A high-throughput assay for screening ʟ- or ᴅ-amino acid specific aminotransferase mutant libraries

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ABSTRACT

Aminotransferases are pyridoxal phosphate-dependent enzymes whose potential for the biocatalytic production of enantiopure amino acids is increasingly recognized. Because of this, there is a growing interest in engineering them to alter their substrate specificity and to increase their catalytic activity. Here, we report the development of a high-throughput assay for screening α-ketoglutarate-dependent aminotransferase mutant libraries. To achieve this, we exploited the ʟ-glutamate dehydrogenase coupled assay that has previously been shown to allow for aminotransferase activity to be monitored in vitro. We adapted this assay to allow screening of mutant libraries of either ʟ- or ᴅ-amino acid specific aminotransferases in a continuous fashion. This assay requiring clarified cell lysates is reproducible, rapid, and sensitive because it allowed for the identification of a catalytically active mutant of *Bacillus* sp. YM-1 ᴅ-amino acid aminotransferase displaying a decrease in k_{cat}/K_M of more than two orders of magnitude. In addition, this assay allowed us to discover a mutant of *Escherichia coli* branched-chain amino acid aminotransferase, F36W, which is approximately 60-fold more specific toward the natural substrate ʟ-leucine than ʟ-phenylalanine as compared with wild type. This result demonstrates the potential of our assay for the discovery of mutant aminotransferases displaying altered substrate specificity, an important goal of enzyme engineering.

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Aminotransferases (EC 2.6.1.X), also called transaminases, are pyridoxal phosphate-dependent enzymes that catalyze the transfer of the amino group from a donor substrate to a ketone acceptor. In vivo, these enzymes are primarily involved in the biosynthesis of amino acids and amino acid-derived metabolites [1], making their donor and acceptor substrates amino and keto acids, respectively. Many aminotransferases displaying different donor substrate specificities have been studied over the years. For example, aminotransferases that are specific for ʟ-aliphatic [2], ʟ-aromatic [3], ʟ-acidic [4], or ᴅ-amino acids [5] have been reported. Recently, aminotransferases that synthesize β-amino acids [6,7] or ω-amino acids [8] have also been discovered. Although these aminotransferases display markedly different donor substrate specificities, many have in common the use of the α-ketoglutarate acceptor substrate, which is a key intermediate in the Krebs cycle and in amino acid metabolism.

The potential of aminotransferases for the biocatalytic production of enantiopure natural and unnatural amino acids is increasingly recognized [9,10]. Because of this, there is a growing interest in engineering aminotransferases to alter their substrate specificity and to increase their catalytic activity. To facilitate the enzyme engineering process, a high-throughput screening assay is required. This assay needs to reliably reflect the activity level of the mutant aminotransferases and should allow screening of various amino acid substrates because increased activity and altered substrate specificity are desirable characteristics of engineered biocatalysts. Assays that have been used to screen aminotransferase mutant libraries are generally low-throughput methods requiring separation of products by liquid chromatography followed by their detection in a discontinuous fashion [11–13]. In contrast, genetic selection methods have also been developed to screen large aminotransferase mutant libraries [14–16], thereby increasing throughput. However, genetic selection has the disadvantage of requiring auxotrophic bacterial strains, special growth media, and genetic engineering, increasing the complexity of the experimental setup. In addition, a requirement for genetic selection is that the amino acid produced by the mutant aminotransferase be essential for cell growth, limiting the use of this methodology in the development of biocatalysts that synthesize unnatural amino acids.

Over the years, numerous continuous coupled enzyme assays have been developed to study the kinetic properties of various aminotransferases [17–21]. These coupled assays allow for the detection of one of the products of the aminotransferase catalyzed reaction, either the amino or the keto acid, through the catalytic action of a coupling enzyme. Typically, in aminotransferase coupled assays, the coupling enzyme transforms the aminotransferase

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product using oxidation/reduction of the NADH[1] (β-nicotinamide adenine dinucleotide, reduced form)/NAD+ (β-nicotinamide adenine dinucleotide, oxidized form) coenzyme, which can be followed spectrophotometrically. Coupling enzymes such as L-glutamate [22], lactate [17], D-2-hydroxyisocaproate [23], leucine [18], malate [17], and (R)-2-hydroxyglutarate [24] dehydrogenases have all been used successfully to follow the catalytic activity of aminotransferases in a continuous fashion. Recently, amino acid oxidase reactions have also been coupled to aminotransferase reactions [19,25] for the detection of the amino acid product. In addition to requiring an amino acid oxidase, this type of assay requires a third enzyme that is typically a peroxidase that uses the hydrogen peroxide produced by the oxidase to oxidize a colorless compound into one that absorbs visible light.

In this study, we report the development of a high-throughput screening assay for any α-ketoglutarate-dependent aminotransferase. To achieve this, we exploited the reliable L-glutamate dehydrogenase (GDH) coupled assay that has previously been shown to allow for aminotransferase activity to be monitored in vitro [22,26]. We adapted this assay to allow screening of mutant libraries of either L- or D-amino acid specific aminotransferases in a continuous fashion. This assay requiring clarified cell lysates is reproducible, rapid, and sensitive because it allowed for the identification of a catalytically active aminotransferase mutant displaying a decrease in k_{cat}/K_M of more than two orders of magnitude. In addition, this assay allowed us to discover a branched-chain amino acid aminotransferase (BCAT) mutant, F36W, with altered donor substrate specificity.

Materials and methods

Materials

All reagents used were of the highest available purity. Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs. Synthetic oligonucleotides were obtained from Integrated DNA Technologies, and Ni-NTA agarose resin was obtained from Promega. All aqueous solutions were prepared using water purified with a Barnstead Nanopure Diamond system. Enzyme substrates and cofactors were purchased from Sigma–Aldrich.

Mutagenesis

Codon-optimized *Escherichia coli* branched-chain amino acid aminotransferase (BCAT) and *Bacillus* sp. YM-1 D-amino acid aminotransferase (DAAT) genes synthesized by Integrated DNA Technologies were subcloned into pET11-a (Novagen) via *Nde*I/*Bam*HI. The plasmids were then transformed into *E. coli* XL-1 Blue (Stratagene). The entire *Nde*I/*Bam*HI fragments, including the whole coding region, were verified by DNA sequencing. Site saturation mutations were introduced into the BCAT and DAAT genes by overlap extension mutagenesis [27] using VentR DNA polymerase. Briefly, external primers were used in combination with sets of complementary pairs of oligonucleotides containing the NNS degenerate codon in individual polymerase chain reactions (PCRs). The resulting overlapping fragments were gel-purified (Omega Biotek) and recombined by overlap extension PCR. The resulting amplicons were digested with *Nde*I/*Bam*HI, gel-purified, and ligated into pET11-a expression vector with T4 DNA ligase. Constructs were verified by sequencing the entire open reading frame.

¹ *Abbreviations used:* NADH, β-nicotinamide adenine dinucleotide, reduced dipotassium salt; NAD⁺, β-nicotinamide adenine dinucleotide hydrate; GDH, L-glutamate dehydrogenase; BCAT, branched-chain amino acid aminotransferase; DAAT, D-amino acid aminotransferase; PCR, polymerase chain reaction.

Preparation of clarified cell lysates

The DNA libraries prepared as described above were transformed into chemically competent *E. coli* BL21-Gold (DE3) cells (Stratagene). Colonies were picked into individual wells of V96 MicroWell polypropylene plates (Nunc) containing 200 μl of medium (LB with 100 μg/ml ampicillin supplemented with 10% glycerol). The plates were covered with a sterile breathable rayon membrane (VWR) and incubated overnight at 37 °C with shaking. After incubation, these mother plates were used to inoculate sterile Nunc V96 MicroWell polypropylene plates ("daughter" plates) containing 300 μl of Overnight Express Instant TB media (Novagen) supplemented with ampicillin per well. Daughter plates were sealed with breathable membranes and incubated overnight (37 °C, 250 rpm). After incubation, the cells were harvested by centrifugation (3000g, 30 min, 4 °C) and the cell pellets were washed twice with phosphate-buffered saline (pH 7.4). Washed cell pellets were resuspended in lysis buffer (100 mM potassium phosphate buffer [pH 8.0] containing 1× Bug Buster Protein Extraction Reagent [Novagen], 25 U/ml Benzonase Nuclease [EMD], and 1 mg/ml lysozyme). The clarified lysate was collected following centrifugation and stored at 4 °C until used in the enzymatic assay.

Protein expression and purification

Proteins were expressed in 0.5-L cultures of *E. coli* BL21-Gold (DE3) cells transformed by a pET11-a vector containing the BCAT or DAAT gene. When the cultures reached an optical density of 0.6 at 600 nm, 1 mM isopropyl β-D-1-thiogalactopyranoside was added to the flasks to induce protein expression and cells were incubated with shaking for an additional 3 h at 37 °C. Following expression, cells were harvested by centrifugation and lysed with an EmulsiFlex-B15 cell disruptor (Avestin). The proteins were then extracted and purified by immobilized metal affinity chromatography according to the manufacturer's protocol. Elution fractions containing the aminotransferases were desalted by gel filtration using EconoPAC 10DG columns (Bio-Rad) into a final buffer solution of 100 mM potassium phosphate buffer (pH 8.0). Protein concentrations were quantified via a modified version of the Bradford assay, where the calibration curve is constructed as a plot of the ratio of the absorbance measurements at 590 and 450 nm versus concentration [28].

Screening assays

All assays were performed in triplicate 200-μl reactions at 37 °C in 100 mM potassium phosphate buffer (pH 8.0). The standard reaction mixture for the BCAT screening assays contained final concentrations of 16 μM pyridoxal phosphate, 5 mM L-leucine, 1 mM α-ketoglutarate, 1 U of GDH from bovine liver (Sigma), and 0.5 mM NAD+. Plates containing the standard reaction mixture were preincubated at 37 °C for 10 min prior to initiation of the reaction by the addition of 25 μl of clarified cell lysates prepared as described earlier. For substrate specificity screening, L-leucine was replaced by 5 mM L-phenylalanine. For the DAAT screening assays, the standard reaction mixture contained final concentrations of 16 μM pyridoxal phosphate, 10 mM D-glutamate, 5 mM pyruvate, 1 U of GDH from bovine liver (Sigma), 15 mM ammonium chloride, and 0.5 mM NADH. Plates containing the standard reaction mixture were preincubated at 37 °C for 10 min prior to initiation of the reaction by the addition of 1 μl of clarified cell lysates prepared as described earlier. For substrate specificity screening, pyruvate was replaced by 5 mM phenylpyruvate. Enzyme reactions were monitored by measuring absorbance of NADH at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) every 12 s for 30 or 60 min in individual wells of 96-well plates (Greiner Bio-One) using an Infinite M1000 plate reader (Tecan).