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Modifying the oligomeric state of cyclic amidase and its effect on enzymatic catalysis

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Abstract

A group of cyclic amidases, including hydantoinase, allantoinase, dihydropyrimidinase, and dihydroorotase, catalyze the reversible hydrolysis of cyclic ureides, such as 5-monosubstituted hydantoins and dihydropyrimidines. These four enzymes carry hydrophobic patches to form dimers. With the exception of dihydroorotase, these enzymes are further dimerized to form tetramers by hydrophobic interactions. This leads us to speculate that the hydrophobic interaction domain may be a significant factor in the catalytic property of these oligomeric cyclic amidases, for which activities are not allosterically regulated. We generated a dimeric D-hydantoinase by mutating five residues in the hydrophobic α-helical interface of a tetramer and analyzed the kinetic properties of the dimeric form of D-hydantoinase. The specific activity of the dimeric D-hydantoinase corresponds to 5.3% of the activity of tetrameric D-hydantoinase. This low specific activity of the dimeric D-hydantoinase indicates that the dimeric interaction to form a tetramer has a significant effect on the catalytic activity of this non-allosteric tetramer.

Keywords: Cyclic amidase; Hydantoinase; Dihydropyrimidinase; Oligomeric enzyme; D-Amino acid production

A group of amidohydrolases, including hydantoinase (HYD), allantoinase (ALN), dihydropyrimidinase (DHP), and dihydroorotase (DHO), belong to a family of cyclic amidases. They catalyze the reversible hydrolysis of the cyclic amide bond (–CO–NH–) in either five- or six-membered rings [1] (Scheme 1).

Multiple sequence alignments were carried out in order to establish the functional and evolutionary relationships among these four kinds of enzymes. Microbial hydantoinases share about 37–42% amino acid identity with mammalian DHP. The amino acid identities of DHP with ALN and DHO were found to be about 19–26 and 7–16%, respectively. ALN shared an identity of 9–20% with DHO [1].

In spite of possessing functional similarity, their mutual amino acid identities appear to be relatively low, except for that between HYD and DHP. Regardless of these low sequence identities among the four enzymes, the conserved regions, which span 75% of full sequence, were located throughout the entire sequence, except for the highly mismatched C-terminal region [1]. The existence of these extended, conserved regions may imply that overall structural conformations are conserved, even though the detailed specificities of each enzyme vary.

Three of the four structures, with the exception being ALN, have currently been determined and they are much more similar to one another than we might have expected, based on the sequence identity data [2–5]. Therefore, it is likely that the overall structure of ALN is very similar to these three known structures [1].

HYD, ALN, and DHP exist as homotetramers in solution and DHO exists as a homodimer [3]. Structural

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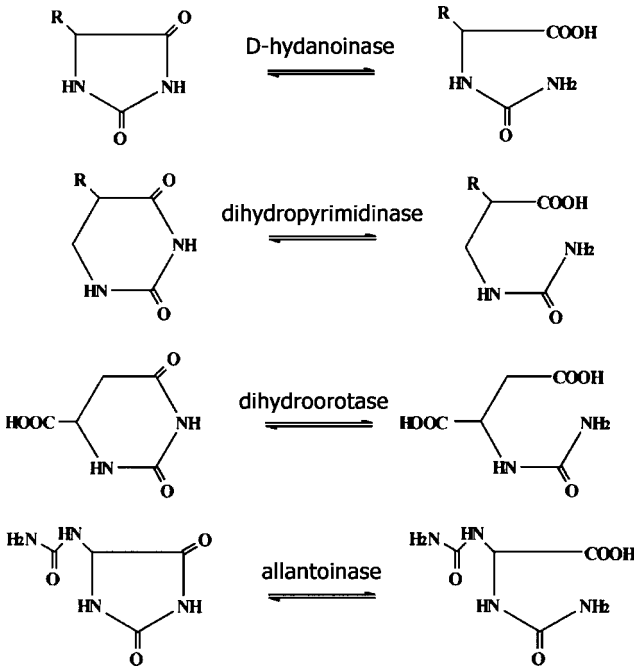

Scheme 1. Reactions catalyzed by the four cyclic amidases [1].

studies for two HYDs [2,4], DHO [5], and DHP [3] established that these four kinds of cyclic amidases exist in homotetrameric (HYD, DHP) or homodimeric (DHO) conformation in solution. In addition, sequence alignment [1] also suggested that ALN possesses the same homotetrameric conformation as HYD and DHP.

In the case of these four enzymes, the monomers of each enzyme form a dimer. DHO therefore exists as a dimer, whereas the other three enzymes are further dimerized to form tetramers. DHO is dimerized mainly by means of hydrophobic interactions using one α-helix and several turns from each monomer [5]. In contrast, a study of the HYD and DHP structures revealed that three α-helices form coiled-coil interactions to generate the dimer interface in the enzymes [2]. The dimeric HYD and DHP are further dimerized to form tetramers, using the β-sheets from the N- and C-terminal domains [2,3].

In the crystal structure of DHO, one subunit was observed to bind to the substrate and the other to bind to the product, suggesting some kind of cooperative action between the two subunits [5], even though this enzyme turned out to be a non-allosteric enzyme. The oligomeric effects for these homodimeric or tetrameric forms of cyclic amidases have not yet been investigated for any of the enzymes.

Microbial D-hydantoinase (D-HYD) is an industrial enzyme. This enzyme has been used for the commercial production of optically pure D or L-active amino acids in biocatalysis [6]. It has also been used for the production of D-amino acid intermediate compounds through the stereo-specific hydrolysis of chemically synthesized cyclic hydantoins [7]. These intermediates are widely used for the synthesis of semisynthetic antibiotics, peptide hormones, pyrethroids, and pesticides. Although the biology of microbial HYD remains largely unexplored and many natural substrates remain unknown, its homologous enzyme in humans is DHP, which plays an essential role in the reductive pathway of pyrimidine degradation. It is essential for the detoxification of pyrimidine analogues that are used as pharmaceuticals, such as 5-fluorouracil [6]. Whether HYD and DHP are identical enzymes (see Scheme 1) remains unclear, even though the two enzymes have currently been assigned to the same enzyme classification (E.C. 3.5.2.2) [8].

Each monomer of homotetrameric D-HYD has a triose phosphate isomerase (TIM) barrel domain with strands of parallel β-sheet flanked on the outer surface by α-helices [5]. The DALI program [9] provided information that the D-HYD TIM barrel domain also shared a homology with urease [10], phosphotriesterase [11], and adenosine deaminase [12], in addition to the family of cyclic amidases [2]. Furthermore, the cyclic amidases belong to a metalloenzyme family, which shares a conserved binuclear metal center [2,5].

The helices, αD, αE, and αG, of one monomer of D-HYD are involved in the coiled-coil interactions with the counterpart monomer (Figs. 1A and B, in this figure, A and H for subunits) [2]. The core residues for the dimeric coiled-coil interactions are Y167, V189, Y192, L193, and L230 (Fig. 1C). We designed a group of mutations, in order to generate a dimeric D-hydantoinase from wild-type tetrameric D-HYD by introducing five mutations on the interface of the coiled-coil interaction. The five residues from the helices, αD (Y167), αE (V189, Y192, and L193), and αG (L230), were selected. These five residues were converted to mutants (Y167S, V189A, Y192C, L193A, and L230G; a mutation of Tyr167 to Ser is named Y167S) cumulatively, such that the functional groups are conserved and only the hydrophobic interactions are reduced or abolished, for example, by reducing the length of the side-chain arms.

In the current report, we discuss the generation of a dimeric D-hydantoinase, by introducing five consecutive mutations on the interface region of the coiled–coil interaction. The resulting dimeric D-hydantoinase was characterized using various biophysical and biochemical tools, and the properties of the oligomeric form of D-hydantoinase, such as its conformation and catalytic activity, were compared with those of wild-type tetrameric D-hydantoinase.

Experimental procedures

Strains, plasmids, and media

The D-hydantoinase gene from *Bacillus stearothermophilus* SD1 [13] was cloned into *Escherichia coli* DH5α and subcloned into *E. coli* BL21 (DE3). Plasmids pET28a (+) (Novagen, USA) and pGEM-easy

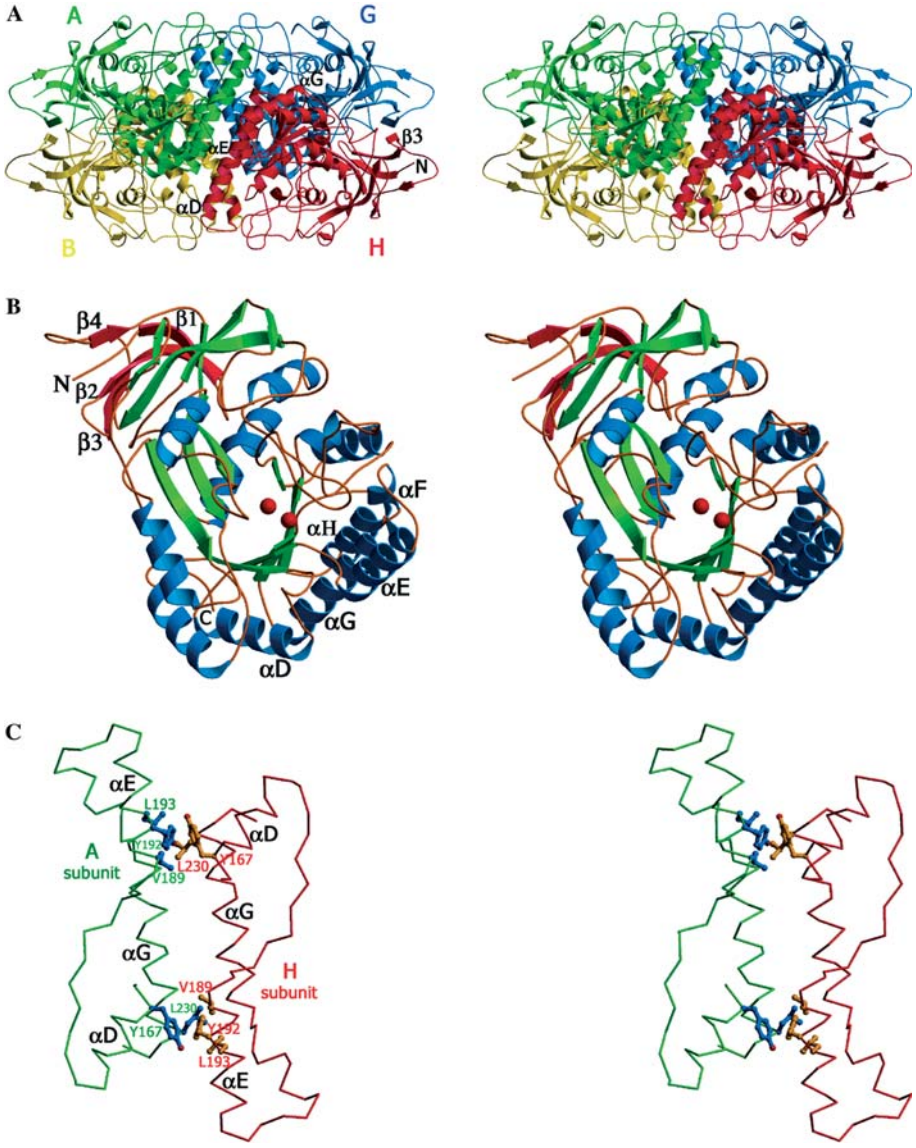

Fig. 1. Overall structure of D-hydantoinase. (A) Tetrameric form of wild-type D-hydantoinase [2]. A, B, G, and H represent the monomeric subunits of tetrameric wild-type D-hydantoinase. N represents the N-terminus of the subunit H. Key secondary structural features of the subunit H at subunit interfaces are indicated. One coiled-coil interaction of dimeric interfaces (A subunit in green and H subunit in red) is shown and the three helices of the subunit H (αD, αE, and αG) involved in the coiled-coil dimeric interaction are indicated. The other coiled-coil dimeric interaction between the third monomer (B subunit in yellow) and the fourth monomer (G subunit in blue) also exists, but it is not clearly visible when viewed from this angle. The other two dimeric interactions by β-strands are shown in the N-terminal regions (one between the subunits G and H, the other one between the subunits A and B, respectively). Only one strand β3 of the subunit H involved in this dimeric interaction at the N-terminus is indicated for clarity. (B) Monomeric presentation of D-hydantoinase [2]. The monomer with the hydrolytic active site is marked with two bound zinc ions (red spheres). The detailed notations of the secondary structures were previously described [2]. The helices αD (163–176), αE (187–199), and αG (214–231) of the monomer are involved in the coiled-coil dimeric interaction. The four β-strands β1 (6–11), β2 (16–23), β3 (27–32), and β4 (42–44) at the N-terminal region are involved in the other dimeric interaction. N and C represent the N- and C-termini of the monomer. (C) Residues involved in the coiled-coil dimeric interaction between the A and H subunits. Five residues of subunit A interact with identical residues of subunit H by forming hydrophobic bonds, but the interacting counterparts are not identical, because the two subunits are positioned by twofold symmetry. Interestingly, Y167 (αD helix) and Y230 (αG helix) from subunit A interact with V189 (αE helix), Y192 (αE helix), and L193 (αE helix) of subunit H. Conversely, V189 (αE helix), Y192 (αE helix), and L193 (αE helix) of subunit A interact with Y167 (αD helix) and Y230 (αG helix) from subunit H. The two patches of hydrophobic interactions at both ends are symmetrical by twofold rotational symmetry, which is reflected in the 222 non-crystallographic symmetry of the crystal packing of the D-hydantoinase structure.

T (Promega, USA) were used for cloning and expression. The cloned *E. coli* cells were grown in Luria–Bertani (LB) medium at 37 °C. Kanamycin (30 μg/ml) and ampicillin (50 μg/ml) were added when necessary.

Site-specific mutagenesis, cloning, and sequencing

The residues (167, 189, 192, 193, and 230) of wild-type D-hydantoinase were mutated by using the overlapping-extension PCR

method. The mutated gene was constructed with the pGEM-easy T vector that used the TA cloning strategy (Promega , USA). Ligated vectors were electro-transformed into *E. coli* DH5α. After screening for the desired transformant cells, DNA sequencings were performed. The transformed plasmids, which were prepared by the alkaline lysis method, were used as templates for the next site-specific mutagenesis.

Expression and purification

The cloned plasmids were treated with the restriction enzyme *Eco*RI and *Hin*dIII. The mutated genes, which had N- and C-terminal sticky ends, were re-ligated with pET28a(+). The cloned gene was transformed into *E. coli* BL21 (DE3) for protein expression. The overnight culture seeds of wild-type D-hydantoinase and the mutated D-hydantoinase (10 ml) were inoculated into 1400 ml LB medium containing kanamycin (30 μg/ml) and grown at 37 °C. When the optical density of the cells at 600 nm reached 0.5, target proteins were induced by adding 0.5 mM IPTG at 37 °C. After the overnight inductions, cells were harvested by centrifugation at 4000*g* for 20 min. The cell pellets were resuspended with 20 mM Tris–HCl (pH 7.4) containing 200 mM NaCl, 1 mM β-mercaptoethanol (βME), 0.5 mM $MnCl_2$, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The suspended cells were sonicated and the supernatants were obtained by centrifugation at 15,000*g* for 20 min.

After each supernatant was loaded onto Ni–NTA resin, the elutes were obtained by using a 50–300 mM concentration gradient of imidazole. The purified fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Each enzyme was dialyzed against 20 mM Tris–HCl (pH 7.4) containing 1 mM βME for the low salt-free condition or 20 mM Tris–HCl (pH 7.4) containing 500 mM NaCl and 1 mM βME for the high-salt condition, respectively.

Determination of oligomeric states

Native-PAGE. Native-polyacrylamide gel electrophoresis (PAGE), also called non-denaturing gel electrophoresis, separates proteins based on their size and charge states. Thus, the oligomeric state of each enzyme was initially examined by native-PAGE. Bovine serum albumin (66 kDa, Amresco, USA) and urease (monomer: 272 kDa, dimer: 545 kDa, Sigma, USA) were adopted as standard markers. Wild type D-hydantoinase was also the reliable standard protein marker in this study [2,14]. To minimize heat generation, gel electrophoresis was carried out in a cold room. The effects of ionic strength were tested under both high-salt and low salt conditions. D-hydantoinase, whose p*I* value is 5.31 (Expasy PI/MW tool), was negatively charged at a pH of 8.8, and thus, gel electrophoresis was performed at pH 8.8.

Gel filtration chromatography. The oligomeric states of the enzymes were also able to be deduced using a fast liquid chromatography system (Amersham, USA) with gel filtration column chromatography using Sephadex G-100 resin (Amersham, USA). The same standard MW markers were used as in the native-PAGE analysis. The column was equilibrated with 20 mM Tris–HCl (pH 7.4) containing 1 mM βME for low salt condition, and 20 mM Tris–HCl (pH 7.4) containing 1 mM βME and 500 mM NaCl for high-salt condition. The elution profiles were analyzed by both SDS–PAGE and native-PAGE. The flow rate of gel filtration chromatography was 0.5 ml/min at 0.5 MPa. The gel filtration experiments were performed in a cold room.

Analytical ultracentrifuge (Sedimentation equilibrium experiment). Analytical ultracentrifugation is one of the most accurate methods for determining the oligomeric states of an enzyme. We used an Optima XL-A analytical ultracentrifuge (Instrument manual, Beckman, USA) to analyze the oligomeric states of D-hydantoinase. The sedimentation equilibrium method provides information on the average molecular weights of macromolecules by allowing them to be equilibrated in the sample cell of an analytical ultracentrifuge.

The absorbances of the enzyme solutions in the sample cells were adjusted to a level of 0.3 of OD_{280}. One hundred and 10-μl of enzyme solution and 110 μl of enzyme-free solution (buffer) were placed into the 12 mm double-sector cells of the XL-A analytical ultracentrifuge. After all of the double-sector cells were balanced, the rotor speed (for example, 8000*g*) was determined by depending upon the presumed molecular weight of the enzyme. Then, macromolecular distribution data were collected by UV absorbance and a computer analysis of these data was carried out. Finally, the molecular weight of the enzymes was calculated as described previously in the literature [15].

Enzyme activity assay

The activities of wild-type D-hydantoinase and mutated dimeric D-hydantoinase were determined by measuring the initial reaction rates [1]. The reaction mixtures containing 20 mM Tris–HCl (pH 7.4), 1 mM βME, and 0.5 mM $MnCl_2$ were used for the determination of the enzyme activities. The substrate concentration varied in the range of 2–50 mM during the initial reaction rate measurements. In order to investigate the catalytic activity of the mutated dimeric D-hydantoinase, we compared the kinetic parameters of wild-type D-hydantoinase with those of mutated dimeric D-hydantoinase. These two enzymes were simultaneously purified as described above. The specific activities of the reaction were measured on a substrate (D-hydantoin) and the concentration of the reaction products was determined (*N*-carbamoyl D-amino acid). Seven hundred and fifty microliters of reaction solution containing an appropriate hydantoin concentration was incubated with 2 μg of the purified enzymes at 37 °C for 5 min. The enzyme reactions were terminated with 6 M HCl containing a color reagent (10% *p*-dimethylaminobenzaldehyde (DAB)). The reaction mixtures were then centrifuged at 15000*g* for 5 min. The amount of product formed was determined by measuring the absorbance of the color reagent DAB at OD_{440}. The standard curve of hydantoic acid (*N*-carbamoyl glycine) was used to convert the absorbance to concentration.

Results and discussion

Identification of residues involved in coiled-coil interaction

Each monomer of wild-type tetrameric D-hydantoinase (HYD-T) is involved in two different dimeric interactions at two different ends: one involves the coiled-coil interaction with the α-helices and the other involves the interaction with the β-sheets (Fig. 1A) [2]. The helices, αD, αE, and αG, of the monomer are involved in one coiled-coil dimeric interaction, and the β-sheets, β1–β4 and β14–β18, are involved in the other dimeric interaction (Fig. 1A). We tried to mutate five residues for the purpose of destroying the coiled-coil dimeric interaction, so that it could generate two dimers from one tetrameric D-hydantoinase. It would be interesting to see to what extent the newly generated dimeric D-hydantoinase (HYD-D) behaves differently from HYD-T.

DHO is dimerized using one α-helix and several turns, of which the dimerizing pattern is similar to that of HYD-T [5]. Unlike homodimeric dihydroorotase, HYD-D is further dimerized to form a tetrameric D-hydantoinase, using the N-terminal β-sheets from its extra domain, a component which does not exist in

DHO. This β-sheet domain includes the N- and C-termini (β1–β4 and β14–β18; Fig. 1B). It is conceivable that the first dimerization step plays a role in the enzymatic activity of both HYD-T and DHO. The oligomeric state may increase the catalytic performance of the two enzymes. In fact, in the crystal structure of homodimeric DHO, one subunit was observed to bind to the substrate, and the other to bind to the product, which suggests that there is cooperative action between the two monomers of dimeric DHO [5]. On the other hand, it was established that neither HYD-T nor DHO follows the kinetics of an allosteric enzyme [14,16,17]. Regardless of the fact that the tetrameric form of D-HYD is not an allosteric enzyme, such mixed states of ligand binding as in DHO could also be important in the D-HYD reaction, because its hydrolytic active site is completely buried (see below).

It is interesting to compare the HYD-T enzyme to HYD-D in view of their different catalytic performances. Therefore, we designed five cumulative mutations (Y167S, V189A, Y192C, L193A, and L230G) in the three α-helices (αD, αE, and αG), in order to abolish the two coiled-coil interactions of the four interfaces of HYD-T (Fig. 1A).

Monomer A of HYD-T interacts with monomer H to generate one coiled-coil interaction of HYD-T and monomer B makes the other coiled-coil interaction with the G monomer (Fig. 1A). In the coiled-coil interface, five residues of the A monomer interact intensively with five residues of the H monomer (Fig. 1C). The five residues of the A subunit interact with identical residues of the H subunit by forming hydrophobic bonds, but the interacting counterparts are not identical, because the two subunits are positioned by non-crystallographic twofold symmetry (Fig. 1C). Based on the results of graphical modeling, it seems possible that if we carry out five mutations cumulatively (Y167S, V189A, Y192C, L193A, and L230G) on one monomer, the coiled-coil dimeric interactions of the mutant may be abolished, resulting in two dimers from one tetrameric D-HYD.

Mutagenesis of five residues in coiled-coil region

It was first necessary to confirm whether the mutated D-HYD in the five residues was expressed in solution. We conducted SDS–PAGE for the mutated D-HYD and wild type D-HYD. Both the mutated D-HYD and wild type D-HYD were over-expressed, as previously reported. Each expression level showed that the mutated D-HYD was expressed almost to the same extent as wild-type D-HYD. It seems that none of the five mutations in the coiled-coil region have any effect on the folding pathways of the mutated D-HYD. Site-specific mutagenesis for the five residues was achieved one by one, resulting in the accumulation of five mutations (Y167S, V189A, Y192C, L193A, and L230G) (Fig. 2).

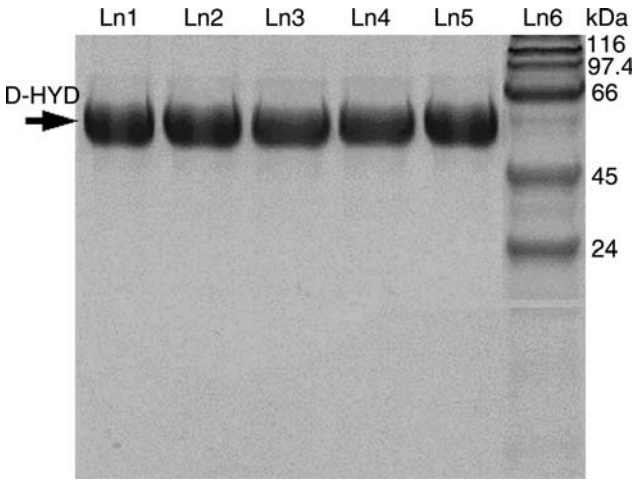

Fig. 2. Purification of wild-type and mutants of D-hydantoinase. Ln1: wild type, Ln2: mutation in residue 167, Ln3: mutations in residues 167 and 189, Ln4: mutations in residues 167, 189, 192, and 193, Ln5: mutations in residues 167, 189, 192, 193, and 230, and Ln6: molecular weight marker (205, 116, 97.4, 66, 45, and 29 kDa, respectively) from the top sequentially. The arrow is for D-hydantoinase.

The mutant proteins in each step were purified by Ni–NTA, following the procedure described in the user manual (Qiagen, USA).

Is mutant D-hydantoinase really a dimer?

Since the mutated D-HYD was expressed in solution, it needed to be verified whether or not the mutated D-HYD existed as a dimer in solution. We first ran native-PAGE for both HYD-T and the mutated D-HYD (the five residue mutated D-HYD, Ln 5 in Fig. 2). On the other hand, we were concerned about whether the ionic

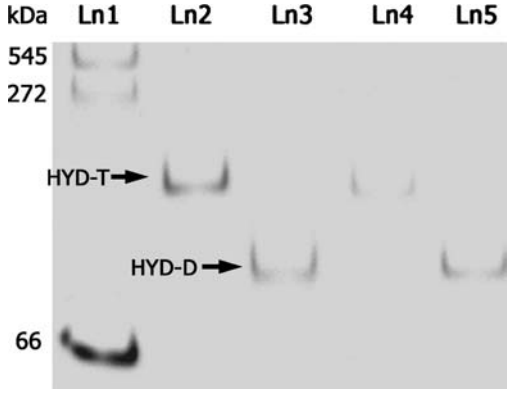

Fig. 3. Native-PAGE for tetrameric and dimeric D-hydantoinases. Ln 2 and 3 are in high-salt and Ln4 and 5 are in low-salt conditions. Ln1: Molecular weight markers (ureases of 545 kDa and 272 kDa, BSA of 66 kDa), Ln2: tetrameric D-hydantoinase in 20 mM, Tris–HCl (pH 7.4), 500 mM NaCl, and 1 mM βME, Ln3: dimeric D-hydantoinase in 20 mM, Tris–HCl (pH 7.4), 500 mM NaCl, and 1 mM βME, Ln4: tetrameric D-hydantoinase in 20 mM Tris–HCl (pH 7.4) and 1 mM βME, and Ln5: dimeric D-hydantoinase in 20 mM Tris–HCl (pH 7.4), 1 mM βME. The arrows represent tetrameric and dimeric D-HYDs.

strength would affect the oligomeric state of D-HYD (depending on the ionic strength, it may exist either as a dimer or as a tetramer). Thus, we ran native-PAGE of D-HYD under both high-salt and low-salt conditions (see Fig. 3. for the gel and salt conditions). Regardless of the salt conditions, the gel patterns of HYD-T and the mutated D-HYD were identical (Fig. 3). It seems evident that the salt conditions did not affect the change in the oligomeric state of D-HYD under our experimental conditions. It was obvious that the location of the mutated D-HYD was positioned below HYD-T, which could indicate that the mutated D-HYD exists as dimeric D-hydantoinase (HYD-D) in solution. However, under high salt conditions, it was noticeable that non-specific aggregates of the two enzymes formed 5 days after purification (data not shown). Thus, care must be taken to use fresh samples of HYD-T and HYD-D for accurate experimental results to be obtained.

Determination of molecular weight of dimeric D-hydantoinase

The molecular weight of D-HYD is about 55 kDa for the monomer, 110 kDa for the dimer, and 220 kDa for the tetramer. It was established that wild-type D-HYD exists as a tetramer, whose molecular weight is 220 kDa. Molecular weights can be deduced from native-PAGE by comparing the relative mobility of the PAGE sample with those of standard molecular markers, such as ureases and BSA [16]. We were able to deduce from native-PAGE (Fig. 4) that the molecular weight of HYD-D was about 110 kDa, by extrapolating the relative migration of HYD-D on the standard curve.

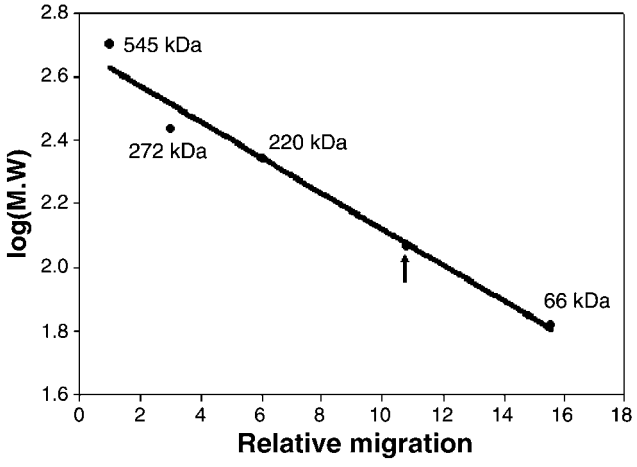

Fig. 4. Measurement of molecular weight for dimeric D-hydantoinase. Standard molecular weight (MW) markers: ureases of 545 and 272 kDa, tetrameric D-hydantoinase of 220 kDa, BSA of 66 kDa. The arrow represents the position of dimeric D-hydantoinase and the relative mobility of the dimeric D-hydantoinase corresponds to a size of about 110 kDa. The filled circles represent each MW nearby.

In addition, we performed gel filtration chromatography to examine the oligomeric states of HYD-T and HYD-D. It was previously reported that HYD-T exists in the tetrameric form [2,14] and thus, HYD-T was used as the standard molecular weight marker. There was a clear difference of molecular sizes between HYD-T and HYD-D in the elution profile of gel filtration chromatography. A mixture of 500 μl of HYD-T (10 mg/ml concentration) and 500 μl of HYD-D (10 mg/ml concentration) was loaded onto the gel filtration column. The elution profile for the mixture of HYD-T and HYD-D was also obtained by SDS–PAGE analysis (Fig. 5A) and native-PAGE analysis (Fig. 5B) for the same eluted fractions, in order to establish whether HYD-T and HYD-D were fractionated depending on their molecular sizes. Fig. 5 clearly shows that HYD-T and HYD-D have different sizes and thus different molecular weights.

Sedimentation equilibrium experiment to determine oligomeric state

The molecular weight data obtained by gel filtration chromatography and native-PAGE provide quite convincing evidence for the dimeric state of HYD-D.

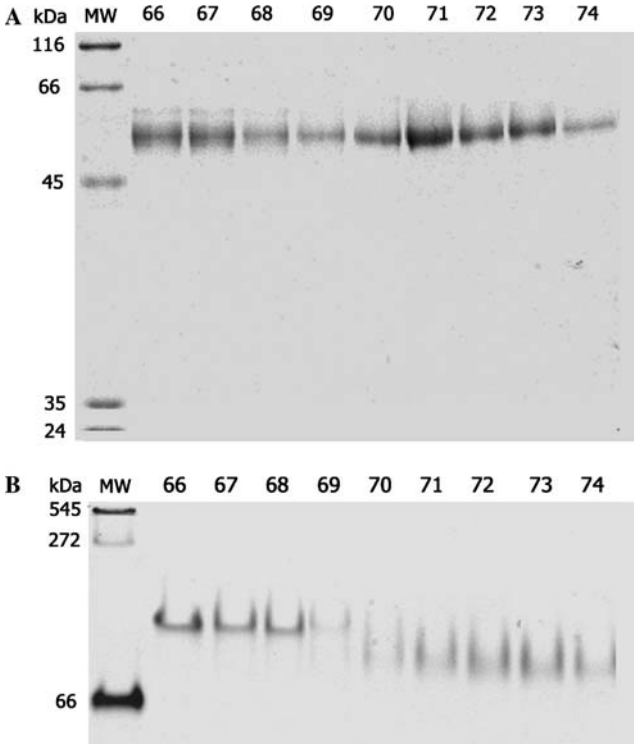

Fig. 5. Elution profiles of gel filtration chromatography for tetrameric and dimeric D-hydantoinases. (A) SDS–PAGE analysis for elutes. (B) Native-PAGE analysis for elutes. Molecular weight markers (marked as MW) are 116, 66, 45, 35, and 24 kDa for SDS–PAGE. The same markers (marked as MW) as those shown in Fig. 3 were used in native-PAGE. In SDS–PAGE, the fractions from 64 to 69 are shown for HYD-T and the fractions from 70 to 74 are for HYD-D. Native-PAGE was performed for the same fractions as SDS–PAGE.

However, these results could be considered as relative measurements. We tried to obtain other, more conclusive evidence for the dimeric state of HYD-D by performing an experiment using analytical ultracentrifugation, which is currently considered to be one of the most reliable methods for molecular weight determination.

In analytical ultracentrifugation, if the ln C (C: concentration) or ln A (A: absorbance at 280 nm) versus R^2 (R: radius of the sample holder in cm) plots are linear, a reasonably accurate average molecular weight can be obtained for a given protein [16] (Beckman XL-A instruction manual). Once centrifugal operation reached sedimentation equilibrium, the resultant data were collected from the computer window terminal of the analytical ultracentrifuge. Sedimentation equilibrium was achieved after 27 h. The centrifugal passages of HYD-T and HYD-D are shown and the distributions of their passages are obviously distinct from one another, which indicates that their oligomeric states are not the same (Fig. 6). The distribution curve of HYD-T was steeper than that of HYD-D, which implies that HYD-D is a smaller macromolecule than wild type D-HYD, because a larger molecule is to be found closer to the bottom of the sample cell at sedimentation equilibrium, i.e., it has a steeper distribution. We were able to calculate the molecular weight from the slopes of the ln A versus R^2 plots, on the basis that the dln C^2/R^2 was proportional to the molecular weight [16]. The slope (dln C^2/R^2) of HYD-T was 43.662 and that of HYD-D was 24.981 with standard errors of 0.011 and 0.013, respectively. The slope of HYD-T is approximately twice that of HYD-D. From the analytical ultracentrifuge experiments, the molecular weight of HYD-D was estimated to be 125.87 kDa. This result was consistent with the native-PAGE analysis, which provided a value of 118 kDa for HYD-D. Taken together, it could be assumed that HYD-D exists as a dimer in solution and that its molecular weight is about 110 kDa, which indeed corresponds to that of a dimer.

Dimeric D-hydantoinase is catalytically active?

The hydrolytic activity of the purified D-HYDs exhibited normal hyperbolic Michaelis–Menten kinetic patterns [17]. The initial reaction rates for the enzyme reactions were obtained at substrate concentrations in the range of 2–50 mM in the case of hydantoin. The kinetic parameters, such as K_m and V_{max}, were calculated for wild-type D-hydantoinase (HYD-T) and dimeric D-hydantoinase (HYD-D) from the Lineweaver–Burk plots as described in the report by Shimizu et al. [17]. Measurements of V_{max} and K_m were carried out three times and the values were averaged. V_{max} and K_m for HYD-T were 0.236 μmol [min mg]$^{-1}$ and 52.6 mM, respectively (standard deviations are 0.020 and 4.0, respectively). V_{max} and K_m for HYD-D were 0.0612 μmol

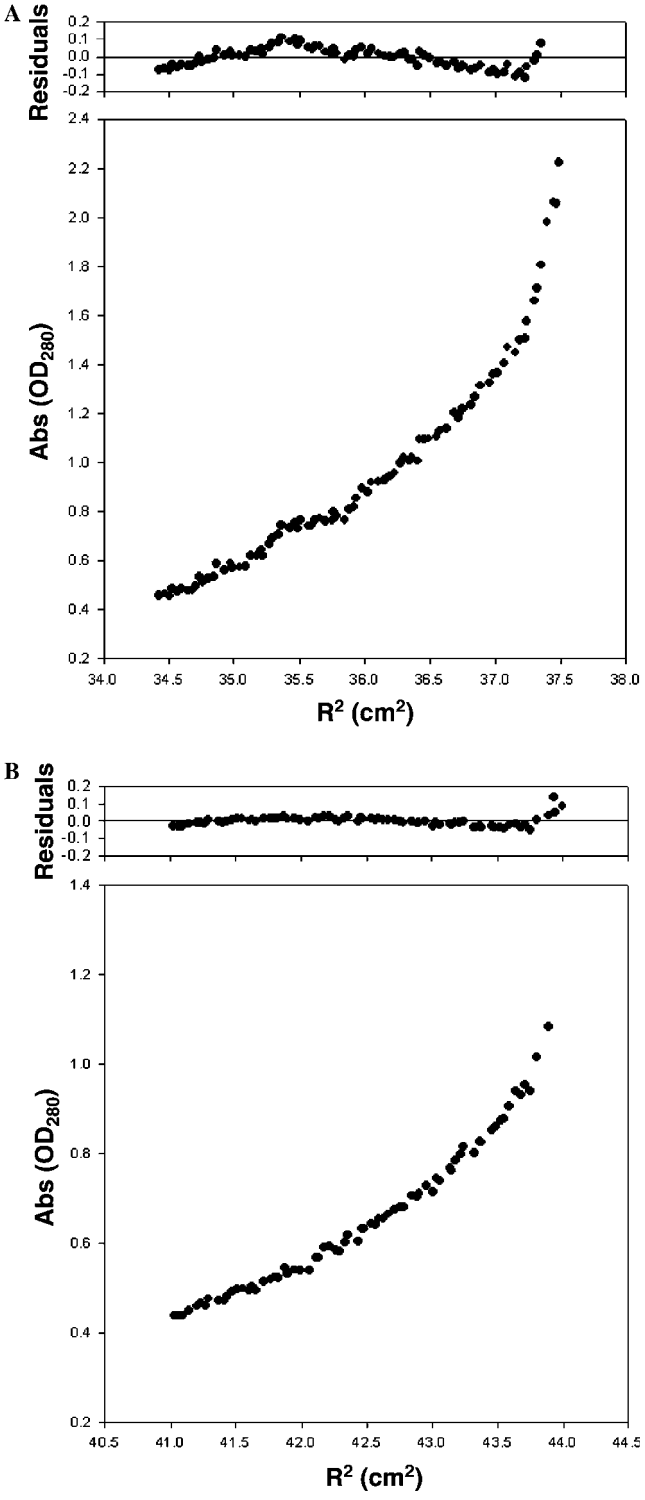

Fig. 6. Profiles of sedimentation equilibrium by analytical ultracentrifugation. (A) Tetrameric D-hydantoinase. (B) Dimeric D-hydantoinase. The sedimentation curve of tetrameric D-hydantoinase is steeper than that of dimeric D-hydantoinase (the values in the horizontal axis are in a different range). Abs represents absorbance and R represents the radius to the absorbance (Abs) peak position. The profiles of Abs distribution show that a clear difference exists between the tetrameric and dimeric D-hydantoinases. Residuals in the top panels represent the difference between actual values and estimated values by non-linear quadratic regression.

[min mg]$^{-1}$ and 190.1 mM, respectively (standard deviations are 0.0031 and 5.3, respectively). The V_{max} value of HYD-D was 26% of that of HYD-D and the K_m value of HYD-D was about four times that of HYD-T.

The specific activity of HYD-D was determined to be 83.3 U/μmol enzyme, which corresponds to 5.3% of the activity of HYD-T. Under these reaction conditions, one unit of enzyme is defined as the quantity of enzyme required to make the product at a rate of 1 μmol/min [17,18]. The low specific activity of HYD-D indicates that its coiled-coil interaction may have a significant effect on the catalytic activity of wild-type tetrameric D-hydantoinase (HYD-T). It may be for analogous reasons that the catalytic activity of monomeric DHO is less than that of dimeric DHO.

Based on the experiment designed to change the oligomeric state of D-HYD, it can be supposed that the catalytic performance of D-hydantoinase is somehow related to the oligomeric structure of the enzymes in D-HYD and DHO, even though their kinetic patterns do not represent allosteric catalysis [5,17,19]. It will also be interesting to see, if we were to design monomeric D-hydantoinase, how much further the change in the oligomeric state would affect its catalytic behavior.

Inferred model of enzymatic catalysis for dimeric D-hydantoinase

The specific activity of HYD-D corresponds to only 5.3% of that of HYD-T. This clearly indicates that the oligomeric state of D-HYD has an influence on its catalytic activity. For various proteins, the assembly to oligomers has a distinct biological function, such as allosteric regulation or cooperativity. However, it was reported that neither D-HYD nor DHO performs such regulatory functions, even though they exist as homotetrameric and homodimeric forms, respectively [1,14,17].

One interesting feature is that the V_m value of HYD-D is one-third of that of HYD-T, whereas its K_m value is four times larger than that of HYD-T, which might indicate that the change of oligomeric state might very much hinder the substrate binding so that it would lower the value of V_m. In the case of DHO, the substrate is bound to one active-site of the dimer and the product is bound to the other active-site [5], which may suggest that one monomer of DHO is linked to the ligand binding of the other monomer. It is apparent that a higher oligomeric conformation is more effective in promoting the substrate binding of D-HYD. In order for the substrate to bind to the active-site of D-HYD, three stereo gate loops have to be moved away from the ceiling of the active-site, because the active-site of D-HYD is completely buried by these three stereo gate loops [2]. Thus, it is conceivable that the change of oligomeric state may have some influence on the movement of these stereo gate loops.

On the other hand, it has been reported that the deactivation of L-HYD resulting from the removal of the metal ions is accompanied by the disassembly of the tetramer leading to the formation of monomers [4]. The metal binding was related to the change of oligomeric state in L-HYD. It may be that HYD-D is more prone to losing its metal binding capability than HYD-T. Another possibility is that the dimeric state of D-HYD changes the conformation by destabilizing the metal binding in the active-site or conformation. Detailed structural research will be needed to elucidate the exact mechanism involved in the catalytic effect of HYD-D.

A group of cyclic amidases, including hydantoinase and dihydropyrimidinase, are very important enzymes in terms of biochemical and industrial aspects. The D-hydantoinase is being used for the industrial production of D-amino acids [20] and the dihydropyrimidinase is a pyrimidine-metabolizing (a base of DNA) enzyme in human [3]. Our study has proposed that they have a distinctive enzymatic feature; enzymatic activity increases in the higher oligomeric state. In addition, the reaction is not controlled by allosteric regulation whereas many oligomeric enzymes generally do their reaction by allosteric fashion [17,19]. Therefore, it will be very significant to continuously investigate how the enzymatic reaction for this group of cyclic amidases is related to oligomeric states, which will be useful for the industrial application of D-hydantoinase and also to understand a new biochemical feature for a group of the oligomeric cyclic amidases.

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