

Electrostatic interaction between two domains of isocitrate dehydrogenase from *Thermus thermophilus* is important for the catalytic function and protein stability

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Abstract The role of electrostatic interaction between Lys⁹⁶ and Glu¹⁴⁷ of isocitrate dehydrogenase from *Thermus thermophilus* was investigated by site-directed mutagenesis. These two residues are located near the active site and involved in the interdomain interaction. Analyses of the catalytic properties and thermostability of the Glu¹⁴⁷Gln mutant revealed that this interaction plays important roles in catalytic function and protein stability.

Key words: Active site; Thermostability; Site-directed mutagenesis; Thermophilic enzyme

1. Introduction

Isocitrate dehydrogenase (ICDH, EC 1.1.1.42) and 3-isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85) belong to a unique enzyme family of bifunctional decarboxylating dehydrogenases. These enzymes act on structurally similar substrates containing a malate moiety, and catalyze chemically equivalent reactions: dehydrogenation at carbon-2 of the malate moiety to form a carbonyl group from a hydroxyl group, and decarboxylation at carbon-3. These two enzymes share a common structural framework and catalytic mechanism [1,2], but have different substrate and coenzyme specificities.

We have cloned and sequenced the gene coding for NADP-dependent ICDH and NAD-dependent IPMDH from an extreme thermophile, *Thermus thermophilus* HB8 [3–5]. The 3-D structures of *T. thermophilus* and *Escherichia coli* IPMDHs and *E. coli* ICDH have been reported [1,2,6]. The primary structure and the folding topology of ICDH, as well as the subunit structure, are similar to those of IPMDH [1–6], suggesting that these enzymes might have diverged from a common ancestral enzyme. The X-ray structure of *E. coli* ICDH-substrate co-crystal [2] revealed that the malate moiety of isocitrate is recognized by three arginine, three aspartate, one lysine, and one tyrosine residues. These are highly conserved in this enzyme family including thermophile ICDH and IPMDH, suggesting that the active site structure is well conserved. This is also supported by our recent mutational analyses [7–10]. However, the reported structures of IPMDH, including the substrate or coenzyme complex, have an open conformation with a wide cleft concerning the active site, while ICDH takes a closed conformation by narrowing the cleft (Fig. 1). The small-angle X-ray scattering analysis showed that IPMDH adopts two distinct intermediate conformations between the open and closed states upon binding of

coenzyme and substrate, respectively, and that it prefers a fully closed conformation in a ternary complex with both coenzyme and substrate [11]. Thus, closure of the active site is necessary to form the Michaelis complex.

In *E. coli* ICDH, Arg¹¹² interacts with Glu¹⁶⁴; the distance between NH of Arg¹¹² and Oε of Glu¹⁶⁴ is less than 3 Å (Fig. 1). This interaction seems to play important roles in the catalytic function and/or protein stability of ICDH. Glu¹⁶⁴ is well conserved in thermophile ICDH (Glu¹⁴⁷), and Arg¹¹² is also conserved as a basic residue, Lys⁹⁶, leading to the expectation that the interdomain interaction may also be present in the thermophile ICDH (Table 1). In contrast, the corresponding residues in IPMDHs are not conserved, and the electrostatic interaction between the two domains cannot be found in the IPMDH structure (Fig. 1). In this study, the role of the interaction between Lys⁹⁶ and Glu¹⁴⁷ in the catalytic function and thermostability was investigated by site-specific amino acid replacement of Glu¹⁴⁷ with Gln.

2. Materials and methods

2.1. Construction of mutant enzymes

DNA manipulating enzymes used in this study were products of either Toyobo, Bethesda Research Laboratories or New England Biolabs. *E. coli* MV1190 ($\Delta(lac-proAB)$, *thi*, *supE*, $\Delta(srl-recA)$ 306::Tn10 (*ter*), F'*[traD 36, proAB, lacZ⁺ lacZ Δ M15]*) was used for DNA amplification and expression of mutated *icd* genes of *T. thermophilus* HB8 [3]. Site-directed mutagenesis was carried out according to the method of Kunkel [12]. The oligonucleotide used for generating mutation was 5'-GAGATGCGGCCTTAAGTCGTG-TACGTC-3' for substituting Glu¹⁴⁷ with Gln. Expression and purification of the wild-type and mutant enzymes were done as described previously [7]. Both enzymes used in this study were purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis.

2.2. Kinetic analysis

Michaelis constant, K_m , for isocitrate and catalytic constant, k_{cat} , were determined in steady-state kinetic experiments at 60°C in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate-NaOH buffer (pH 7.8) containing 5.0 mM MgCl₂, 5.0 mM NADP. The isocitrate concentration was varied in the range of 5–500 μ M. Initial velocities were determined by monitoring the formation of NADH at 340 nm. In order to determine K_m for NADP, the concentrations of coenzymes were varied in the range of 5–500 μ M employing a fixed isocitrate concentration (1.0 mM). It has been confirmed that no substrate inhibition occurred under these conditions.

2.3. Circular dichroism measurement

Circular dichroism (CD) measurements were carried out with a JASCO J-720 spectropolarimeter. The 0.1-cm cell was used for the far-UV CD measurement. The temperature of the sample solution in the cell was controlled by a programmable temperature controller and monitored by a thermocouple in the cell. The scan rate was about 1.0 deg/min and the concentrations of the wild-type and the mutant enzymes which resolved in 20 mM potassium phosphate buffer (pH 7.8) were 0.20 mg/ml and 0.21 mg/ml, respectively.

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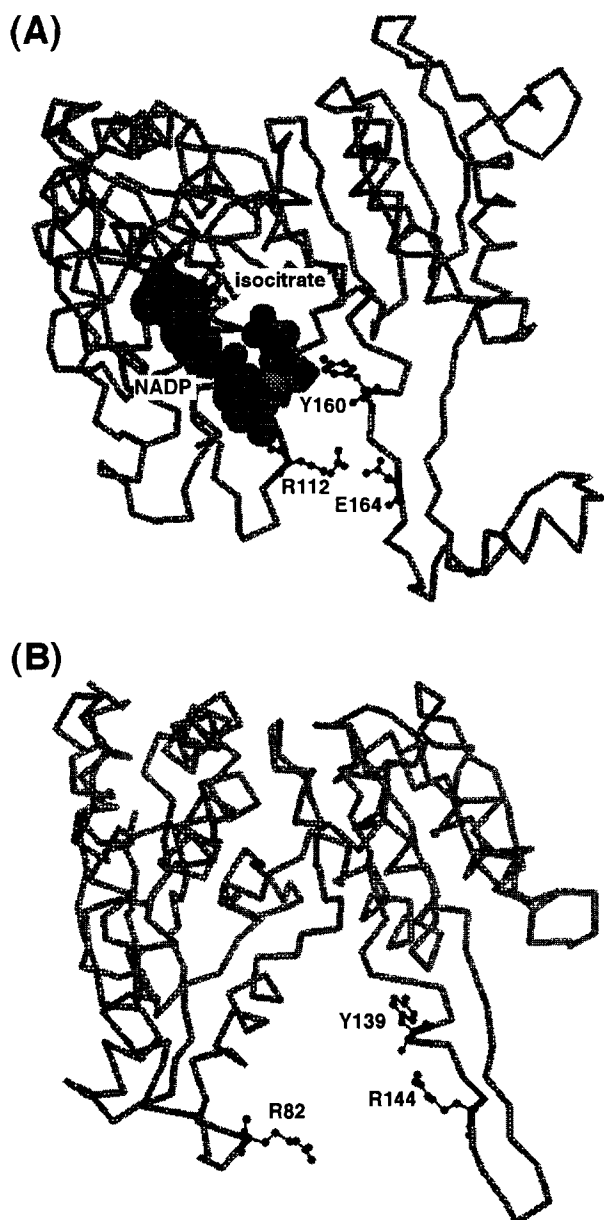


Fig. 1. Comparison of the Ca backbone structures of *E. coli* ICDH (A [17]) and *T. thermophilus* IPMDH (B [1]). The substrate, isocitrate, and the coenzyme, NADP, bound to ICDH are indicated for convenience in Fig. 1A.

3. Results and discussion

3.1. Thermostability of the wild-type and mutant enzymes

The far-UV CD spectrum of the mutant, Glu¹⁴⁷Gln, at

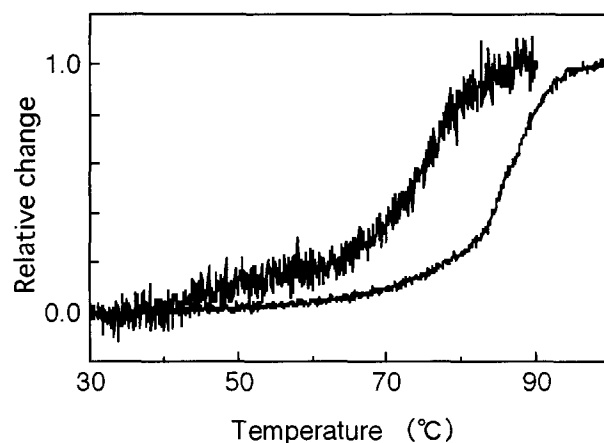


Fig. 2. Comparison of the thermal denaturation curves of the wild-type and the mutant ICDHs monitored by CD at 222 nm. Thin and thick lines represent the normalized denaturation curve of the wild-type enzyme and that of the mutant enzyme, respectively.

25°C was identical with that of the wild-type, suggesting no significant change in their secondary structures (data not shown). However, the CD melting curves suggested that the thermostability of the mutant enzyme was significantly decreased as compared with that of the wild-type ICDH (Fig. 2). Half denaturation temperatures of the wild-type and the mutant enzymes measured by CD at 222 nm (Fig. 2) were equal to those calculated by remaining activity after heat treatment (Fig. 3): 85°C for the wild-type and 73°C for the Glu¹⁴⁷Gln mutant. These results suggest that the interdomain interaction between Lys⁹⁶ and Glu¹⁴⁷ is involved in the protein stability.

3.2. Catalytic properties of the wild-type and mutant enzymes

The kinetic parameters at 60°C of the wild-type and the mutant enzymes are shown in Table 2. The k_{cat} value of the mutant enzyme was about 1/10 of that of the wild-type, while the K_m values for substrate and coenzyme did not change largely. Arrhenius plots of the wild-type and the mutant enzymes (Fig. 4) revealed discontinuities in slope at 30°C. From the plots, activation energies of the wild-type enzyme were calculated to be 13.9 kcal/mol and 20.8 kcal/mol, above and below 30°C, respectively. Those of the mutant enzyme both increased slightly, 15.7 kcal/mol and 33.7 kcal/mol, respectively.

3.3. Role of the interaction between Lys⁹⁶ and Glu¹⁴⁷

The present results indicate that the interaction between Lys⁹⁶ and Glu¹⁴⁷ concerning the interdomain interaction of thermophile ICDH plays important roles in both the catalytic function and the protein stability. The marked destabilization

Table 1
Multiple sequence alignment of ICDHs

| Source | Sequence | Ref. | |
|------------------------|---------------------------|---------------------------------------|------|
| | 96 | 147 | |
| <i>T. thermophilus</i> | TPVGYGE K SANVTLRK | RENVED L YAGIE H MQTPSV | [3] |
| <i>S. cerevisiae</i> | TPIGKG R SRLNLTLRK | RENTEGE Y SGIE H IVCPGV | [14] |
| <i>E. coli</i> | TPVGGG I RSLNVALRQ | RENSEDI Y AGIE W KAD-SA | [15] |
| <i>B. subtilis</i> | TPVGGG I RSLNVALRQ | RENTEDI Y AGIE Y AKG-SE | [16] |

Residue numbers are based on the thermophile ICDH sequences. Ser⁹⁷ and Asn⁹⁹ are involved in substrate recognition [7,8], and Tyr¹⁴³ is expected to have catalytic function [9,13].

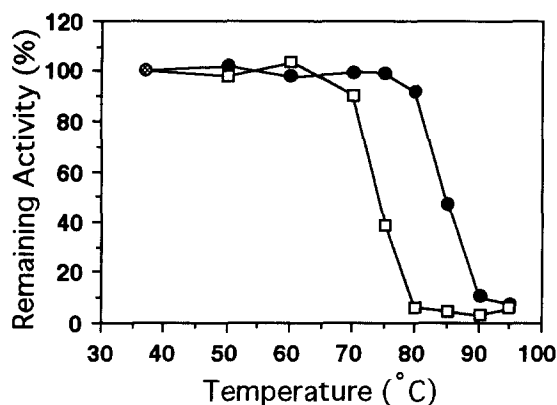


Fig. 3. Remaining activity of the wild-type (closed circles) and the mutant (open squares) enzymes after heat treatment. Each enzyme (0.2 mg/ml in 20 mM potassium phosphate buffer, pH 7.8) was incubated for 10 min at each temperature, and then the remaining activity was determined. The value at 37°C was taken as 100% for each enzyme.

of the mutant enzyme may be caused by breaking this inter-domain interaction which is attributable to a closed conformation of ICDH. Further studies are under way to elucidate the mechanism of the stability of thermophile ICDH.

Recently, the 3-D structure of the intermediate reaction complex of *E. coli* ICDH has revealed that Tyr¹⁶⁰ (corresponding to Tyr¹⁴³ of thermophile ICDH; Table 1) is involved in the catalytic function of the dehydrogenation step of the enzyme reaction [13]. Because inspection of this structure suggests that the salt bridge between Arg¹¹² and Glu¹⁶⁴ contributes the closed conformation of the ES complex of

Table 2
Kinetic parameters of the wild-type and mutant ICDHs at 60°C

| Enzyme | K_m (μ M) | | k_{cat} (s^{-1}) | k_{cat}/K_m ($s^{-1} \mu M^{-1}$) | |
|------------------------|------------------|------------|------------------------|---------------------------------------|------------|
| | NADP | isocitrate | | NADP | isocitrate |
| Wild-type | 7.2 | 8.9 | 71 | 9.9 | 8.0 |
| Glu ¹⁴⁷ Gln | 8.6 | 9.1 | 5.2 | 0.6 | 0.6 |

E. coli ICDH, the corresponding electrostatic interaction between Lys⁹⁶ and Glu¹⁴⁷ of thermophile ICDH (see Table 1) is probably involved in keeping the active site in closed conformation. Thus, the decrease in the k_{cat} and the loss of thermostability of the mutant enzyme (Table 2 and Figs. 2 and 3) would be caused by breaking this electrostatic interaction, which eventually leads to the destabilization of the closed conformation of the active site cleft and the breakage of the interaction between the catalytic residue, Tyr¹⁴³, and the substrate in thermophile ICDH. On the other hand, in the case of *T. thermophilus* IPMDH, the corresponding electrostatic interaction between the two domains does not exist, in spite of the involvement of Tyr¹³⁹, corresponding to Tyr¹⁴³ of thermophile ICDH, in the catalytic function [9]. Thus, although ICDH and IPMDH have essentially the same catalytic mechanism, the strategy of stabilization of the Michaelis complex of IPMDH may be slightly different from that of ICDH.

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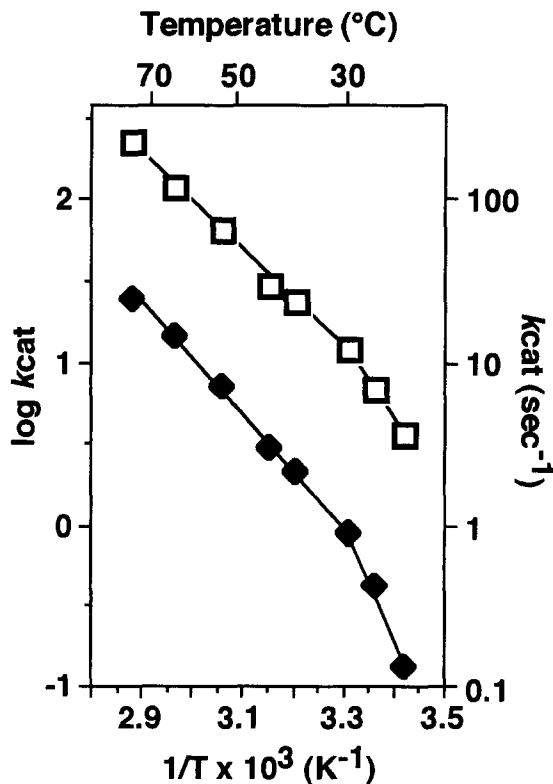


Fig. 4. Temperature dependence of the reaction. The activities of the wild-type (open squares) and the mutant enzymes (closed squares) were measured at the various temperatures indicated.