A Monomeric L-Aspartase Obtained by *in Vitro* Selection*

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By mimicking the partial spatial structure of the dimer of the L-aspartase subunit, the central ten-helix bundle, and an "active site" between the cleft of domain 1 (D1) and domain 3 (D3) from different subunits, we designed L-aspartase variants, in which D1D2 and D2D3 were ligated with a random hexapeptide loop. As expected, we obtained the variant with the highest activity (relative activity is 21.3% of the native enzyme, named as drAsp017) by *in vitro* selection. The molecular weight of this variant, obtained from size-exclusion column chromatography, is about 81 kDa, which indicates that it is indeed a monomer, whereas native L-aspartase is a tetramer. The activity-reversibility of drAsp017 (10⁻⁷ m) was 80% after incubation for 30 min at 50 °C, while native enzyme only retained about 17% under the same conditions. Reactivation of drAsp017 denatured in 4 m guanidine HCl was independent of protein concentration at up to 20 × 10⁻⁸ m at 25 °C, whereas the protein concentration of native enzyme strongly affected its reactivation under the above conditions. The sensitivity of drAsp017 (10⁻⁷ m) to effective factors in the fumarate-amination reaction compared with native enzyme was also determined. Half-saturating concentrations of the activator L-aspartate and Mg²⁺ for drAsp017 (0.8 and 0.5 mM, respectively) are much higher than that of the native enzyme (0.10 and 0.15 mM, respectively). The data show that a monomeric L-aspartase is obtained by *in vitro* selection. Thus, the conversion of oligomeric proteins into their functional monomers could have important applications.

L-Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible conversion of L-aspartate to fumarate and NH₃⁺ (1). L-Aspartase from *Escherichia coli* W is composed of four identical subunits that are arranged with the point symmetry P₂₁₁₂ (2). The monosubunit of native L-aspartase has no catalytic function (3). Each subunit consists of three domains oriented in an elongated S-shape: D1, D2, and D3 (see Fig. 1a). The central domain (D2), containing more than half of the total residues, is the most conserved domain in the aspartase-fumase structural family. The central core of D2 is made up of five long helices, which are all slightly bent and are nearly parallel to each other. In maintaining the active conformation, it is important to account for most of the intersubunit contacts in L-aspartase; the five-helix bundles of D2 from four subunits form a stable structure (a 20-helix cluster) in the tetramer (4) (see Fig. 1b).

L-Aspartase is an important enzyme used in industry. However, the practical application for L-aspartase in aspartate synthesis has been limited, mostly due to its relatively poor stability (5). As a tetramer protein, L-aspartase dissociates easily under industrial production conditions, and this is accompanied by the loss of its catalytic activity. In the presence of 0.4 M guanidine HCl, 45% of the native activity was observed when the L-aspartase tetramer had been reversibly dissociated into dimer. Fluorescence and CD studies show that only a small change occurred in the secondary and tertiary structures of the dimer compared with the native tetramer (6). These results indicate that the dimer of the L-aspartase subunit possesses the spatial structure of an active site(s), although not as perfect as the tetramer. Most likely, the reverse arrangement of the two subunits determines the proximity of D1 and D3 to each other in space, forming an active site between the cleft of D1 and D3 between different subunits. If so, by mimicking part of the spatial structure of the dimer of the L-aspartase subunit, the central ten-helix bundle, and an "active site" as above, we designed the L-aspartase variants, D1D2 and D2D3, which were ligated by a hexapeptide loop to form a monomer while maintaining the active site in the native dimer of the L-aspartase subunit. The most appropriate method is randomization of the inserted sequence between D1D2 and D2D3, followed by *in vitro* selection for L-aspartase activity (7–27). We selected the variant with the highest activity (relative activity is 21.3% of the native enzyme, drAsp017) from a combinatorial library. The molecular mass of this variant is about 81 kDa, as determined by size-exclusion chromatography, which indicates that it is indeed a monomer. Furthermore, this monomer properties that are more suitable for applications in industry than does the native enzyme.

MATERIALS AND METHODS

Materials and Plasmids—Restriction endonucleases, *Pfu* DNA polymerase, and the PCR system were purchased from MBI Fermentos. The DNA marker pUCM-2000 and λ DNA/HindIII were bought from TaKaRa Biotech. Ultrapure dNTPs and agarose for gel electrophoresis were obtained from Promega. The DNA recovery Kit (silver beads) and primers were from Sangon Biotech (Shanghai, China). Sephadex G-150 was ordered from Amersham Biosciences. Sequencing was performed in Sangon. All the other chemicals were of analytical grade.

*E. coli* J5 containing the plasmid-carrying L-aspartase gene was stored in our laboratory. *E. coli* JR1476 (aspA) strain was a generous gift of Dr. J. R. Guest (University of Sheffield, Sheffield, UK). B21 (DE3), T7-based expression vector pET-22b(+), and the affinity chromatography system for purifying His₆-tagged proteins were purchased from Novagen.

Construction of the Pool of Recombinant Plasmids pUCDRA—PCR amplification was performed with 1 µg of DNA template of the L-aspartase gene from *E. coli* J5, 0.2 mM dNTPs, 1 × PCR buffer, 1.5 mM MgCl₂, 2 µM primer, and 2 units *Pfu* polymerase in a total volume of 100 µL. Reaction conditions were heating at 95 °C for 5 min followed by 30 cycles of 1 min at 95 °C, 2 min at 42 °C, 3 min at 72 °C, and a final cycle of 1 min at 95 °C, 2 min at 42 °C, 15 min at 72 °C. Primers used for PCR were as follows: P₁D₁₂ (forward), 5'-TCGTGAGCTCGGGTATTCGGTC-
GATGCAG-3’; P_{D2D3} (reverse), 5’-GTCGAGCTCAGCCGTTAATGC-3’; P_{nris} (forward), 5’-GCATTACGACATCGGCATTAAC(N)_{n}TCCACTAACGGCAGGCTACCCGACG-3’; P_{D3D3} (reverse), 5’-TCGCGGTCTGTAGATGATGCA-3’ (where N = A, C, G, or T).

PCR products were purified from low melting point agarose following the manufacturer’s instruction. The two kinds of purified fragments were then combined using overlap extension under identical conditions to the first reaction. The resulting fragments were purified as before and then cloned into pUC18 vector with restriction enzymes SacI and BamHI.

**Selection Method**—The selection medium (5) that contained 0.5% L-aspartic acid as the sole nitrogen source, 1% glucose, 1% (NH_4)_2SO_4, 0.7% KH.PO_4, 0.3% KH.PO_4, and 0.01% MgSO_4.7H_2O was used for Asp-N selection. The pool of recombinant plasmids pUCDRA was transformed into E. coli JRG1476 (aspA-) competent cells. Colonies that grew faster or larger on the Asp-N selection agar plates were transferred into 3 ml of 2YT liquid medium (1.6% bacto-tryptone, 1% bacto-yeast extract, and 0.5% NaCl) and cultured at 37 °C overnight. The activities of the crude enzymes of these colonies were measured. The mutants with higher L-aspartase activity of were selected for further analysis.

**Cloning the Selected Genes of drAsp into pET-22b(+)**—The genes were amplified by PCR under the conditions as before and then subcloned into T7-based expression vector pET-22b(+) with restriction enzymes SacI and XhoI. The primers were 5’-TCGTGAGCTCAGCTGTAATGC-3’ and 5’-TCGCGGTCTGTAGATGATGCA-3’.

**Activity Assay**—The activity of L-aspartase was routinely determined spectrophotometrically by measuring the formation of fumarate following the increase in absorption at 240 nm (5). The standard assay system consists of 100 mM sodium L-aspartate (pH 7.0), 6 mM MgCl_2, 100 mM Tris-HCl (pH 7.0), and the enzyme. In the assay of the fumarate-amination reaction, the activity was monitored spectrophotometrically by measuring the decrease in the absorbance at 240 nm caused by the disappearance of fumarate in 1.0 ml of an assay mixture that consists of 50 mM TAPS-NaOH buffer (pH 8.5), 5 mM fumarate, 100 mM NH_4Cl, the enzyme, and various concentrations of MgCl_2 and L-aspartate.

**Activity-Reversibility**—The enzymes (10^-7 M) were incubated for 30 min at each temperature at different temperatures from 20 to 65 °C, and then the remaining catalytic activities were detected under the standard enzyme assay conditions. Relative activity was represented as percentage of maximum activity.

**Denaturation and Renaturation**—L-Aspartase or evolved enzyme was denatured by incubation of the enzyme in a solution of 4 M guanidine HCl, which consists of 25 mM potassium phosphate (pH 6.8), 50 mM KC1, 2.5 mM 2-mercaptoethanol, and 0.5 mM EDTA for 30 min to 2 h at room temperature. Enzyme-guanidine HCl solutions were prepared by diluting a 1:1 solution of the enzymes into 8 M guanidine-HCl. For determination of the recovery of activity, the enzymes denatured in 4 M guanidine HCl were renatured by a rapid 100:1 dilution of the above mixture into potassium phosphate-KCl buffer (50 mM potassium phosphate [pH 6.8], 0.1 M KCl, 5 mM 2-mercaptoethanol, and 1 mM EDTA).

**The Sensitivity to Effective Factors**—The activating effects of L-aspartate and Mg^{2+} ions (fumarate-amination reaction) were examined by the following methods. The concentration of the assay mixture containing l-aspartase (or mutant enzyme) was 10^-7 M, and the concentration of Mg^{2+} and l-aspartate was up to 6 mM, respectively.

1. The abbreviation used is: TAPS, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid.
RESULTS

At the genetic level, the genes of D1D2 (gD1D2) and D2D3 (gD2D3), respectively, were obtained by PCR amplification with two pairs of primers that were designed to introduce a random sequence into the genes (see Fig. 2a). The two purified fragments were then combined using overlap extension to construct drAsp genes that were a combinatorial library of variants with six random codons inserted between gD1D2 and gD2D3 (see Fig. 2b).

We cloned the genes for drAsp into the pUC18 (Promega) vector and transformed the pool of recombinant plasmids into E. coli JGR1476 (aspA⁻/H11002) competent cells. Without L-aspartase activity, JGR1476 cells grow very slowly in the selection medium that contains L-aspartate as the sole nitrogen source (Asp-N selection medium) (5). The considerable growth of some clones on the Asp-N selection agar plates indicates that plasmids encoding functional drAsp variants were introduced into these cells. The diameters of colonies are positively related to the L-aspartase activity of the drAsp variants expressed in the cells. From thousands of colonies, 23 clones were selected, of diameters larger than 1 mm from a selection agar plate at 37 °C for 36 h at various pH values (pH 6.5–8.5). To determine the oligomeric states of the proteins, we transferred the pool of selected genes to the T7-based expression vector pET-22b (+) (Novagen) and isolated individual clones for further analysis.

His₆-tagged proteins were purified by affinity chromatography, and their molecular weights were determined by size-exclusion column chromatography on a Sephadex G-150 column (0.8 × 90 cm). All 23 selected drAsp variants eluted from the gel filtration column as single peaks and had lower apparent molecular weights than the theoretical dimer. 18 of the selected variants showed evidence that they were mixtures of monomeric and...
dimeric species, since the ratio of the observed molecular weight to that calculated from the amino acid sequence varied from 1.20 to 1.60. This situation indicated that the 18 variants could not form stable dimers, although they aggregated in a certain extent, and there were rapid equilibria between the monomers and dimers. Differing from this pattern, the other five selected proteins eluted from the size-exclusion column with retention times expected for monomeric species. Their molecular masses were about 81 kDa (Fig. 3 shows the elution curve of drAsp017 as an example).

The corresponding genes of the monomeric drAsp variants were sequenced (Table I). The inserted hexapeptides were EYTDKT, DSGISH, YPDTGG, TLPGNK, and VSGSGN, respectively. The secondary structure of the monomeric variants were predicted by the GOR I method (28). The results showed that all the inserted sequences of the five monomers would generate turn structures. The presence of the appropriate turn between D1D2 and D2D3 may be necessary for yielding monomeric proteins with catalytic activity. However, as a result of disruption of the quaternary structure and diminution of the active sites, the relative activities of these monomeric variants were all reduced significantly, compared with the native L-aspartase (kcat = 180 s−1, Km = 1.2 × 10−3 M) under the standard assay conditions.

We selected the mutant enzyme with the highest activity from a combinatorial library, which we termed drAsp017, to examine in detail. As expected for a monomeric enzyme, the activity-reversibility of drAsp017 is higher than that of the native L-aspartase. For example, after being incubated for 30 min at 50 °C, the native enzyme conserved about 17% of its activity, while drAsp017 retained about 80% of that (see Fig. 4).

Reactivation of the native L-aspartase and drAsp017 denatured in 4 M guanidine HCl was examined under various protein concentrations at 25 °C. The results are shown in Fig. 5, which indicate that the reactivation of the native enzyme is a function of protein concentration up to 20 mM, and thereafter it gradually decreases as the protein concentration increases. In contrast the protein concentration of drAsp017 did not significantly affect its reactivation because it is a monomeric enzyme. After reversible denaturation and renaturation, the recovery of activity of drAsp017 was higher than that of the native enzyme.

It has been observed that the L-aspartase from E. coli W exists as a pH-dependent equilibrium between two forms. The higher pH form of L-aspartase has an absolute requirement for L-aspartate and divalent metal ions, while the lower pH form does not require any effectors for catalytic activity (29). The high pH form of the enzyme appears to be inactive unless L-aspartate and divalent metal ions are bound to the active site. To determine whether the activator site in drAsp017 was partially destroyed, and its affinity for L-aspartate and Mg2+ ions was decreased considerably.

DISCUSSION

In this study, we considered that the conversion of the L-aspartase tetramer into its functional monomer would be facilitated by in vitro selection. We identified the intersubunit contacts that were required for L-aspartase activity and designed an L-aspartase variant in which D1D2 and D2D3 were ligated to form a stably folded monomer. One of the active sites was retained from the native L-aspartase by mimicking the spatial structure of its dimer. Through a systematic structure-based analysis, we changed the domain sequence of L-aspartase from D1D2D3 to D1D2D2D3 by duplicating the central domain and introducing a random hexapeptide between the repeated D2 units. In fact, the loop was substituted for D1 and D3 from different subunits of the dimer of the L-aspartase subunit (see Fig. 7a). Generally, protein domains are the smallest functional and folding elements and often serve as modules (30). Therefore, the domains in the artificial domain-rearranged L-aspartase (drAsp) variants will not alter their basic structural character. The two five-helix bundles from the repeated D2 units will interact with each other just like those from different subunits. If the inserted hexapeptide loop adopts appropriate conformation, a 180° turn could be induced that would allow D2D3 (or D1D2) to fold back and displace other subunits to form a ten-helix bundle; it is similar to the central structure in L-aspartase dimer (see Fig. 7a). As shown in Fig. 7c, if the interdomain contacts occur between the D1 and D3, drAsp will form one potential active site.

It is not surprising that drAsp variants have diminished or no activity due to the sensitivity of catalytic efficiency to structural perturbations. However, through long range tertiary interactions, the residues inserted between the repeated D2 units can dramatically influence the potential active site, a small fraction of which will possibly be beneficial to the enzyme’s catalytic activity, presumably by restoring proper interactions for folding, stability, and adjusting the spatial position and orientation of residues in the potential active site. Given the uncertainties in predicting the optimal inserted sequence with exacting conformational control required for efficient enzyme catalysis, the most appropriate method is randomization of the inserted sequence, followed by a directed selection for the activity of L-aspartase. Since changes have occurred in the structure of drAsp variants, it is likely that some of them form monomers, without aggregation to form a 20-helix cluster structure.

By mimicking the partial spatial structure of the native dimer of the L-aspartase subunit, we obtained its functional monomer. Such structure-based rational approaches to protein engineering, combined with the randomization insert sequence, have given rise to the enzyme with altered oligomeric states. Converting oligomeric proteins into their functional monomers could have important applications, since monomeric proteins do not dissociate into the non-active form and have advantages for activity-reversibility and lower immunogenicity in protein medicine (31). The monomeric L-aspartase and the method described here may serve as model system for studying the mechanism by which oligomer proteins change to the monomeric form.

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