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**Effects of the combined substitutions of amino acid residues on thermal properties of cold-adapted monomeric isocitrate dehydrogenases from psychrophilic bacteria**

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**Abstract** In the two cold-adapted monomeric isocitrate dehydrogenases from psychrophilic bacteria, *Colwellia maris* and *Colwellia psychrerythraea* (*CmIDH* and *CpIDH*, respectively), the combined substitutions of amino acid residues between the Leu693, Leu724 and Phe735 residues of *CmIDH* and the corresponding Phe693, Gln724 and Leu735 residues of *CpIDH* were introduced by site-directed mutagenesis. A double mutant of *CmIDH* substituted its Leu724 and Phe735 residues by the corresponding ones of *CpIDH*, *CmL724Q/F735L*, and the triple mutant of *CpIDH*, *CpF693L/Q724L/L735F*, showed the most decrease and increase of activity, respectively, of each wild-type and its all mutated enzymes. In the case of *CmIDH*, the substitutions of these three amino acid residues resulted in the decrease of catalytic activity and thermostability for activity, but the combined substitutions of amino acid residues did not necessarily exert additive effects on these properties. On the other hand, similar substitutions in *CpIDH* had quite opposite effects to *CmIDH*, and the effects of the combined substitutions were additive. All multiple mutants of *CmIDH* and *CpIDH* showed lower and higher catalytic efficiency ( $k_{\text{cat}}/K_{\text{m}}$ ) values than the respective wild-type enzymes. Single and multiple mutations of the substituted amino acid residues in the *CmIDH* and *CpIDH* led to the increase and decrease of sensitivity to tryptic digestion, indicating that the stability of protein structure was decreased and increased by the mutations, respectively.

**Key words** Isocitrate dehydrogenase · Cold-adapted enzyme · Site-directed mutagenesis · *Colwellia maris* · *Colwellia psychrerythraea*

## Introduction

Isocitrate dehydrogenase (IDH; EC 1.1.1.41(42)) catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate and CO<sub>2</sub> coupled with the reduction of NAD(P)<sup>+</sup> in the central metabolic pathway, TCA cycle, and is ubiquitously distributed in a wide variety of organisms. In addition, this enzyme plays an important role in controlling metabolic flux between the TCA cycle and the glyoxylate shunt. Most bacteria possess NADP<sup>+</sup>-specific IDHs. On the basis of subunit structure, bacterial NADP<sup>+</sup>-specific IDHs can be classified into two types, homodimer consisting of about 40-45 kDa subunits and monomer with a molecular mass of 80-100 kDa. Many bacteria possess only one of either type IDH, but both of the two type IDHs are known to be present in several bacteria such as *Colwellia maris* (Ochiai et al. 1979; Ishii et al. 1987), *Colwellia psychrerythraea* NRC1004 (Maki et al. 2006), *Ralstonia eutropha* (Wang et al. 2003) and a psychrotrophic bacterium, *Pseudomonas psychrophila* (Matsuo et al. 2010).

Monomeric IDHs of psychrophilic bacteria, *C. maris* (Yumoto et al. 1998; Takada et al. 1979) and *C. psychrerythraea* NRC1004 (D'Aoust and Kushner 1972) (*CmIDH* and *CpIDH*, respectively), show the maximum activity at 20°C and 25°C, respectively, and marked thermolability, indicating that the two IDHs are typical cold-adapted enzymes (Ochiai et al. 1979 and 1984; Maki et al. 2006). *CpIDH* showed high degrees of homology in amino acid sequence to *CmIDH* (77% of identity), but specific activity of the former IDH was much lower than that of the latter IDH (about 15% by the comparison of the maximum activities at respective optimum temperatures).

Mesophilic monomer-type IDH from a nitrogen-fixing bacterium, *Azotobacter vinelandii*, (*AvIDH*) shows a high degree of sequential homology in

amino acid to the *CmIDH* (69.5% identity) (Sahara et al. 2002). From the crystal structure of *AvIDH* resolved by Yasutake et al. (2002 and 2003), this enzyme was found to contain Domain I, consisting of N- and C- terminal segments (Region 1 and 3, respectively), and Domain II, corresponding to the intermediate segment (Region 2). Studies on chimeric enzymes exchanged each region between *CmIDH* and *AvIDH* revealed that the respective C-terminal Region 3 of the two enzymes is involved in their thermal properties such as optimum temperature for activity and thermostability (Watanabe et al. 2005).

On the other hand, a preliminary study on chimeric enzymes exchanged each region between *CpIDH* and *CmIDH* suggested that the respective C-terminal Region 3 of these enzymes was responsible for their distinct catalytic abilities (Maki et al. 2006). Several differences of amino acid sequence were found in Region 3 of *CpIDH* and *CmIDH*. To identify the amino acid residues of this Region involved in catalytic activities at low temperature and thermostability for activities of *CpIDH* and *CmIDH*, Yasuda et al. (2013) introduced the substitutional mutations of ten amino acid residues between the two IDHs by site-directed mutagenesis and examined several properties of the mutated enzymes. Among them, the exchange between the three amino acid residues, Leu693, Leu724 or Phe735, in *CmIDH* and the corresponding Phe693, Gln724 or Leu735 residues in *CpIDH* resulted in significant changes in the thermal properties of the two enzymes. The mutated *CmIDHs*, in which the respective three amino acid residues were substituted by the corresponding ones of *CpIDH*, showed lower specific activity and thermostability for activity than the wild-type *CmIDH*. In contrast, the corresponding substitutions of *CpIDH* by the amino acid residues of *CmIDH* had quite opposite effects on the thermal properties of *CpIDH*.

In this study, to examine effects of the combined substitutions of these three

amino acid residues on catalytic activities at low temperature and thermostability for activities of *CmIDH* and *CpIDH*, variously combined substitutions of the three amino acid residues were introduced to the two IDHs by site-directed mutagenesis, and several properties of the wild-type IDHs and their single mutants were compared with those of the double and triple mutants.

## **Materials and methods**

### Bacteria, plasmids and growth media

A mutant of *Escherichia coli* defective in IDH, DEK2004 (Thorsness et al. 1987), was used as a host for the expression of wild-type and mutated genes for *CmIDH* and *CpIDH*. Plasmid pTrcHisB (Invitrogen) was used as a vector for conferring N-terminal (His)<sub>6</sub>-tag on the expressed IDH proteins. Plasmids pHis*CmIDH* (Watanabe et al. 2005; termed pHis*CmWT* in the paper) and pHis*CpIDH* (Maki et al. 2006; termed pHis*CpIDH-M* in the paper), harboring the *CmIDH* and *CpIDH* genes into the *Bam*HI-*Sac*I site of pTrcHisB, respectively, were used as templates of PCR for the site-directed mutagenesis. Luria-Bertani medium (Sambrook and Russell 2001) or Super broth medium (Watanabe et al. 2005) was used for growth of these *E. coli* transformants. When necessary, ampicillin was added to the culture media at a concentration of 0.1mg/ml.

### Construction of the mutated IDH genes by site-directed mutagenesis

Mutated IDH genes were constructed by three times PCRs as described

previously (Yasuda et al. 2013) except for the following modifications. A reaction mixture for PCR (50  $\mu$ l) contained 50 ng pHis*Cm*IDH or pHis*Cp*IDH as a template and 20 pmol forward and reverse primers, and PCRs were carried out in a DNA thermal cycler 2400 (Perkin-Elmer) or Veriti 96 well Thermal Cycler (Applied Biosystems).

### Overexpression and purification of His-tagged IDHs

According to the methods of Yasuda et al. (2013), The wild-type and mutated *Cm*IDH and *Cp*IDH overproduced in the *E. coli* DEK2004 cells were purified by Ni-NTA agarose column chromatography (Qiagen) except for the following modifications. The cell suspension added hen-egg lysozyme was gently shaken for 1 h at 4°C. After the shaking, phenylmethylsulfonylfluoride (PMSF) was added to the cell suspension at a final concentration of 1 mM and then the cells were disrupted by an ultrasonic oscillation. The same concentration of PMSF was added to each supernatant after the following two centrifugations. The respective elutants containing IDH activity were concentrated with polyethylene glycol #10,000 and dialyzed against buffer consisting of 20 mM sodium phosphate buffer (pH 8.0), 2 mM MgCl<sub>2</sub>, 0.5 M NaCl, 5 mM sodium citrate, 1 mM dithiothreitol (DTT) and 50% (v/v) glycerol. All His-tagged recombinant IDHs were stored at -30°C until use.

### Enzyme assay

Unless otherwise stated, the IDH activity was assayed at 20°C as described

previously (Yasuda et al. 2013). One unit of enzyme activity was defined as the amount of the enzyme catalyzing the reduction of 1  $\mu\text{mol}$  of  $\text{NADP}^+$  per min. To examine thermostability for IDH activity, all purified recombinant IDHs (1 mg/ml) were dialyzed overnight at  $4^\circ\text{C}$  against 20 mM sodium phosphate buffer (pH 8.0) containing 2 mM  $\text{MgCl}_2$ , 100 mM  $\text{NaCl}$ , 10 % (v/v) glycerol and 1 mM DTT. After incubation for 10 min at indicated temperatures, the enzyme was immediately cooled on ice for 10 min. Then, the residual activity was assayed at  $20^\circ\text{C}$ . Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. All data for activity and kinetic parameters are the mean values of at least two independent experiments.

#### Digestion of IDH proteins with trypsin

The purified recombinant IDHs were dialyzed overnight at  $4^\circ\text{C}$  against 0.1 M  $\text{NaHCO}_3$  (pH 8.1). The enzyme samples (1 mg/ml) were incubated for various times at  $25^\circ\text{C}$  with trypsin (final concentration of 100 ng/ml). Tryptic digestions of the enzyme samples were terminated by the addition of an equal volume of 2 $\times$ SDS-PAGE sample buffer (100 mM Tris-HCl (pH 6.8), 20% glycerol, 4% sodium dodecyl sulfate, and 0.2% bromphenolblue) and the subsequent boiling of the samples for 3 min. Then, each sample containing 18  $\mu\text{g}$  of protein was analyzed by SDS-PAGE (Laemmli, 1970) with 10% gel at 40 mV. After electrophoresis, the proteins on the gel were stained with Coomassie Brilliant Blue R250.



## Results

### Construction and purification of mutated IDHs

Double and triple substitutional mutations of the corresponding three amino acid residues in the Region 3 between *CmIDH* and *CpIDH* (Leu693, Leu724 or Phe735 of the former IDH and Phe693, Gln724 or Leu735 of the latter one) were introduced in the two IDH genes by site-directed mutagenesis as described in Materials and methods. The double and triple mutants of *CmIDH* were designated as *CmL693F/L724Q* (the two Leu693 and Leu724 residues of *CmIDH* were substituted by the corresponding Phe693 and Gln724 residues of *CpIDH*), *CmL693F/F735L*, *CmL724Q/F735L* and *CmL693F/L724Q/F735L*, respectively. On the other hand, *CpF693L/Q724L*, *CpF693L/L735F*, *CpQ724L/L735F* and *CpF693L/Q724L/L735F* were three double and a triple mutants of *CpIDH*.

pTrcHisB carrying these mutated genes, pHis*CmIDH* and pHis*CpIDH* were introduced into *E. coli* DEK2004. Then, these His-tagged wild-type and mutated IDH proteins were overexpressed in the *E. coli* DEK2004 cells and were purified as described above. From SDS-PAGE of the final elutant of Ni-NTA column chromatography, the purified enzyme samples were found to contain a major protein with a molecular mass of about 80 kDa (data not shown), corresponding to those of the *CmIDH* and *CpIDH* proteins (Ishii et al. 1987; Maki et al. 2006). In this study, a protease inhibitor, PMSF, was added at several times during the purification. Such an improvement of the purification procedure were appeared to result in the increased specific activities of IDHs, compared to those of IDHs previously purified in the absence of PMSF (Yasuda et al. 2013).

## Temperature dependence of wild-type and mutated IDH activities

To examine the influence of the multiple substitution of amino acid residues on the catalytic function of *Cm*IDH and *Cp*IDH, activities of wild-type and mutated IDHs were assayed at various temperatures (Figs. 1 and 2). It has been reported that the His-tagging to the N-terminals of *Cm*IDH and *Cp*IDH proteins has no significant effect on their thermal properties including optimum temperature and thermostability for activity (Yoneta et al. 2004; Maki et al. 2006). The His-tagged wild-type *Cm*IDH showed the maximum activity (80 unit/mg protein) at 25°C (Fig. 1). At almost all temperatures tested, specific activities of all multiple *Cm*IDH mutants were lower than those of wild-type enzyme and its all single mutants. At the optimum temperature of each enzyme, *Cm*L693F/L724Q, *Cm*L693F/F735L and the triple mutant showed only 15, 48 and 9% of wild-type enzyme activity, respectively. In particular, the largest loss of specific activity at optimum temperature (4% of the wild-type enzyme activity) was observed in *Cm*L724Q/F735L. Furthermore, except the triple mutant, all double mutants were appeared to lower the optimum temperatures for activities to about 15-20°C.

On the other hand, as reported previously (Maki et al. 2006), wild-type *Cp*IDH showed the maximum activity (2.9 unit/mg protein) at 25°C and its activity was much less than that of *Cm*IDH (at the respective optimum temperatures, only about 4% of *Cm*IDH activity) (Fig. 2). Except for *Cp*F693L/Q724L, optimum temperatures for activities of all multiple *Cp*IDH mutants were shifted up to about 30-35°C. In contrast to the case of *Cm*IDH, specific activities of all multiple *Cp*IDH mutants were higher at almost all

temperatures tested than those of wild-type enzyme and its all single mutants. In addition, specific activities of all double mutants at the respective optimum temperatures were about 10-16 folds higher than that of wild-type *CpIDH*. Specific activity of the triple mutant, *CpF693L/Q724L/L735F*, was 64 unit/mg protein at the optimum temperature for activity, 35°C, at which wild-type *CpIDH* loses completely the activity, and was higher than those of these double mutants. These results indicate that the combined substitutional mutations of these amino acid residues cause additive increment of the specific activities in the *CpIDH*.

#### Thermostability of wild-type and mutated IDH activities

After incubation for 10 min at the indicated temperatures, remaining activities of the wild-type and mutated *CmIDH* and *CpIDH* were assayed at 20°C (Figs. 3 and 4). All multiple *CmIDH* mutants lost larger activities after incubation at 25 and 30°C than wild-type enzyme, but the effects of the combined mutations on the thermostability of *CmIDH* activity were not necessarily additive (Fig. 3). In contrast, all double and triple *CpIDH* mutants showed higher thermostability for activity than wild-type *CpIDH* and its single mutants (Fig. 4). Even after incubation at 25°C, all double mutants maintained over 40% of their activities and the triple mutant kept the activity completely, while remaining activity of wild-type enzyme was only about 6%. Thus, the combined substitutional mutations of *CpIDH* were found to cause additive enhancement of thermostability for enzyme activity.

## Kinetic parameters of wild-type and mutated IDHs

Table 1 summarizes the values of  $K_m$  for isocitrate,  $k_{cat}$  and  $k_{cat}/K_m$  of wild-type and mutated IDHs at 20°C. In the case of *Cm*IDH, the values of catalytic efficiency,  $k_{cat}/K_m$ , of all multiple mutants were lower than that of wild-type enzyme, and the values of *Cm*L693F/L724Q, *Cm*L693F/F735L, *Cm*L724Q/F735L and *Cm*L693F/L724Q/F735L were decreased to 20%, 30%, 6% and 20% of that of wild-type enzyme, respectively. The decrease of  $k_{cat}/K_m$  values were confirmed to result from the decreased  $k_{cat}$  values of all multiple mutants, in addition to the increased  $K_m$  values of *Cm*L693F/F735L and *Cm*L724Q/F735L. On the other hand, in the case of *Cp*IDH, all multiple mutants showed higher  $k_{cat}/K_m$  values than wild-type enzyme, and these values of *Cp*F693L/Q724L, *Cp*F693L/L735F, *Cp*Q724L/L735F and *Cp*F693L/Q724L/L735F were about 6, 9, 6 and 7-folds of that of wild-type enzyme, respectively. In contrast to *Cm*IDH, these rises of  $k_{cat}/K_m$  values were found to be attributable to the increased  $k_{cat}$  values.

## Sensitivity of mutated IDHs to tryptic digestion

Previous study on chimeric enzymes between *Cm*IDH and *Av*IDH revealed that sensitivity of these enzyme proteins to tryptic digestion is significantly correlated to the thermostability of their protein structures (Watanabe *et al.*, 2005). Therefore, the sensitivity of wild-type and mutated *Cm*IDHs and *Cp*IDHs to tryptic digestion was examined to estimate their structural stability (Fig. 5). At 25°C, wild-type *Cm*IDH was little digested after incubation for 60 min with trypsin (about 90% of this enzyme protein was remained), while wild-type

*Cp*IDH was rapidly digested by incubation for 30 min (diminished to about 38%). All single and double mutants of *Cm*IDH were found to be more sensitive to tryptic digestion than wild-type enzyme when the digestion was carried out for more than 30 min. The triple mutant of *Cm*IDH was most sensitive to tryptic digestion of all wild-type and mutated *Cm*IDHs, and was rapidly digested even after 5 min (decreased to about 61%). In contrast to *Cm*IDHs, all single and multiple mutants of *Cp*IDH were more resistant to tryptic digestion than wild-type enzyme. These results indicate that single and multiple mutations of the substituted amino acid residues in *Cm*IDH and *Cp*IDH have quite opposite effect on their stability of protein structure, that is, the decrease and increase of stability, respectively. However, it was appeared that the combined mutations in *Cm*IDH and *Cp*IDH did not necessarily have additive influences on stability of protein structure. In addition, sensitivity of single or multiple mutants of *Cm*IDH and *Cp*IDH to tryptic digestion was not strictly correlated to thermostability for their activities (Figs. 3 and 4).

## **Discussion**

In this study, the combined substitutions of the corresponding three amino acid residues between *Cm*IDH and *Cp*IDH were introduced in the two enzymes and several thermal properties, including temperature dependence and thermostability for activity, of mutant enzymes were examined. The double mutant of *Cm*IDH, *Cm*L724Q/F735L, showed lowest specific activity of wild-type and all mutated *Cm*IDHs, and its optimum temperature for activity was 15°C while that of wild-type IDH was 25°C (Fig. 1). Moreover, the specific activities of another multiple mutants of *Cm*IDH, *Cm*L693F/L724Q and

*CmL693F/L724Q/F735L*, were much lower than wild-type *CmIDH* and its single mutants. On the other hand, specific activities of *CmL693F/F735L* at low temperatures below 20°C were higher than those of the single mutant, *CmF735L*. In addition, after incubation at 25°C and 30°C, all double and triple mutants of *CmIDH* maintained more activities than the single mutants (Fig. 3). These results indicated that the substitutions of these amino acid residues in *CmIDH* certainly led to the decrease of the catalytic activity and thermostability for activity but the effects of the combined substitutions are not necessarily additive. On the other hand, the specific activities of all double mutants of *CpIDH* at the respective optimum temperatures were higher than those of wild-type *CpIDH* and its single mutants (Fig. 2). Furthermore, at 35°C, the triple mutant, *CpF693L/Q724L/L735F*, showed highest specific activity of wild-type and all mutated *CpIDH*. In addition, thermostability of the *CpIDH* activity was improved by the substitutions of three amino acid residues, and the enzyme activity became more stable with the increase of number of the introduced mutation (Fig. 4). Therefore, in contrast to *CmIDH*, the effects of the mutations in *CpIDH* were additive. Furthermore, the multiple mutants of *CpIDH* enhanced the activity, but wild-type *CmIDH* was still better than the mutants in specific activities. These results imply that other amino acid residue(s) except for 693rd, 724th and 735th may be involved in different effects of the three substituted amino acid residues between *CmIDH* and *CpIDH* and their catalytic activities, and further study is required to confirm this possibility. The molecular models of the wild-type *CmIDH* and *CpIDH* and their mutants, which were built with a homology modeling program, SWISSPDB VIEWER, were compared each other (Fig. 6). However, no clear structural difference could be found out among them.

The  $k_{cat}/K_m$  values of all multiple mutants of *CmIDH* and *CpIDH* were lower and higher than those of their wild-type enzymes, respectively, and such changes

were found to result from the  $k_{\text{cat}}$  values altered by mutations (Table 1). Although structural bases of the altered  $k_{\text{cat}}$  values by the mutations could not be elucidated from their molecular models (Fig. 6), an increment of the  $k_{\text{cat}}$  values is known to be a strategy to improve the catalytic efficiency of cold-adapted enzymes (Zecchinon et al. 2001). However, the triple mutants of *CmIDH* and *CpIDH* showed  $k_{\text{cat}}/K_m$  values similar to the respective double mutants. These results suggest that the introduction of multiple mutations does not necessarily exert additive effects on kinetic parameters.

In the experiment of tryptic digestion to estimate the structural stability of wild-type and mutated IDH proteins, the triple *CmIDH* mutant was most sensitive to tryptic digestion of all wild-type and mutated *CmIDHs*, but showed thermostability for activity similar to wild-type enzyme (Figs. 3 and 5). In addition, the triple *CpIDH* mutant showed highest thermostability for activity of all wild-type and mutated *CpIDHs* but was sensitive to tryptic digestion rather than several other mutants (Figs. 4 and 5). These results suggest that sensitivity of wild-type and mutated *CmIDH* and *CpIDH* to tryptic digestion did not completely correspond to thermostability for their activities. The two double mutants, *CmL693F/L724Q* and *CpF693L/Q724L*, showed quite opposite sensitivity to tryptic digestion, that is, after incubation for 30 min with trypsin, *CmL693F/L724Q* and *CpF693L/Q724L* were much more sensitive and resistant than the respective wild-type enzymes, respectively. However, their single mutants, both *CmL724Q* and *CpQ724L*, showed high resistance to tryptic digestion. These results imply that interaction(s) between the 724th amino acid residue and other ones except for 693rd and 735th residues might contribute to structural stability of the *CmIDH* and *CpIDH* proteins.

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## Legend to figures

**Fig. 1** Effect of temperature on activities of wild-type and mutated *Cm*IDHs. Wild-type *Cm*IDH (\*), *Cm*L693F (■), *Cm*L724Q (▲), *Cm*F735L (◆), *Cm*L693F/L724Q (□), *Cm*L693F/F735L (△), *Cm*L724Q/F735L (◇) and *Cm*L693F/L724Q/F735L (○).

**Fig. 2** Effect of temperature on activities of wild-type and mutated *Cp*IDHs. Wild-type *Cp*IDH (\*), *Cp*F693L (■), *Cp*Q724L (▲), *Cp*L735F (◆), *Cp*F693L/Q724L (□), *Cp*F693L/L735F (△), *Cp*Q724L/L735F (◇) and *Cp*F693L/Q724L/L735F (○).

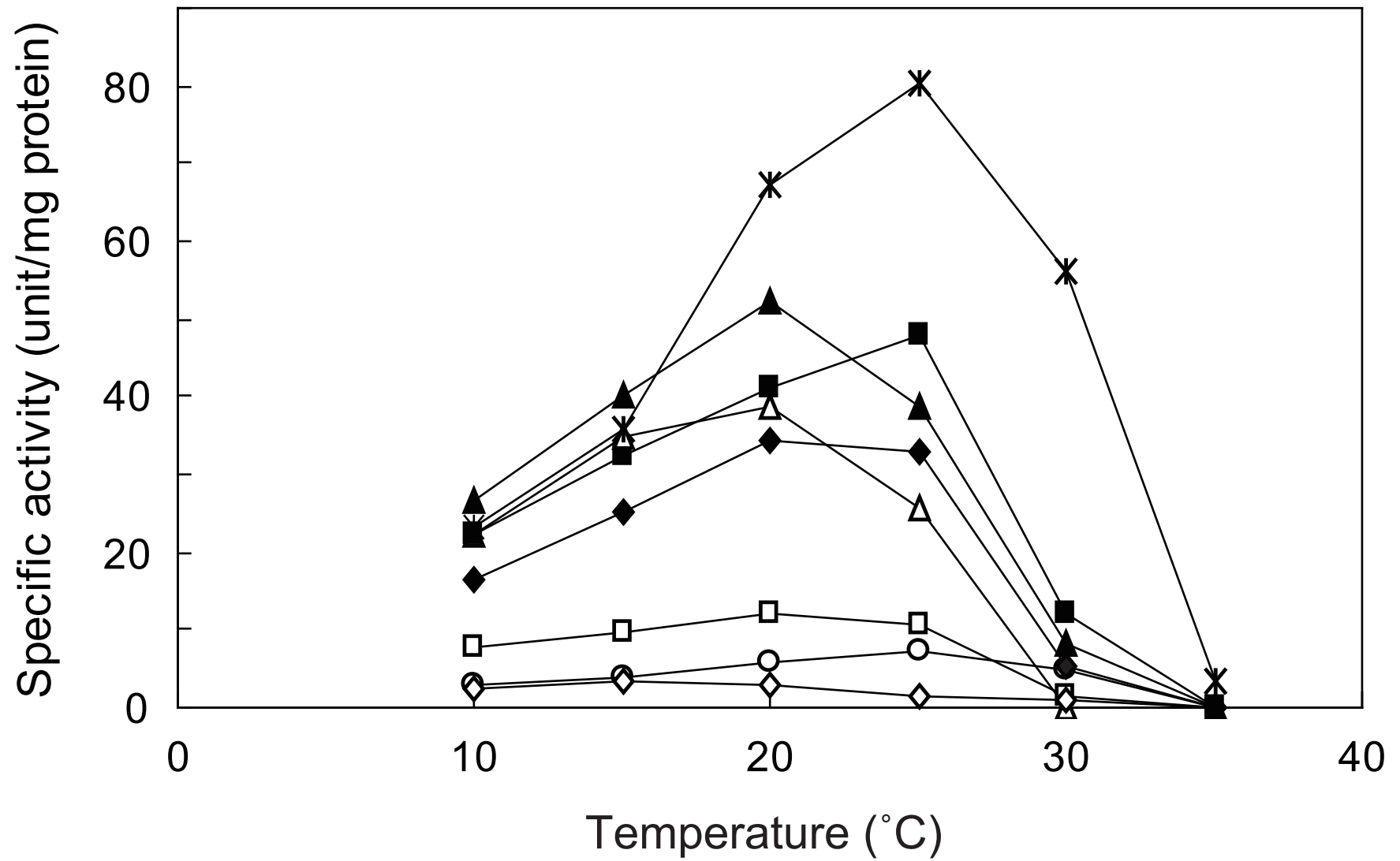
**Fig. 3** Thermostability of wild-type and mutated *Cm*IDH activities. Residual activity after incubation for 10 min at the indicated temperatures is represented as a percentage of that without the incubation. Symbols used in this figure are the same as Fig. 1.

**Fig. 4** Thermostability of wild-type and mutated *Cp*IDH activities. Residual activity after incubation for 10 min at the indicated temperatures is represented as a percentage of that without the incubation. Symbols used in this figure are the same as Fig. 2.

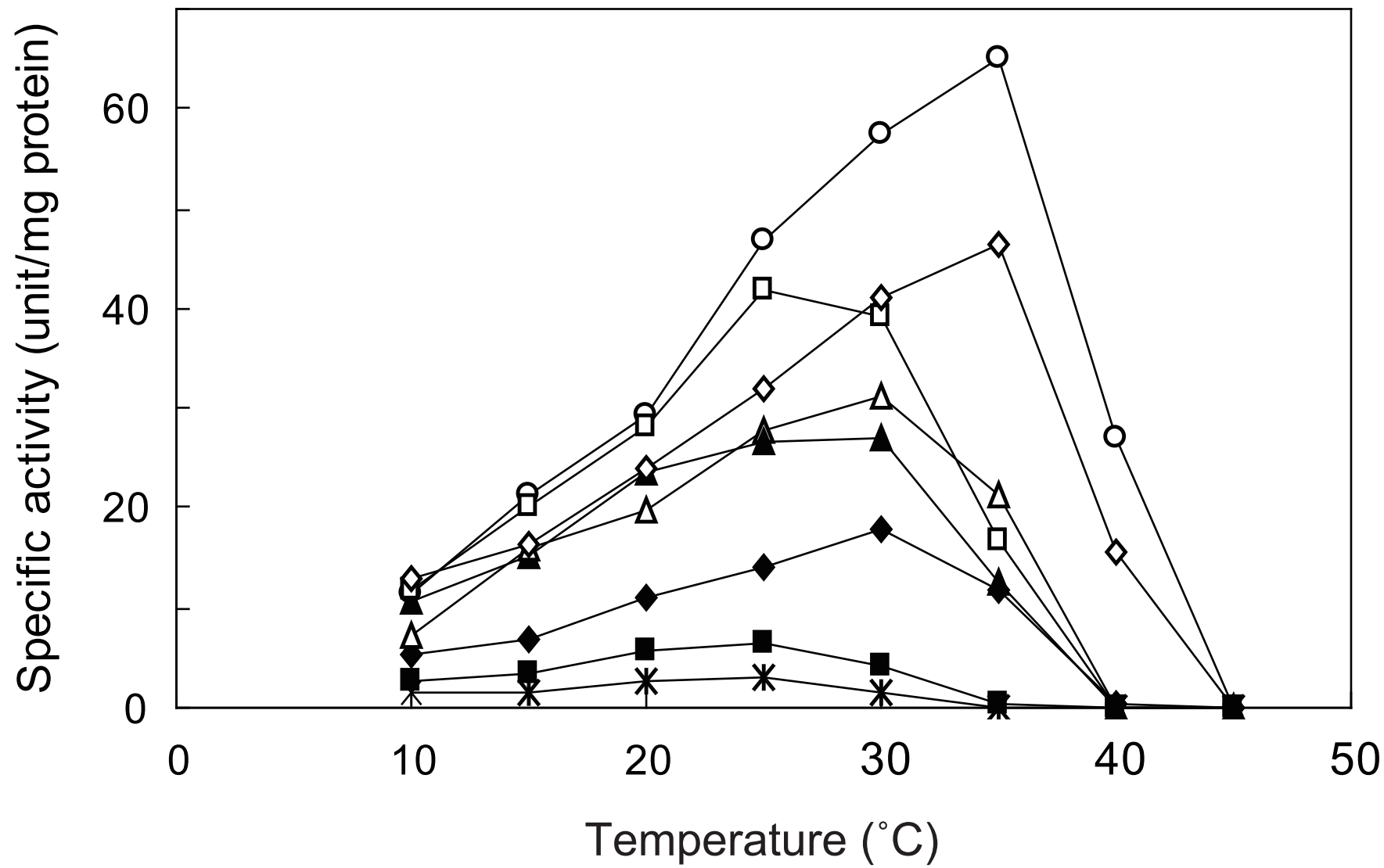
**Fig. 5** Digestion of wild-type and mutated IDHs with trypsin. Each enzyme was incubated for various times at 25°C with trypsin. After digestion with trypsin, enzyme samples were analyzed by SDS-PAGE.

**Fig. 6** Molecular models of *Cm*IDH and *Cp*IDH. The models were built using

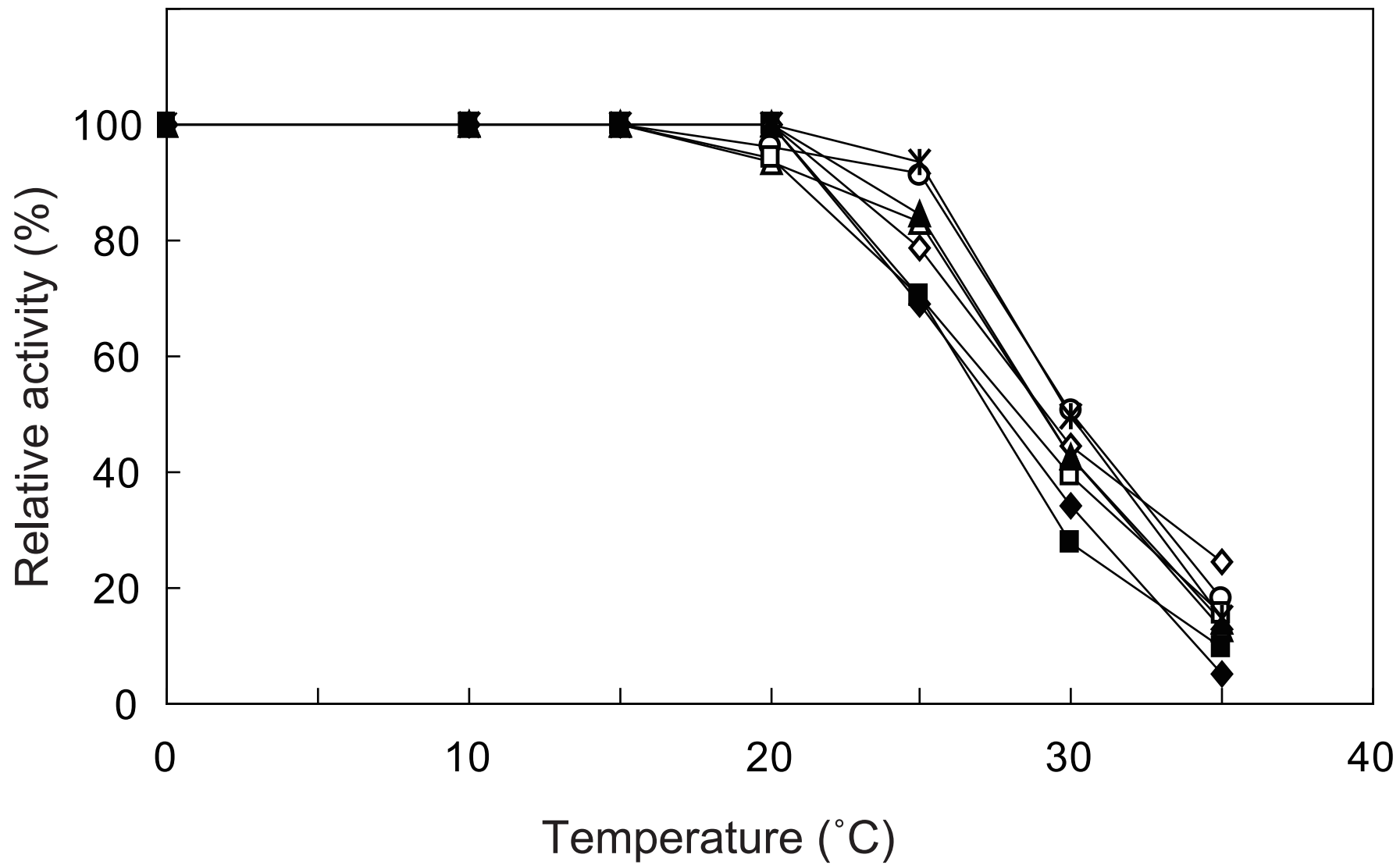
*Av*IDH (PDB No. 1ITW) as a homology model with a program SWISSPDB VIEWER (<http://spdbv.vital-it.ch>). (A) Front view and (B) left view of *Cm*IDH. (C) Left view of *Cp*IDH. The amino acid residues involved in the binding of isocitrate,  $Mn^{2+}$  and  $NADP^+$  are indicated by green. The amino acid residues substituted in this study are indicated by orange and red in *Cm*IDH (A and B) and *Cp*IDH (C), respectively. The structure of *Cm*IDH (D) and *Cp*IDH (E) near the amino acid residues substituted in this study. The side chains of amino acid residues were indicated. Oxygen, hydrogen and sulfur in the side chains are indicated by red, blue and yellow, respectively. Hydrogen bonds are shown by green dashes.



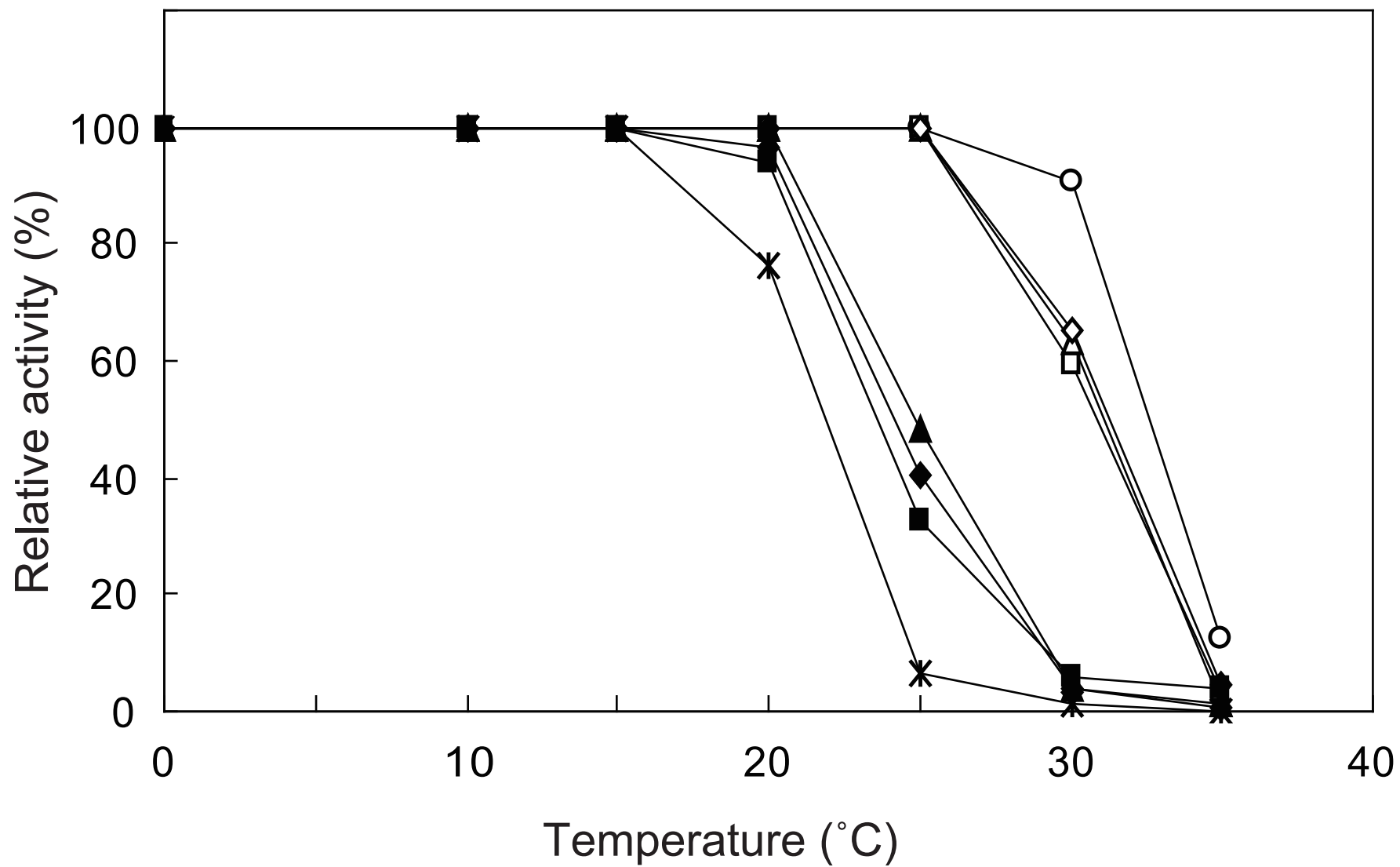
**Fig. 1**



**Fig. 2**

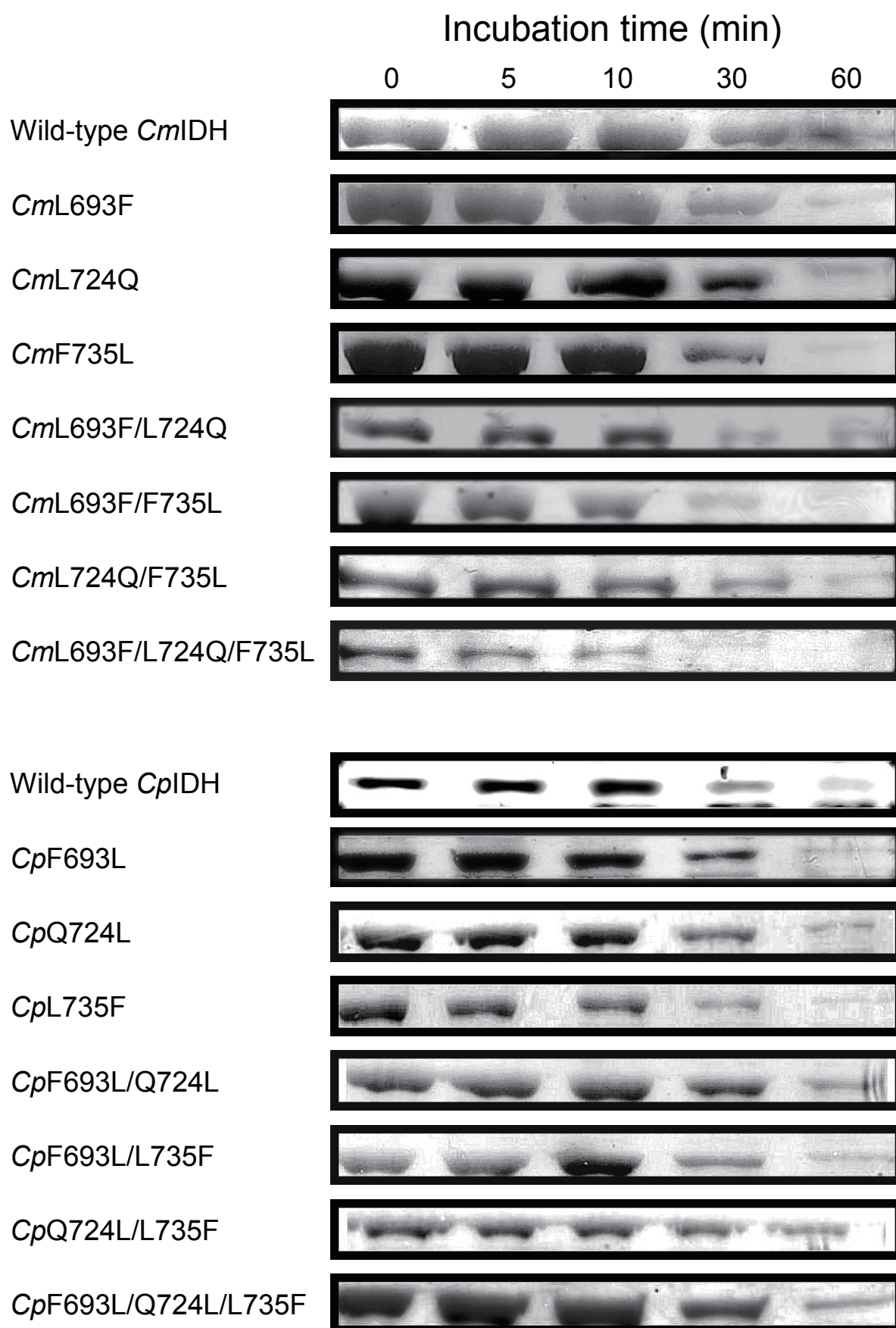


**Fig. 3**

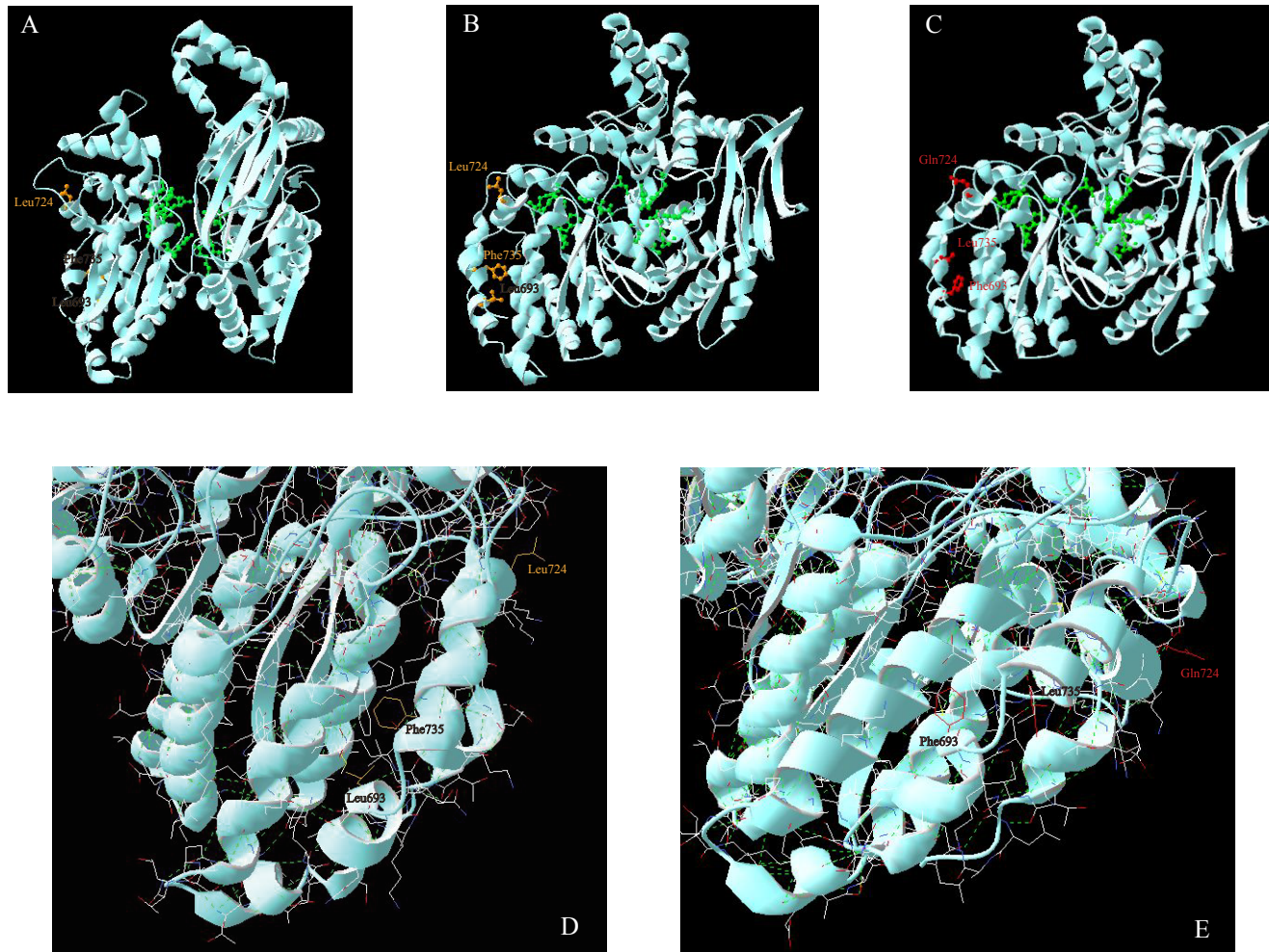


**Fig. 4**





**Fig. 5**



**Fig. 6**

**Table 1** Kinetic parameters at 20°C

		$K_m$ ( $\mu\text{M}$ )*	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{s}^{-1}\text{M}^{-1}$ )*
	Wild-type	41.1	92.0	$22.4 \times 10^5$
	<i>Cm</i> L693F/L724Q	35.6	16.0	$4.5 \times 10^5$
<i>Cm</i> IDH	<i>Cm</i> L693F/F735L	87.3	58.7	$6.7 \times 10^5$
	<i>Cm</i> L724Q/F735L	95.2	12.8	$1.3 \times 10^5$
	<i>Cm</i> L693F/L724Q/F735L	29.3	13.1	$4.5 \times 10^5$
	Wild-type	15.4	4.2	$2.7 \times 10^5$
	<i>Cp</i> F693L/Q724L	26.6	50.0	$16.9 \times 10^5$
<i>Cp</i> IDH	<i>Cp</i> F693L/L735F	20.8	51.1	$24.7 \times 10^5$
	<i>Cp</i> Q724L/L735F	22.1	37.2	$17.1 \times 10^5$
	<i>Cp</i> F693L/Q724L/L735F	26.6	51.4	$19.3 \times 10^5$

\*  $K_m$  values for isocitrate are indicated.