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High-Level Expression and One-Step Purification of Cyclic Amidohydrolase Family Enzymes

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The cyclic amidohydrolase family enzymes, including hydantoinase, dihydropyrimidinase, allantoinase and dihydroorotase, are metal-dependent hydrolases and play a crucial role in the metabolism of purine and pyrimidine in prokaryotic and eukaryotic cells. With the increasing demand for the elucidation of enzyme structures and functions, along with industrial applications, the research on the family enzymes has recently been proliferating, but the related enzymes had been purified conventionally by multistep purification procedures. Here, we reported the expression in *Escherichia coli* cells of maltose-binding protein-fused family enzymes and their one-step purification. The expression levels of the fusion proteins account for 20–35% of the total protein in *E. coli*, allowing approximately 2–3 mg of the purified proteins by affinity chromatography to be obtained per 0.3 L of bacterial culture. As more promising results, their nascent biochemical properties, after the cleavage of the fusion proteins with Factor Xa, in terms of oligomeric structure, optimal pH, specific activity, and kinetic property, were also conserved as those from the native enzymes. The availability of the family enzymes to fusion strategy shows potential as a convenient procedure to recombinant protein purification and accelerates the structure–function study of the related family enzymes.

Key Words: cyclic amidohydrolase; MBP-fusion; nascent properties; affinity chromatography.

Cyclic amide bonds are ubiquitously found in a variety of biomolecules in living organisms (1, 2). Evidence suggesting that enzymes acting on cyclic amide bonds are structurally and functionally related at molecular level, primarily participating in nucleotide metabolism, has been accumulated (3–5). Direct experiments on dihydroorotase and hydantoinase deduced the metal binding sites and signature sequence of the enzyme (6, 7). With this signature sequence and rigidly conserved regions, recent study on the related family enzymes, including dihydroorotase, allantoinase, dihydropyrimidinase, and hydantoinase, proposed a cyclic amidohydrolase family (6, 8). Microbial hydantoinase has been presumed to be a microbial counterpart of the eukaryotic dihydropyrimidinase (EC 3.5.2.2), which is involved in the catabolic degradation of pyrimidine and also catalyzes the hydrolysis of a variety of hydantoins. Dihydroorotase catalyzes the reversible cyclization of carbamyl aspartate to form dihydroorotate, the third step in *de novo* pyrimidine biosynthesis (9, 10). Allantoinase, the first enzyme involved in the degradation of allantoin, which is an intermediate in the catabolic pathway of purines, was studied from the various sources (11–13). Including these typical family enzymes, the superfamily was further extended by a remote homology search based on the substructure of urease (4). These findings support the hypothesis that in various organisms, conserved signature sequences and residues might be the useful probes for identifying the related enzymes. These assumptions were strongly supported by identification of two cyclic amidohydrolase genes from *Escherichia coli* encoding allantoinase and a novel phenylhydantoinase, both of which were also found to conserve the structural and functional properties from the evolutionary process (14). In this

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context, a recent study showing the distinct fusion ability of hydantoinase also indicated that the related enzymes may be tolerable to a functional fusion of other proteins (15). Furthermore, human adenine deaminase (16), a novel member of the family, was reported to be easily isolated from an MBP[2] fusion protein, implying that the functional fusion can be extended to other enzymes in the cyclic amidohydrolase family.

In this paper, for application of a fusion strategy to cyclic amidohydrolase family enzymes, we constructed the MBP fusion proteins by simple end-to-end fusion of whole open reading frames. Investigation of the resulting fusion proteins showed that the family enzymes were highly expressed in the soluble fraction, which was purified efficiently by amylose resin affinity chromatography. Moreover, the isolated cyclic amidohydrolases from their corresponding fusion proteins after the Factor Xa cleavage retained their nascent properties, as did those from wild-type enzymes. The relevant studies of the functional fusion also provided an interesting implication that the functional expression of the fusion proteins with correct folding could broaden the potential use of the family enzymes.

MATERIALS AND METHODS

Strains and Media

Derivatives of *E. coli* K-12 were used as sources for the cyclic amidohydrolase genes, allantoinase (14, 17) and dihydroorotase (10), and *Bacillus thermocatenulatus* GH2 was the source for hydantoinase (18). *E. coli* strain JM109 was used as a cloning host of the MBP fusion proteins. *E. coli* cells were transformed by electroporation, and the resulting transformants harboring recombinant genes encoding the fusion and wild-type proteins were grown at 30 or 37°C in Luria–Bertani (LB) medium. Ampicillin (50 μg/mL) was supplemented when needed.

Construction and Expression of Cyclic Amidohydrolase Enzymes with MBP Fusion

Chromosomal DNA was isolated from *E. coli*, or *B. thermocatenulatus* GH2, by using a genomic DNA purification kit (Promega), and the genes encoding the corresponding family enzymes were cloned using standard recombinant DNA techniques (19). Two sets of primers were designed to span the genes encoding the allantoinase (1362 bp) and dihydroorotase (1047 bp) from *E. coli*, and a set of primers for hydantoinase (1419 bp) from *B. thermocatenulatus* GH2 (Table 1). A restriction site *Bam*HI was inserted into the C-terminal primer of *E. coli* allantoinase. In other cases, restriction sites, *Eco*RI and *Pst*I, were introduced into the N- and C-terminal primers, respectively. The amplified DNA fragments encoding the family enzymes were isolated from agarose gel (0.8%) using the GeneClean II kit (Bio 101 Inc.) and then cloned into the *Eco*RI/*Pst*I (or *Eco*RI/*Bam*HI) site of pMAL-c2X (New England Biolabs).

TABLE 1

Primers Used in This Study

Primer name	Sequence[a]
ALLN	5′-AGGAATTCGTTATGTCTTTTGATTTAATCATT-3′
ALLC	5′-GGGGATCCTTACTGCTGATGTTTAAGGATAA-3′
DHON	5′-ATGAATTCACTGCACCATCCCAGGTATTAAAGAT-3′
DHOC	5′-ATCTGCAGTTATTGTTTAACGGACCAGCGTACC-3′
HYDN	5′-TAGAATTCATGACAAAATTGATAAAAAATG-3′
HYDC	5′-TACTGCAGTTAGGACATTTTCACCACATCT-3′

[a] Underlines indicate the restriction sites introduced in the synthetic oligonucleotides.

For the expression of the MBP fusion proteins, *E. coli* JM109 cells were transformed by electroporation in 10% glycerol with the construct. Expression of three fusion proteins was achieved by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C when the optical density at 600 nm reached about 0.4–0.6.

Purification of Fusion Proteins

After the cultivation of induced cells (300 mL) for 3 h, the cells were harvested by centrifugation at 6000*g* for 10 min. The resulting pellets were resuspended in a total volume of 10 mL Tris–HCl buffer (20 mM, pH 8.0) containing 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1% Triton X-100. The suspended cells were disrupted by sonication, and the supernatant was obtained after centrifugation at 18,000*g* for 30 min. The resulting solution and cell pellets were assayed directly for enzyme activity, and protein expression was analyzed by using SDS–PAGE. For biochemical characterizations, the protein was further purified to apparent homogeneity using the standard chromatographic procedures. The fusion proteins in the soluble fraction were absorbed onto an amylose resin, and then washed three or four times with a buffer containing 20 mM Tris (pH 8.0) and 200 mM NaCl. After complete washing, proteins were eluted with an elution buffer (20 mM Tris, 200 mM NaCl, 10 mM maltose). The purified fusion proteins were further concentrated by using a Centriprep 10 (Amicon), and the recombinant enzyme (cyclic amidohydrolase) was separated from the MBP domain by treatment with

[2] Abbreviations used: MBP, maltose binding protein; LB, Luria–Bertani; IPTG, isoprophyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione *S*-transferase; DHO, dihyroorotase.

Factor Xa for 25–30 h at 8°C. The cleaved enzyme was isolated from the fusion partner, MBP, by reapplying it onto the affinity resin. Pooled enzyme solution was concentrated by dialysis against 20 mM Tris–HCl buffer (pH 8.0) containing 10% glycerol, and then stored at −20°C.

For the comparative study, the family enzymes were also purified in their native state as conventional method with slight modification (6, 14, 20). All purification steps were conducted by three or four chromatographic steps and performed by using a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech) at room temperature.

Gel Filtration Chromatography

The oligomeric structures of the family enzymes were determined on a FPLC system with a gel filtration column (Superose-12 HR10/30). The flow rate of the mobile phase containing 20 mM Tris–HCl and 150 mM NaCl was 0.3 mL/min. The column was calibrated using native protein markers (Amersham Pharmacia Biotech). A molecular mass standard curve was established by plotting the elution profile of the protein markers versus the known molecular masses on semilog paper. Aliquots of each eluted fraction were confirmed by SDS–PAGE and enzyme assay.

Enzyme Activity Assay

The activities of the family enzymes were determined at 40°C for 30 min with constant shaking. The enzyme reaction was carried out with the purified enzyme (10 μg) in 1 mL of reaction mixture containing 100 mM Tris–HCl (pH 8.0), 0.5 mM DTT, and the predetermined concentration of cyclic ureide (allantoin, 10 mM; dihydroorotate, 1 mM; hydroxyphenylhydantoin, 15 mM). A decrease in the concentration of the cyclic ureide used as a substrate and an increase in the production of the corresponding *N*-carbamyl compound were analyzed, respectively, using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC)(21, 22). The amount of product formed was also determined by using either HPLC or color reagent *p*-dimethylaminobenzaldehyde. All assays were carried out in duplicate. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of cyclic ureide per minute under the specified conditions. Kinetic parameters were determined according to the reported methods (14, 21).

RESULTS

Construction of the MBP-Cyclic Amidohydrolase Fusion Proteins

Previously, we reported that a microbial hydantoinase, as a typical cyclic amidohydrolase family enzyme, from *B. stearothermophilus* SD1 permits a functional fusion with MBP and thus easy purification by one-step affinity chromatographic procedure (15). Based on this finding and close evolutionary relationship, we also noticed a possibility that the family enzymes might be functionally expressed under conditions irrespective of the presence of a larger polypeptide, such as MBP or GST, fused at their N-terminus (15).

To confirm the possibility, we constructed the MBP fusion proteins of three typical family enzymes, a hydantoinase (HYD) from *B. thermocatenulatus* GH2 and two enzymes, allantoinase (ALN) and dihydroorotase (DHO) from *E. coli*. We used a commercially available vector pMAL-c2X expressing *E. coli* maltose binding protein, which is controlled by the strong *P*tac promoter. Two genes of family enzymes were subcloned downstream of the *malE* gene using a set of restriction enzyme sites, *Eco*RI and *Pst*I. The natural sequence of the allantoinase gene has a unique *Pst*I site in its open reading frame (14). Therefore, in the case of allantoinase, the gene cloned into a set of restriction site, *Eco*RI and *Bam*HI. The linear fusion of MBP to the N-terminus of family enzymes was attempted without an additional peptide linker to evaluate their own fusion ability. The strategy was also intended to minimize the remained artificial residues when the cyclic amidohydrolase domains were cleaved and isolated from the fusion partner MBP. After the constructs confirmed by DNA sequencing, we expressed the fusion proteins in a general host *E. coli* JM109 at various conditions.

Expression and Purification of MBP-Fusion Proteins

When the *E. coli* cells harboring fusion proteins were plated on the activity staining plate containing each typical substrate (8), we clearly detected the enzyme activities comparable to those of wild-type enzymes, whereas inhibition of growth was negligible in each case. No distinct difference was observed when the recombinant cells were grown at various temperatures (25, 30, and 37°C). We further confirmed the functional expression of three fusion proteins with the cells grown in flask cultures containing 300 mL of LB medium at 30°C. When *E. coli* cells harboring the recombinant genes were induced with 0.5 mM of IPTG for 3 h, all fusion proteins, MBP-ALN, MBP-DHO, and MBP-HYD, were markedly overproduced with the calculated molecular masses (Fig. 1A). SDS–PAGE analysis of the crude extracts indicated that the fusion proteins accounted for 20–35% of the total cell protein and was mainly detected in the soluble fraction (>80%). In most of cases, the fragments derived from proteolysis were negligible, and thus improper bands resulting from high-molecular-weight aggregate or truncated fragment were not observed.

To compare the structural and biochemical properties

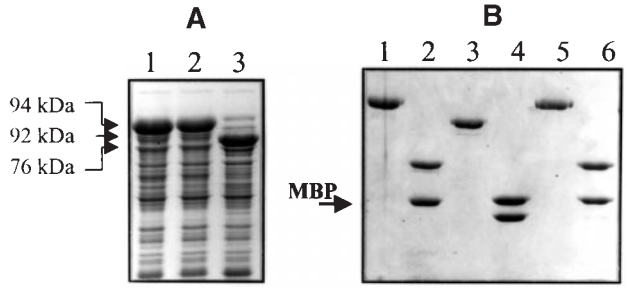

FIG. 1. High-level expression and purification of the cyclic amidohydrolase enzymes with MBP fusion. The enzymes were fused with maltose-binding protein at their N-terminus by simple end-to-end fusion. (A) SDS–PAGE analyses of crude extracts of *E. coli* cells expressing the MBP-fused proteins. Lane 1, MBP-ALN (92 kDa); lane 2, MBP-HYD (94 kDa); lane 3, MBP-DHO (76 kDa). Soluble fractions of the crude extracts were analyzed after 3 h induction with 0.5 mM of IPTG at 30°C. (B) Factor Xa cleavage of the MBP-fused family enzymes. The purified fusion proteins (250 μg were cleaved with Factor Xa at 8–10°C for 24 h, and an aliquot (3 μg) was analyzed on 11% SDS–PAGE. SDS–PAGE analysis of the fusion proteins before and after the digestion with Factor Xa: MBP-ALN (lanes 1 and 2); MBP-DHO (lanes 3 and 4); MBP-HYD (lanes 5 and 6).

with those of the wild-type enzymes, the fusion proteins were further purified to apparent homogeneity with the amylose resin (Fig. 1B). About 2–3 mg of the fusion proteins were recovered from a 300-mL culture of the induced cells with 0.5 mM of IPTG, and purified enzymes showed an expected migration rate corresponding to calculated molecular masses. For further purification, the fusion proteins were cleaved with Factor Xa. As shown in Fig. 1B, the MBP-fused family enzymes were efficiently cleaved and separated to their respective domains, which indicates that both domains fold independently and are easily accessible to site-specific protease. In general, misfolded or aggregated fusion protein is resistant against protease attack. The Factor Xa-cleaved proteins were reapplied onto the amylose resin, and the unbound family enzymes were collected. The resulting enzyme pools were concentrated appropriately (1 mg/mL) for further analyses. Gel electrophoresis under denaturing conditions showed homogeneous enzymes with their corresponding molecular masses. The affinity purification procedure and yields are summarized in Table 2.

TABLE 2

Affinity Purification of Three Cyclic Amidohydrolases with MBP Fusion

Enzyme	Total protein (mg)	Purified protein (mg)	Yield (%)
MBP-ALN	89	2.8	71
MBP-DHO	74	1.9	83
MBP-HYD	81	2.5	75

The Separated Cyclic Amidohydrolases Retained Their Nascent Properties

To analyze whether the kinetic properties of the purified enzymes were changed, we compared the affinity-purified enzymes with their nascent wild-type enzymes. The activity of the purified enzyme toward typical substrate was determined and found to be almost similar to those of the wild-type ones (Table 3), only a minor difference was found in the dihydroorotase (DHO). The specific activity of the affinity-purified DHO toward dihydroorotate was about 1.2-fold higher than that of the native enzyme, whereas the specific activities of two other enzymes (allantoinase and hydantoinase) were almost comparable to those of the wild-type enzymes. The K_m values were also determined to analyze whether the fusion strategy affected on substrate affinity. Two of the affinity-purified enzymes, dihydroorotase and hydantoinase, exhibited no drastic difference in their affinity values with respect to the wild-type enzymes (Table 3). In the case of allantoinase, about 1.8-fold increase in K_m value was observed, but overall catalytic efficiency of the affinity-purified enzyme was also similar to wild-type enzyme (data not shown). We also determined the pH optimum for the hydrolysis of the corresponding substrate. The pH dependency was analyzed in 0.1 mM boric acid–NaOH, 0.1 mM Tris–HCl, and 0.1 mM sodium phosphate buffer at pH ranging from 8.5 to 10.5, 7.5 to 8.5, and 5.5 to 7.5, respectively. The affinity-purified enzymes showed an optimum pH at about 7.5–8.5, as in the case of the corresponding wild-type enzymes (14).

To examine the oligomeric structure of the affinity-purified enzymes, the gel filtration column chromatography was performed. From the elution profile of the gel filtration column chromatography, three family enzymes, as expected, maintained their nascent oligomeric structure as those of the wild-type enzymes (Fig. 2), as observed with a family enzyme in our previous work (15). Two enzymes, allantoinase and hydantoinase, from affinity purification were coeluted with an apparent molecular mass between 200 and 220 kDa, whereas dihydroorotase was detected in fractions corresponding to a molecular mass between 70 and 75 kDa (Fig. 2). SDS–PAGE analysis of the eluted fractions also supported our observation. The respective subunit masses were calculated to be around 51–53 kDa for allantoinase and hydantoinase, whereas the dihydroorotase was estimated to be about 35–37 kDa. Thus, the quaternary structures for these enzymes were predicted to be a homotetramer for allantoinase and hydantoinase and homodimer for dihydroorotase. These results clearly indicate that their nascent oligomeric structure was conserved, as were those of wild-type enzymes (14, 18, 20).

TABLE 3

Comparison of the Nascent Properties between the Affinity-Purified and Wild-Type Enzymes

	ALN		DHO		HYD	
	Affinity-purified	Wild-type	Affinity-purified	Wild-type	Affinity-purified	Wild-type
Specific activity[a] (U/mg protein)	17.8	19.9	40.3	34.9	45.3	49.2
K_m[b] (mM)	7.4	4.2	0.062	0.053	23.9	25.6
Oligomer[c]	Tet	Tet	Di	Di	Tet	Tet

[a] Enzyme activity was determined using the purified enzymes (10 μg) in the presence of corresponding substrate (allantoin, 10 mM; dihydroorotate, 1 mM; hydroxyphenylhydantoin, 15 mM) in a 1 ml of 100 mM Tris–HCl (pH 8.0) buffer. The reaction products were analyzed either by HPLC or a color reagent.

[b] The kinetic constants were determined in 1 ml of 100 mM Tris–HCl (pH 7.8) buffer, using appropriate substrate concentrations ranging from 0.5 to 15 mM for allantoinase, 0.01 to 0.5 mM for dihydroorotase, and 0.5 to 15 mM for hydantoinase, respectively.

[c] Tet and Di denote tetramer and dimer, respectively.

DISCUSSION

Reasonable classification of a protein family based on the functional and structural traits is expected to stimulate progress in understanding the structure and biochemical function of family proteins (4), because it is believed that the biophysical and/or biochemical properties of proteins from a common origin could be conserved through the evolutionary process, despite a high sequence divergence (23). Therefore, proteins belonging to a structural family with functional relatedness are assumed to share a structural scaffold, permitting different substrate specificity or stability within family proteins even with a subtle change in their common scaffold. As a consequence, unification of protein families deduced from higher structural resemblance and relevant function, especially recently explored by the structural remote homology approach, leads to broadening the scope of a protein family in the shared properties. Uncovering this kind of trait is valuable and useful for elucidation of protein structure and establishment of a general procedure for protein manipulation. In this context, evolutionary relatedness of the cyclic amidohydrolase family defined by experimental and computational methods may provide some useful information (4, 6). Recently, with the predicted three-dimensional fold and active site architecture, those family members were extended to comprising dihydroorotases, allantoinases, hydantoinases, AMP-, adenine-, and cytosine deaminases, imidazolonepropionase, and proteins involved in animal neuronal development (4). The family enzymes shared a common motif and structural domain, which will confer a clue on the prediction of unidentified proteins. From current knowledge, a structural family with high functional similarity is proposed to have a shared trait of enzymes and thus provide an uncovered functional trait. This uncovered functional or structural trait as a common property still remains to be discovered in cyclic amidohydrolase family enzymes (14). Therefore, it is noteworthy that more biochemical and biophysical traits are conserved in the family enzymes evolved from a pivotal ancestor as observed in this work.

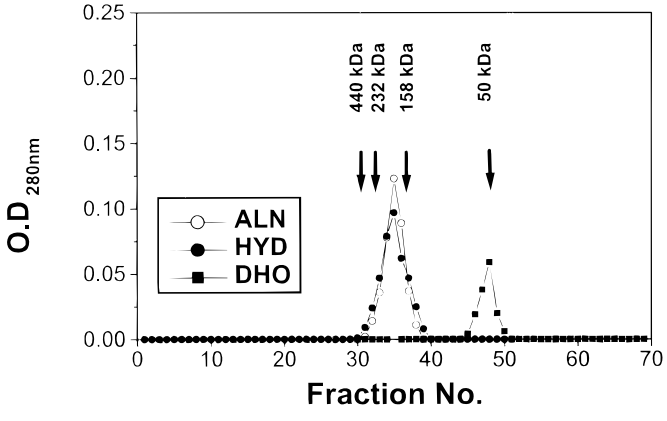

FIG. 2. Oligomeric structures of the affinity-purified enzymes. The purified proteins (50–125 μg) were analyzed on a Superose-12 gel filtration column. Molecular mass of each purified protein was estimated from the elution profile of the standard protein markers: blue dextran, 2000 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; Fab fragment, 50 kDa; MBP, 42 kDa. All experiments were repeated three times at different protein concentrations. The shift in elution time was negligible (<0.2 min).

We have demonstrated that the cyclic amidohydrolase family enzymes can be functionally expressed in MBP fusion state, and thus efficiently purified by affinity chromatography in one-step procedure. Even in the high-level expression under explosive induction conditions, the family enzymes folded well and thus retained their nascent properties, which strongly suggests that the family enzymes have a high folding ability and stability to oligomerization, although the MBP is generally known to be amenable to structural change within fusion protein and thus well addresses the correct folding of the joined domain (24). These suggestions were partly supported by recent works conducted with microbial hydantoinases and adenine deaminases (15, 16, 25). With the complete genome sequences available from various sources, it is very interesting that the putative

cyclic amidohydrolases are ubiquitously distributed in eukaryotic and prokaryotic cells, but most of the enzymes still remain to be elucidated for their functional roles (14, 26). Demonstration of the exact role and investigation of the functional defect in the engineered proteins would address some crucial information regarding the structure and function relationship of the family enzymes. Currently, the research on the cyclic amidohydrolase family enzymes has been proliferating (3, 6, 14, 21, 27). However, the relevant enzymes were used after purification through conventional procedures consisting of four or five consecutive steps, mainly due to the absence of an established method. Therefore, the finding that the family enzymes are tolerable and thus can be functionally expressed with a fusion partner would be applied to isolation of the family enzymes by simple affinity chromatographic procedures, and it stimulated the biochemical and biophysical study on the related family of enzymes.

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