−PAGE. MDH was expressed in MG204 and purified as described elsewhere (*5*).

Activity Assays. AATase was routinely assayed at 25 °C in 200 mM HEPES, pH 7.5, 100 mM KCl, 30 mM L-aspartate, 4 mM α-ketoglutarate, 150 μM NADH, 0.4 μM MDH (i.e., 8.75 U/1 mL of assay), and 20 μM PLP. NADH oxidation was followed at 340 nm. One unit of activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of product/min.

Preparation of PPL−Asp WT AATase. The PLP form of freshly purified WT AATase (2 mg/mL; 46 μM) was converted to the PMP form via an overnight reaction with cysteine sulfinate (100 mM) in 200 mM HEPES, pH 7.5, and 100 mM KCl at 4 °C (*6, 7*). The 360 and 430 nm PLP enzyme absorption bands were converted to the 330 nm absorbance of the PMP form. Apoenzyme was prepared by performing multiple precipitations with ammonium sulfate at low pH (*8*). Specifically, (i) a saturated solution of

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[1] Abbreviations: AATase, aspartate aminotransferase; CS, citrate synthase; GPDH, glycerol-3-phosphate dehydrogenase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; OAA, oxaloacetate; PEG, poly(ethylene glycol); PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PPL−Asp, *N*-(5′-phosphopyridoxyl)−L-aspartic acid; TCA, tricarboxylic acid; WT, wild-type.

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Table 1: Determination of the Intracellular Concentration of AATase in *E. coli* HB101

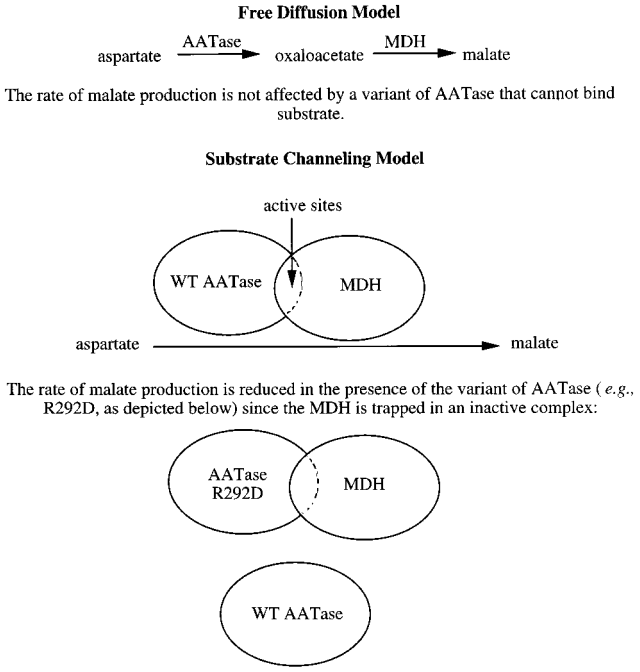
phase of culture during harvest	net U/mg[a]	ng of AATase/μg of total protein in crude extract[b]	no. of viable cells in culture $\times 10^{-10}$	μM AATase in each bacterial cell[c]
exponential	0.15	0.8	17	6 (Western blots) 6 (activity)
exponential	0.17	1.1	14	12 (Western blots) 11 (activity)
lag	0.14	1.6	2.2	40 (Western blots) 22 (activity)
lag	0.15	1.0	1.7	24 (Western blots) 23 (activity)

[a] Net U/mg = (activity in HB101 crude extract − activity in MG204 crude extract). The activity from the MG204 extract is attributed to NADH-mediated reduction of metabolites in the crude extract. [b] The ng of AATase/μg of total protein in crude extract was determined by densitometric analysis of Western blots. [c] The values indicated for cultures harvested in the lag phase are overestimates of the intracellular concentration of AATase. See text.

ammonium sulfate prepared in 100 mM potassium acetate, pH 4.9, was added to PMP-AATase to 40% saturation. (ii) Ammonium sulfate was then added to 75% saturation after 30 min on ice. (iii) The sample was centrifuged and resuspended in 100 mM potassium acetate, pH 4.9, after an additional 30 min on ice. Steps i−iii were repeated three times. The final sample was exhaustively dialyzed against 10 mM Tris, pH 7.0, and 1 mM EDTA. The apo form of AATase was reconstituted with PPL−Asp by incubating a 46 μM solution of the enzyme with 5 mM PPL−Asp in 50 mM HEPES, pH 7.5, at room temperature for 2 h, followed by a 2.5-h incubation on ice. Excess PPL−Asp was removed by dialysis against 50 mM HEPES, pH 7.5, and 1 mM EDTA. The formation of PPL−Asp WT AATase was confirmed by the appearance of an absorption peak at 330 nm. Although 100% of the apoenzyme appeared to be converted to the PPL−Asp form according to spectral analyses, the PPL−Asp AATase preparation exhibited 2% of WT activity, making it unsuitable for use in the channeling tests. This activity is presumably due to incomplete separation of PLP or PMP from the original WT AATase. The activity level was further reduced through the following procedure: PPL−Asp WT AATase (5 μM) was incubated with $NaBH_3CN$ (100 mM) in 50 mM HEPES−KOH, pH 7.5, and 1 mM EDTA for 4 h at room temperature. Following an overnight dialysis against 50 mM HEPES−KOH, pH 7.5, and 1 mM EDTA, the sample exhibited 0.01% of WT AATase activity in the coupled assay with MDH. A repeat of the above procedure reduced the activity to 0.006% of that of the WT enzyme activity. No NADH oxidation was observed in the absence of added aspartate.

Determination of the in Vivo Concentration of AATase. *E. coli* strains HB101 (F^-, *thi*-1, *hsdS*20 (r_B^-, m_B^-), *supE*44, *recA*13, *ara*-14, *leuB*6, *proA*2, *lacY*1, *rpsL*20 (Str^r), *xyl*-5, *mtl*-1) and MG204 were grown in 2xYT media and harvested by centrifugation while in the lag and exponential phases. The viable cell count was determined by plating dilutions of the cultures on LB media and by counting the colonies formed after 24 h of incubation at 37 °C. The values collected in Table 1 represent the number of viable cells present immediately prior to harvest. Crude extracts were prepared by sonication and ultracentrifugation. They were subjected to Bradford analyses, activity assays at 37 °C with the MDH coupled system described above, SDS−PAGE, and Western blotting. The primary and secondary antibodies were mouse anti-*E. coli* AATase (polyclonal, generated in this laboratory) and affinity-purified goat anti-mouse HRP conjugate (Bio-Rad), respectively. The ECL System (Amersham) was used for detection. Densitometric analyses of the Western blots were performed with the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) with several concentrations of pure WT AATase as internal standards. In the calculation of the concentration of AATase in each HB101 cell, the intracellular volume of *E. coli* was taken as 1.038×10^{-15} L (*9*, *10*).

Scheme 1: Two Models Describing the Transfer of OAA from AATase to MDH



RESULTS

Approach. The following method was developed to prove or disprove channeling definitively (Scheme 1): AATase and MDH were added to an assay mixture that included aspartate, α-ketoglutarate, and NADH. The rate of malate production was determined by monitoring the change in absorbance at 340 nm per unit time. Several subsequent assays were performed in which the concentrations of WT AATase and MDH were held constant, and increasing concentrations of inactive mutant AATase were added. The mutant should compete with the wild-type form of AATase for association with MDH, thereby sequestering the latter in a nonproductive

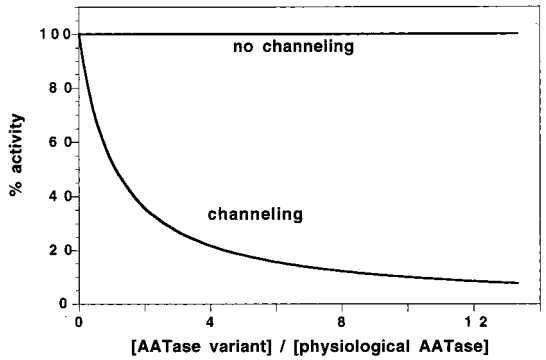


FIGURE 1: Theoretical plot depicting the two possible effects of an inactive variant of AATase on the initial rate of malate production in the channeling test. The channeling simulation was calculated for an AATase·MDH dissociation constant (K_d) equivalent to the physiological concentration of AATase. Lower values of K_d would yield a sharper decrease in activity while significantly higher values would be meaningless *in vivo*.

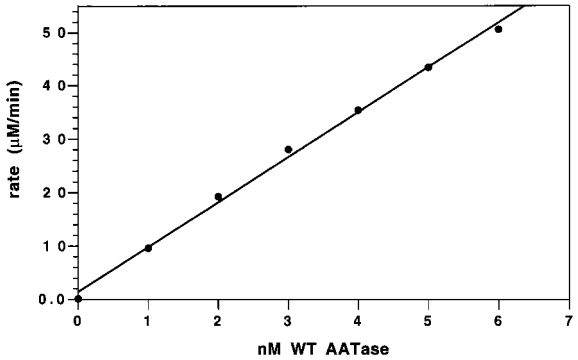


FIGURE 2: Linear dependence of the initial rate of malate production on the concentration of WT AATase. Assays were performed at pH 7.5 in 200 mM HEPES, 100 mM KCl, 30 mM L-aspartate, 4 mM α-ketoglutarate, 150 μM NADH, 10 nM MDH, and 20 μM PLP. The assay temperature was 37 °C, and NADH oxidation was monitored at 340 nm. The standard deviations were typically ±0.8 μM/min.

complex. Therefore, the rate of malate formation should decrease upon the addition of the site-directed mutant if channeling occurs (Figure 1). The presence of the mutant will have no effect on the rates in the absence of channeling. A lower limit for the dissociation constant (K_d) of the AATase/MDH complex can be determined in the absence of observed inhibition since extremely high concentrations of the mutant AATase may be added. The result of this experiment, whether positive or negative, thus provides a conclusive and quantitative answer as to the existence of channeling: if the lower limit of the K_d value is much greater than the *in vivo* concentrations of either AATase or MDH, channeling does not occur.

Two site-directed mutants of AATase were employed in this study: (i) R292D, which does not bind the dicarboxylic acid substrates. The k_{cat}/K_m value associated with this mutant enzyme is ~500 000-fold less than that reported for WT AATase (*3*). (ii) K258E$_Q$, which is a cysteineless version of AATase with the essential active site lysine converted to glutamate. The K_d for aspartate is 0.2 μM, a value that is 15 500 times *less* than the corresponding value with WT AATase, but the maximal rate of the half-transamination reaction with AATase-K258E$_Q$ is 1×10^{-7} that of WT (*4*). AATase-K258E$_Q$ thus binds the substrate aspartate tightly but does not transform it to OAA, making it an excellent mimic of the WT enzyme−substrate complex.

These variants are logical choices for the trap in the channeling tests because they are almost completely inactive. It is theoretically possible, however, that the part of the surface presented to MDH differs from that of the WT, making the R292D and K258E$_Q$ forms of AATase unsuitable selections. For example, substrate may need to be bound to AATase for proper recognition by MDH; this situation can be established adequately by AATase-K258E$_Q$ but not the R292D variant. To address this possibility further, the PPL−Asp form of WT AATase was prepared. PPL−Asp is an analogue of the substrate−coenzyme complex. The crystal structure of AATase containing this reduction product of the imine formed between PLP and aspartate has been solved. The β-carboxylate group of PPL−Asp is hydrogen-bonded to Arg292 and the indole nitrogen of Trp140 (*11*). Overall, the structure of this variant is very similar to that of the PMP form of AATase. Thus, it was possible to employ additionally a protein with the WT AATase structure as a competitive inhibitor in the channeling test.

In Vivo Concentration of AATase. While a decrease in NADH oxidation on addition of the catalytically deficient mutant of AATase may be construed as providing definitive evidence for direct OAA transfer from WT AATase to MDH, the conclusion from the negative result is only secure if the concentration of mutant employed is significantly greater than the physiological WT AATase concentration. There is no report of this value in the literature. *E. coli* HB101 was selected for this determination as it is a cross between the K-12 and B strains that have been extensively characterized. The AATase concentrations in cells in lag and in exponential phases of growth were determined by activity assays and Western blotting. The two methods yielded nearly identical results (Table 1). The crude extracts from the cultures contained 0.8−1.6 ng of AATase/μg of total cellular protein, independent of the phase of the culture. The AATase activity was approximately 0.15 U/mg in all cases. Therefore, the AATase concentration in exponentially growing cells is 6−12 μM. The micromolar values given in Table 1 for the lag phase cells are certainly an overestimate because, for the purposes of the calculations, all of the AATase present was assumed to originate solely from viable cells. Unlike exponentially growing cultures, lag phase cultures contain a large number of nonviable cells that likely contain AATase, making this micromolar value unreliable (*12*). Consequently, the concentration values obtained for the exponentially growing phase are considered more reliable.

Experimental Details of the Test for Channeling of OAA between AATase and MDH. The experimental rationale requires that mutant AATase compete with WT AATase for the putative docking site on MDH; therefore, the observed velocity must be dependent on AATase concentration. The data of Figure 2 show that this relationship is obeyed for 0−6 nM WT AATase with 10 nM MDH. Thus, a concentration of WT AATase within this range (2 nM) was employed in all subsequent tests for channeling. Additionally, the rate of malate production is independent of MDH concentration; identical rates were obtained in the presence of 10 and 50 nM MDH with 2 nM WT AATase. The other conditions are given in the legends to Figures 2 and 3.

Evidence for Lack of Channeling of OAA from Studies with Site-Directed Mutants of AATase. The OAA direct

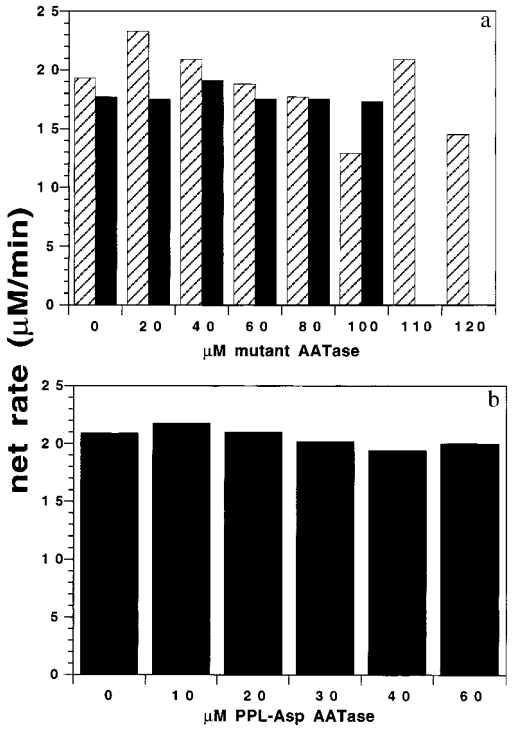


FIGURE 3: (Panel a) Tests for channeling of OAA using site-directed mutants of AATase as MDH scavengers. Solid bars, AATase-K258E$_Q$; hatched bars, AATase-R292D. (Panel b) Test for channeling with PPL−Asp WT AATase as a trap. The conditions were the same as depicted in Figure 2 except the WT AATase concentration was held constant at 2 nM and, in the assays containing PPL−Asp AATase, the residual PLP concentration was 50 pM rather than 20 μM. Net rate = (initial rate of malate production measured for an assay containing MDH, WT AATase, and AATase variant) − (initial rate due to MDH and the AATase variant).

transfer hypothesis was first investigated with the AATase-R292D trap as described above. The net rate of malate production is not significantly diminished by the addition of this mutant enzyme to concentrations greater than 10 times the physiological concentration of AATase (Figure 3a). The same result was obtained when the aminotransferases were preincubated with MDH at 25 °C for 10 min prior to assay (data not shown). Preincubation of the dehydrogenase with only the mutant or with only WT AATase also did not effect the net rate (data not shown). AATase-K258E$_Q$ also had no effect, plus or minus preincubation, when added to 11 times the physiological concentration of AATase (Figure 3a).

It is conceivable that an unknown cofactor in the *E. coli* cell is necessary to "cement" the AATase/MDH complex. To test this hypothesis, assays were carried out in the presence of crude extract of MG204 cells that were harvested from the exponential phase of growth. As shown in Figure 4, neither the R292D mutant nor the K258E$_Q$ mutant of AATase impacted the net initial rates of malate production in these assays, even when present at 60 μM.

Excess PPL−Asp WT AATase Does Not Depress the Rate of Conversion of L*-Aspartate to Malate.* The final test for channeling was performed in the presence of AATase containing the coenzyme−substrate adduct PPL−Asp. This experimental construct presents the WT AATase to MDH. The net rate of malate production is insensitive to added PPL−Asp WT AATase (Figure 3b). Preincubation studies in which this AATase variant and MDH were mixed and

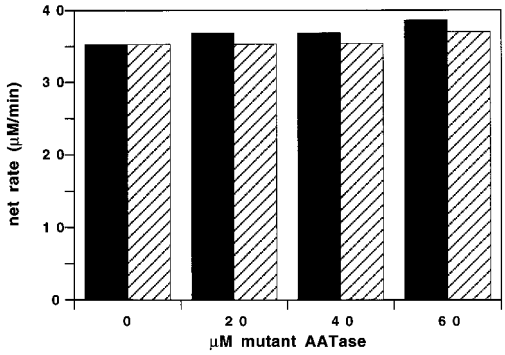


FIGURE 4: Tests for channeling in the presence of crude extracts of *E. coli* MG204 cells (0.36 mg of total cellular protein/1 mL of assay) to investigate the possibility that an unknown cellular cofactor is essential for channeling of OAA. Extracts were prepared by sonicating cells that were harvested in the exponential growth phase and subjecting the lysates to ultracentrifugation. The concentration of total protein in the supernatant was determined by Bradford assay. Channeling tests in the presence of this supernatant were performed as in Figures 2 and 3 except that WT AATase was present at a concentration of 5 nM and the only source of MDH was that contributed by the crude extract. Solid bars, assays in the presence of AATase-K258E$_Q$; hatched bars, AATase-R292D.

allowed to set at 25 °C prior to assay yielded the same results (data not shown).

DISCUSSION

Mechanisms of Substrate Channeling. Two basic, general mechanisms for substrate channeling have been proposed (*13*, *14*). In the direct transfer mechanism (i.e., a "perfect" channel), the intermediate is passed directly to the second enzyme of an enzyme−enzyme complex. This mechanism can occur with both stable and transiently formed enzyme complexes. A reviewer commented that channeling within an enzyme−enzyme complex that is *highly* unstable would escape detection by the scheme described herein. This hypothesis can be excluded for the AATase/MDH system on two grounds. (i) It requires that OAA dissociation from AATase be slow in the absence of MDH; however, the rate constant is 3.2×10^4 s^{-1}, and the kinetics describing the association of AATase with OAA are nearly diffusion-controlled (*2*). The putative capturing enzyme cannot accelerate a diffusion-controlled reaction. (ii) The formation of the thermodynamically unstable enzyme−enzyme complex requires that the reactions be first order with respect to *each* enzyme. This was not observed as the reaction is independent of MDH concentration (see Results).

The other general mechanism, sometimes referred to as the proximity mechanism or "leaky" channel, is operative for any coupled reaction where the second enzyme is locally concentrated near the first (for example, where they are bound at high density on a surface or are loosely associated in a large aggregate). The intermediate dissociates from the first enzyme but has a high probability of being captured by the proximal second enzyme. In both mechanisms, the diffusion of intermediates into the bulk fluid is impeded by the juxtaposition of the active sites and/or by steric hindrance.

Conclusive Examples of Channeling. Although substrate channeling has been difficult to prove unambiguously, a few clear examples do exist. Most of those cases involve single, multifunctional enzymes, and the three-dimensional structures have been solved. In tryptophan synthase, the two active

sites on different subunits are connected by a 25-Å hydrophobic tunnel through which the insoluble and uncharged intermediate, indole, passes (*15*). A second clear demonstration of channeling is provided by glutamine phosphoribosylpyrophosphate amidotransferase. The crystal structures of the inactive and active forms of this enzyme have been solved, and they indicate that activation results from the formation of a 20-Å hydrophobic channel that permits the transfer of ammonia between the two active sites (*16*). The channel is reported to be completely solvent inaccessible, thereby protecting the substrate, phosphoribosyl phosphate, from spontaneous hydrolysis and preventing the protonation of NH_3. Carbamoyl phosphate synthetase, another amidotransferase, provides a third well-established example (*17*). A 96-Å pathway can be visualized through the structure of this enzyme that traverses the three active sites. This channel provides safe haven for carboxyphosphate, carbamate, and ammonia; all of which are chemically unstable or volatile. Additional solution chemical evidence for channeling in this enzyme includes site-directed mutants that show complete uncoupling of the reactions (e.g., ref *18*).

Previous Channeling Studies Involving AATase and/or MDH. The channeling of metabolites produced by pig heart cytosolic AATase reactions has been investigated. Bryce et al. (*19*) reported two results that support direct transfer of OAA from AATase to MDH: (i) No lag phase was observed in the coupled reaction and (ii) the transfer of radioactivity from aspartate to malate was undiluted by unlabeled OAA in a rapid quench experiment. Manley et al. (*20*), however, reported that they were unable to reproduce the results of this earlier report, thereby casting doubt on the existence of channeling in this system and illustrating some of the difficulties routinely associated with such investigations. Channeling has also been investigated with the mitochondrial forms of AATase and glutamate dehydrogenase. Salerno et al. (*21*) observed a lag phase that was shorter in duration than predicted from the individual kinetic parameters thus supporting the channeling of α-ketoglutarate in this system. Fahien et al. (*22*) measured the steady-state rates of the glutamate dehydrogenase reaction under conditions where most of the α-ketoglutarate was bound to AATase. The observed rates were greater than those that could be accounted for by the concentration of free ligand.

Several channeling studies involving mitochondrial MDH and CS have also been reported. One approach used poly(ethylene glycol) (PEG) to aid the formation of complexes of the two enzymes. The inability of excess AATase (*23, 24*) and oxaloacetate decarboxylase (*24*) to compete effectively with CS for OAA in these PEG solutions provided evidence for channeling of OAA between MDH and CS. Datta et al. (*23*) also attempted to use lag phase studies for channeling detection but report inconclusive results. Lindbladh et al. (*25*) found that the OAA transient time in a MDH/CS fusion protein was less than that extant for the free enzymes and that AATase was a poor competitor for OAA in the assays with the fusion protein relative to those employing the free forms of the enzymes. OAA was therefore assumed to be less sequestered in the free enzyme system relative to the fusion construct. Simulations with a model of the fusion protein indicate that OAA may be channeled via an electrostatic mechanism (*26*). Recent ionic strength variation studies support this suggestion (*24*).

Difficulties Associated with Channeling Tests. Several *in situ*, *in vivo*, and *in vitro* methods have been developed to define the quantitative importance of channeling. Many of them, however, have limitations that cannot easily be overcome generally. Fusion proteins, for example, are artificial and may have their active sites oriented in an unphysiological fashion. Trap studies, like those described for CS and MDH, are not always feasible since a metabolite that is putatively channeled is not always used as substrate by more than one enzyme. In fact, most metabolites have only one cellular destiny (*27*). X-ray structure determinations are not always possible. The interpretation of lag phase and isotope dilution studies is problematic (see above). Many of the difficulties associated with channeling studies are readily illustrated by the glycerol-3-phosphate dehydrogenase/lactate dehydrogenase (GPDH/LDH) enzyme pair where direct transfer of NADH has been postulated (*28*) from the results of experiments carried out at high enzyme concentration where the free NADH concentration is low. The observed steady-state rates were too high to be satisfied by the concentration of free NADH. Moreover, the observed rate constant for the equilibration of NADH between GPDH and LDH is less than that predicted for a diffusion mechanism by numerical integration of the on and off rate constants associated with NADH and the enzymes. Later investigations refuted these conclusions based *inter alia* on uncertainties in the constants used by the Bernhard group (*29*). The question of channeling between phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase probed by a similar experimental rational is also controversial (*30, 31*).

Concluding Remarks. The new diagnostic described herein provides a valuable tool for channeling studies since, unlike methodologies such as the enzyme buffering approach, both the positive and the negative results are definitive. Only inactive mutants and an approximate knowledge of the *in vivo* concentration of at least one of the enzymes are required. A reduction in the net rate of malate production in the presence of the AATase variants should have been readily detected by this test if OAA were channeled between AATase and MDH, e.g., if the K_d for the AATase/MDH complex were equal to the *in vivo* concentration of 10 μM (Table 1), then the rate of the channeled reaction would be reduced by ~91% in the presence of 100 μM mutant (Figure 1). Although theoretical advantages exist from channeling OAA between AATase and MDH, this may be insufficient to dictate an imperative. It is possible, for example, that the steady-state level of OAA provides a means of controlling the flux through the tricarboxylic acid (TCA) cycle and amino acid biosynthetic pathways by fractionation through competing CS and MDH reactions, respectively. Furthermore, the fact that AATase provides an anaplerotic pathway for replenishing OAA in the TCA cycle needs to be considered. The absence of channeling of OAA between AATase and MDH may allow this anaplerotic reaction to proceed more easily.

The importance of substrate channeling has been established clearly in some cases, but the extent of penetration of this elaboration of metabolism needs further investigation. The new, conclusive assay described herein should facilitate future probes into this important problem.

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