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Stabilization of D-hydantoinase by intersubunit cross-linking

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Abstract

It was observed that tetrameric D-hydantoinase from *Bacillus stearothermophilus* SD1 is dissociated into monomers under operational conditions, resulting in a detrimental loss of its catalytic activity. As an approach to reduce the dissociation of subunits and to maintain its catalytic activity, intersubunit cross-linking was attempted by using EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride). The cross-linking conditions were optimized in terms of stabilization and catalytic activity of the recovered enzyme. Cross-linked D-hydantoinase showed a four-fold longer half-life under operational conditions and was very stable even at an elevated temperature, whereas the native enzyme was almost completely deactivated. In addition, intersubunit cross-linking of D-hydantoinase also led to stabilization of the enzyme in the presence of 20% methanol and under acidic conditions. The cross-linked enzyme was more efficient in the conversion of substrate, which seems to be due to the increased stability of enzyme.

Keywords: D-hydantoinase; *Bacillus stearothermophilus*; Intersubunit cross-linking; EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride)

1. Introduction

Enzymes or proteins are inactivated via several molecular mechanisms [1], and the overall physical process may be presented simply by two steps. The first step involves reversible conformational changes, followed by a second irreversible unfolding step in which the enzyme or the protein losses its activity.

As an approach to stabilize proteins or enzymes by reducing irreversible unfolding, intra- or intermolecular cross-linking has been attempted. Many studies have shown that intramolecular cross-linking is effective in stabilization of proteins or enzymes [2–5]. In the case of multimeric enzymes or proteins, on the other hand, intermolecular cross-linking has been introduced, leading to protein stabilization by preventing the dissociation of subunits from occurring. White et al. conducted intersubunit cross-linking on hemoglobin with *bis*(3,5-dibromosalicyl) fumarate, and showed that a single cross-link between only two of the four subunits could result in a more stable protein [6]. Recently, streptavidin, a tetrameric protein composed of two weakly associated, stable dimers, was cross-linked between subunits by EDC (1-ethyl-3-(3-dimethylaminopropyl) carboii-mide, hydrochloride), a zero-order cross-linker [7]. The covalently linked streptavidins showed higher biotin-binding ability than the parent streptavidin even after heat treatment.

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In our previous work, we isolated, characterized, and overexpressed a D-hydantoinase from *Bacillus stearothermophilus* SD1 [8–10]. D-hydantoinase is currently used in the production of D-amino acids which are intermediates for the synthesis of semi-synthetic antibiotics, peptide hormones, and pesticides [11]. We observed that homotetrameric D-hydantoinase from *B. stearothermophilus* SD1 is subject to dissociation to a monomer under operational conditions, and dissociation of subunit is accelerated under acidic conditions. The dissociated monomer was very unstable, in agreement with previously reported results [12]. Based on the above observation, we hypothesized that intersubunit cross-linking of tetrameric D-hydantoinase from *B. stearothermophilus* SD1 would reduce the dissociation of subunits, leading to the stabilization of enzyme.

In this paper, we demonstrate that tetrameric D-hydantoinase is stabilized by intersubunit cross-linking using a chemical cross-linker. Details are reported herein.

2. Experimental

2.1. Materials

EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride), β-mercaptoethanol, *p*-dimethylaminobenzaldehyde, hydantoin, and hydantoic acid were purchased from Sigma (St. Louis, MO). *p*-Hydroxyphenyl hydantoin (HPH) was obtained from TCI (Japan). Water and acetonitrile were from Burdict and Jackson Lab (Muskegor, MI). All other reagents were analytical grade.

2.2. Expression and purification of D-hydantoinase

The D-hydantoinase gene from *B. stearothermophilus* SD1 was expressed in an *E. coli* XL1-Blue strain containing a pUC18-based recombinant plasmid, pHU183, as described in our previous work [10]. *E. coli* cells were cultured overnight in Luria–Bertani medium containing 100 μg/ml ampicillin at 37°C. D-hydantoinase was purified as previously described [9].

2.3. Cross-linking of D-hydantoinase

It has been known that degree of cross-linking is significantly affected by pH, and thus cross-linking reaction was first conducted at different pH levels. The purified D-hydantoinase (6 μM) was cross-linked with 20 mM EDC in individual buffered solutions ranging from pH 5 to 10 for 30 min at room temperature. The excess EDC was quenched by adding 20 mM β-mercaptoethanol as reported by Grabarek et al. [13]. The cross-linked enzyme was dissolved in loading buffer containing 0.1% SDS and analyzed with native PAGE to investigate the cross-linking state. The activity of the cross-linked enzyme was determined as previously reported [9].

Once the optimized pH was determined, the D-hydantoinase was cross-linked in the presence of different EDC concentrations for various reaction times. Both the optimal concentration of EDC and reaction time were determined on the basis of activity assay and PAGE analysis.

2.4. Purification of the cross-linked D-hydantoinase

After cross-linking reaction, the reaction mixture containing 0.1% SDS was loaded onto a native polyacrylamide gel composed of 5% stacking gel and 9% resolving gel, respectively. After electrophoresis, gel was washed with 50 mM Tris–Boric acid buffer (pH 8.7) and loaded into Whole Gel Eluter (Bio-Rad). The intersubunit cross-linked enzyme was separated from native enzyme by applying a current of 100 mA and for 27 min in 50 mM Tris–Boric acid buffer (pH 8.7), followed by applying reverse current for 20 s. The eluted fractions of cross-linked enzyme were rerun with SDS-PAGE and detected by Coomassie blue stain. The fractions containing only the cross-linked enzyme were concentrated and dialyzed against 20 mM Tris–HCl buffer (pH 8.0) containing 1 mM $MnCl_2$.

2.5. Characterization of the cross-linked D-hydantoinase

2.5.1. Activity and optimal condition

The activity of the native or the cross-linked enzymes was determined by using the colorimetric

method described in our previous work [9]. The purified enzyme (0.5 μg) was added to the reaction mixture containing 100 mM hydantoin and 0.5 mM $MnCl_2$ in 1 ml of 100 mM Tris–HCl buffer (pH 8.0) after nitrogen flushing. The reaction mixture was incubated for 30 min in a 55°C shaking water bath, and the reaction was stopped by adding 333 μl of 6 N HCl solution. One unit of D-hydantoinase was defined as the amount of enzyme required to produce 1 μmol of *N*-carbamoyl-D-glycine from hydantoin per min under specified condition.

In order to determine the optimal pH, several buffers were used: 50 mM sodium acetate buffer for pH 5–6, 50 mM sodium phosphate buffer for pH 6–7, 100 mM Tris–HCl buffer for pH 7–9, and 50 mM boric acid-NaOH buffer for pH 9–10 were used. The reaction mixture consisted of 0.5 μg purified enzyme, 100 mM hydantoin and 0.5 mM $MnCl_2$ in a total volume of 1 ml of the appropriate buffer solution.

2.5.2. Determination of enzyme stability

Native or cross-linked enzyme (25 μg/ml) was incubated in 20 mM Tris–HCl buffer (pH 8.0) containing 0.5 mM $MnCl_2$ for 60 min at different temperatures, and then residual activity was determined. To trace the enzyme activity as a function of time, aliquots of the enzyme solution were taken at various time intervals, and the residual activity was assayed.

The stability of enzyme in the presence of an organic solvent was investigated by incubating 25 μg/ml enzyme in 20 mM Tris–HCl buffer (pH 8.0) containing 20% methanol and 0.5 mM $MnCl_2$ at 55°C. For this, the aliquots were removed at 30 min intervals and residual enzyme activity was measured.

2.6. Conversion of HPH to N-carbamoyl-D-hydroxyphenyl glycine (NC-HPG) using the cross-linked enzyme

Either native or cross-linked enzyme was incubated in a reaction mixture containing 100 mM HPH as a substrate, 0.5 mM $MnCl_2$, and 100 mM Tris–HCl (pH 8.0) at 55°C. Aliquots were taken at various time intervals and analyzed using HPLC as described below.

2.7. Analysis

The concentrations of HPH and *N*-carbamoyl-D-hydroxyphenyl glycine (NC-HPG) were determined using an HPLC (Shimadzu, Kyoto, Japan) with a UV-VIS detector set at 214 nm. An ODS HPLC column (YMC, Kyoto, Japan) was used with a 10% (vol./vol.) acetonitrile (1 ml/min). Analytical SDS-PAGE in slab gels was performed according to the method of Laemmli [14]. Acrylamide gels were stained with Coomassie brilliant blue G250. The protein concentration was determined by the method of Bradford [15] using bovine serum albumin as a standard.

3. Results and discussion

3.1. Dissociation of tetrameric D-hydantoinase

To investigate whether the catalytic activity of D-hydantoinase is linked with the oligomeric structure of enzyme, we followed the change in the tertiary structure of enzyme with time by both monitoring the enzyme activity and the dissociation of the subunits with PAGE. The purified enzyme was incubated in 50 mM sodium acetate buffer (pH 5.0), and aliquots were removed at various intervals for structure analysis and residual activity assay. For the oligomeric structure, enzyme solution was treated with 20 mM EDC for intersubunit cross-linking and then analyzed on SDS-PAGE. Since only a small portion of the oligomeric enzymes is cross-linked by EDC, most of the remaining oligomers are dissociated into monomers and thus appear as monomer on SDS-PAGE. The portion of the cross-linked enzyme is proportional to the amount of oligomeric enzymes in solution, thus the change in the oligomeric structure of the enzyme can be traced as a function of time. As shown in Fig. 1A, the band corresponding to the oligomeric protein became fainter with longer incubation time and completely disappeared after 5 h. Concurrently, a decrease in the enzyme activity was observed with time (Fig. 1B). These results indicate that the catalytic activity of D-hydantoinase is associated with its oligomeric structure.

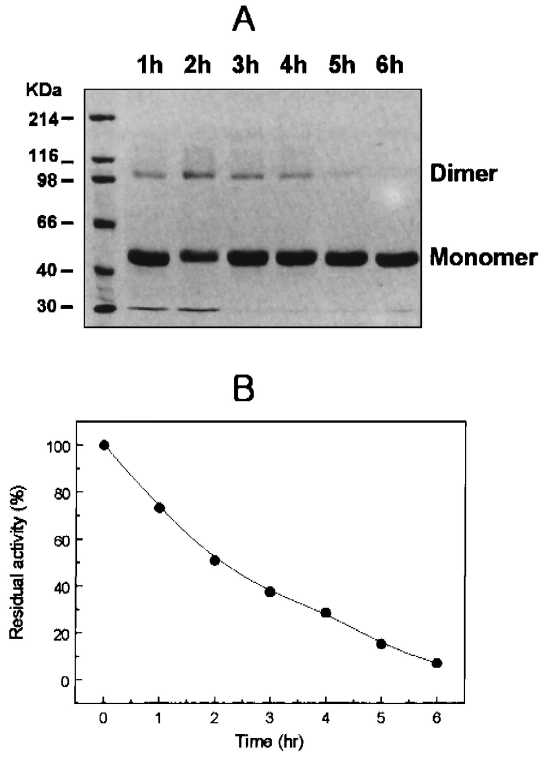

Fig. 1. Dissociation of oligomeric D-hydantoinase. The purified enzyme (30 μg) was incubated in 50 mM sodium acetate buffer (pH 5.0), and aliquots were taken at intervals to quantify the portion of oligomeric enzymes and catalytic activity. (A) Analysis on SDS-PAGE after cross-linking reaction. (B) Residual enzyme activity. Data represent an average of duplicate experiments.

3.2. Optimal condition for cross-linking

It has been reported that the cross-linking of carboxyl group and amine group within a protein by EDC is significantly affected by pH, and that the cross-linking reaction is favorable under acidic conditions [16]. Meanwhile, deactivation of D-hydantoinase due to the dissociation of subunits was observed to be accelerated as the pH was lowered [17]. We first investigated the effect of pH on the degree of cross-linking and the recovery of enzyme activity. The purified D-hydantoinase was incubated with 20 mM EDC in different buffers ranging from pH 6 to 8, and an aliquot of the reaction mixture, mixed with loading buffer containing 0.1% SDS, was analyzed with native PAGE. Since the native D-hydantoinase dissociates into monomers in the presence of 0.1% SDS, the cross-linked enzymes can be separated from the dissociated monomers on a native polyacrylamide gel when loaded with 0.1% SDS. As a result, the portion of cross-linked enzyme was very low at pH 8, even though the activity of the cross-linked enzyme was about 90% of the native enzyme (data not shown). The degree of cross-linking increased as pH was lowered with a corresponding serious loss in catalytic activity. At neutral pH, the degree of cross-linking was significant, and the loss of enzyme activity was less than 15% when compared to that of the native enzyme. Based on these observations, the optimal pH for cross-linking was determined to be 7.0.

Under the optimal pH of 7.0, the effect of EDC concentration on the initial activity and the stability of the enzyme were examined. For stability test, the reaction mixture was incubated at 70°C for 1 h after the cross-linking reaction, and the residual activity was determined. As shown in Fig. 2, the residual activity increased as the EDC concentration increased until a slightly decrease occurred after 10 mM EDC. The native enzyme exhibited about 5% of the initial activity, meanwhile the cross-linked enzyme retained 30% of its initial activity. After the cross-linked enzyme was incubated at 70°C for 1 h, the reaction mixture was analyzed on native PAGE, and

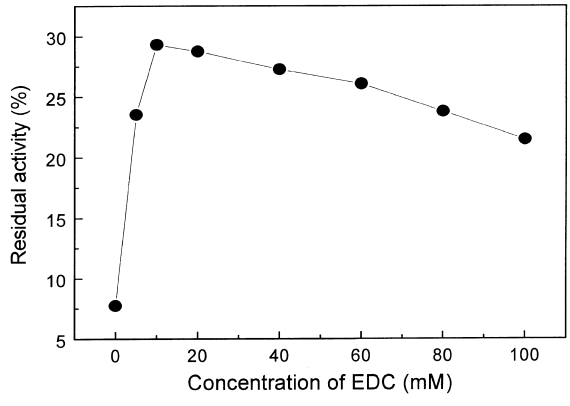

Fig. 2. Effect of EDC concentration on the activity of D-hydantoinase. After the cross-linking reaction, the enzyme solution was incubated at 70°C for 1 h, and then the residual activity was determined. Data represent an average of duplicate experiments.

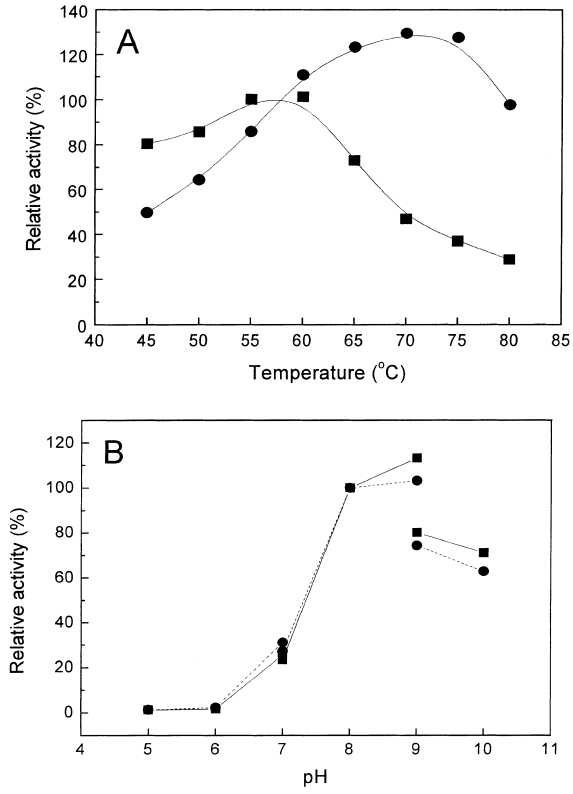

Fig. 3. Effect of temperature (A) and pH (B) on the specific activity of the native (■) and the cross-linked (●) enzymes. Data indicate an average of duplicate experiments.

dissociated subunits were detected to be negligible (data not shown). Intermolecular cross-linking between the tetrameric enzyme molecules was observed to be negligible under the experimental condition. From these results, it seems that the multimeric D-hydantoinase was significantly stabilized by cross-linking, which is due to a reduction in the dissociation of subunits.

3.3. Characteristics of the cross-linked D-hydantoinase

The intersubunit cross-linked D-hydantoinase was purified from non-intersubunit cross-linked enzymes by using a Whole Gel Eluter (Bio-Rad) after native gel electrophoresis as described in experimental section, and used for further characterization.

The specific activity of the cross-linked D-hydantoinase was determined to be 120 units/mg protein, which corresponds to about 85% of the native

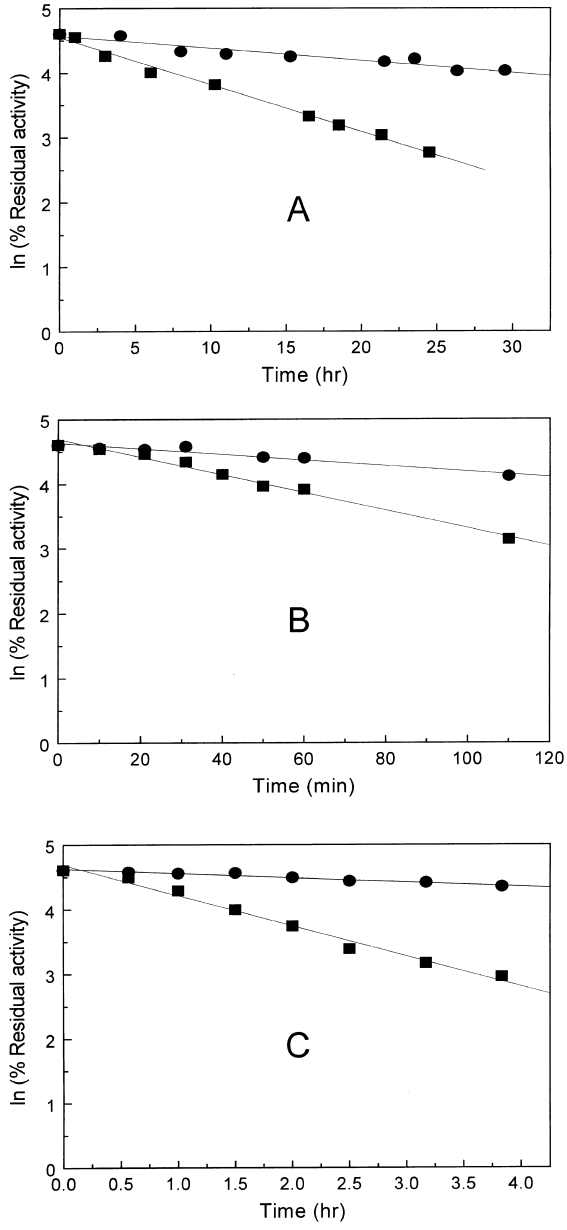

Fig. 4. Stability of the native (■) and the cross-linked (●) enzymes under different experimental conditions. (A) pH 8.0; (B) pH 6.0; (C) in the presence of 20% methanol in 100 mM Tris–HCl buffer (pH 8.0). Data represent an average of duplicate experiments.

enzyme. The cross-linked enzyme showed maximal activity around 70°C, which is much higher than native enzyme (Fig. 3A). This can be attributed to an increase in the stability of the cross-linked enzyme. No significant difference in the dependency on pH was observed between the cross-linked and the native enzymes (Fig. 3B).

To investigate the stability, the cross-linked enzyme was incubated at 55°C, and the residual activity was traced as a function of time. As can be seen in Fig. 4A, the cross-linked enzyme maintained its activity for a longer time than the native enzyme. The half-life of the cross-linked enzyme was calculated to be about 37 h, when inactivation was assumed to follow first-order kinetics. In the case of native enzyme, the first-order deactivation constant was determined to be 9.5 h. The cross-linked enzyme was very stable even at temperature higher than 70°C, whereas the native enzyme rapidly lost its activity (Fig. 5).

As mentioned earlier, dissociation of the subunits became more serious at acidic condition, resulting in the rapid inactivation of the enzyme. We investigated the stability of the cross-linked enzyme at pH 6.0. As shown in Fig. 4B, the cross-linked enzyme remained more stable even under this slightly acidic condition when compared to the native enzyme.

Since the 5-mono-substituted hydantoin derivative has a relatively low solubility in water, addition of an organic solvent to the enzyme reaction solution is expected to improve the performance of reaction due to the increased solubility of the substrate. We chose methanol by taking into consideration the effect on the enzyme activity and substrate solubility. When methanol was added in the reaction mixture containing 10 g/l of HPH particles, the solubility of HPH was linearly increased in proportion to the concentration of methanol. In the presence of 20% methanol, the solubility of HPH increased about three-fold. The cross-linked enzyme maintained its activity even in the presence of 20% methanol, while native enzyme lost its activity very fast (Fig. 4C). The half-life of the cross-linked enzyme was seven times longer than that of the native enzyme.

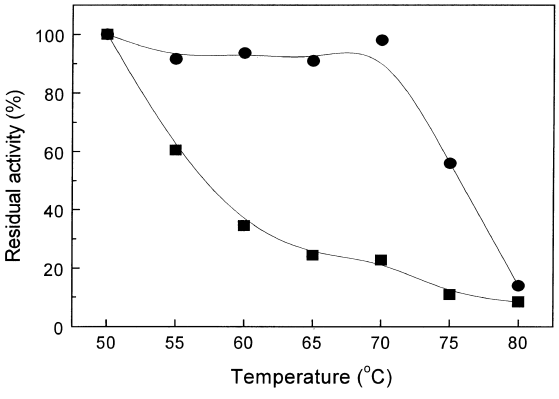

Fig. 5. Thermal stability of the native (■) and the cross-linked (●) enzymes. The enzyme was incubated at different temperatures for 60 min, and the residual activity was determined. Data indicate an average of duplicate experiments.

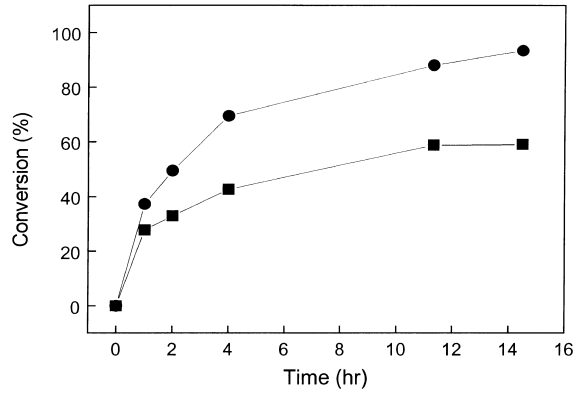

Fig. 6. Production of *N*-carbamoyl-D-hydroxyphenyl glycine from HPH using the native (■) and the cross-linked (●) enzymes. Data indicate an average of duplicate experiments.

3.4. Conversion of HPH using the cross-linked enzyme

To test the applicability of the cross-linked enzyme for the production of D-amino-acids, conversion of HPH was conducted using the cross-linked and the native enzymes. As shown in Fig. 6, the production rate of the *N*-carbamoyl-D-amino acid by the cross-linked enzyme was faster than by the native enzyme. This seems to be due to the corresponding increased stability of the cross-linked enzyme.

We have demonstrated that the tetrameric D-hydantoinase can be stabilized by chemically induced intersubunit cross-linking and that this approach is expected to be applicable to other multimeric enzyme systems.

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