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* (*Cm*IDH and *Cp*IDH, respectively), the combined substitutions of amino acid residues between the Leu693, Leu724 and Phe735 residues of *Cm*IDH and the corresponding Phe693, Gln724 and Leu735 residues of *Cp*IDH were introduced by site-directed mutagenesis. A double mutant of *Cm*IDH substituted its Leu724 and Phe735 residues by the corresponding ones of *Cp*IDH, *Cm*L724Q/F735L, and the triple mutant of *Cp*IDH, *Cp*F693L/Q724L/L735F, showed the most decrease and increase of activity, respectively, of each wild-type and its all mutated enzymes. In the case of *Cm*IDH, 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 *Cp*IDH had quite opposite effects to *Cm*IDH, and the effects of the combined substitutions were additive. All multiple mutants of *Cm*IDH and *Cp*IDH showed lower and higher catalytic efficiency (k_{cat}/K_m) values than the respective wild-type enzymes. Single and multiple mutations of the substituted amino acid residues in the *Cm*IDH and *Cp*IDH leaded 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_2 coupled with the reduction of $NAD(P)^+$ 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 $NADP^+$-specific IDHs. On the basis of subunit structure, bacterial $NADP^+$-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) (*Cm*IDH and *Cp*IDH, respectively), show the maximum activity at 20^{o}C and 25^{o}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). *Cp*IDH showed high degrees of homology in amino acid sequence to *Cm*IDH (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*, (*Av*IDH) shows a high degree of sequential homology in

amino acid to the *Cm*IDH (69.5% identity) (Sahara et al. 2002). From the crystal structure of *Av*IDH 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 *Cm*IDH and *Av*IDH 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 *Cp*IDH and *Cm*IDH 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 *Cp*IDH and *Cm*IDH. To identify the amino acid residues of this Region involved in catalytic activities at low temperature and thermostability for activities of *Cp*IDH and *Cm*IDH, 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 *Cm*IDH and the corresponding Phe693, Gln724 or Leu735 residues in *Cp*IDH resulted in significant changes in the thermal properties of the two enzymes. The mutated *Cm*IDHs, in which the respective three amino acid residues were substituted by the corresponding ones of *Cp*IDH, showed lower specific activity and thermostability for activity than the wild-type *Cm*IDH. In contrast, the corresponding substitutions of *Cp*IDH by the amino acid residues of *Cm*IDH had quite opposite effects on the thermal properties of *Cp*IDH.

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 *Cm*IDH and *Cp*IDH, 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 *Cm*IDH and *Cp*IDH. Plasmid pTrcHisB (Invitrogen) was used as a vector for conferring N-terminal $(His)_6$-tag on the expressed IDH proteins. Plasmids pHis*Cm*IDH (Watanabe et al. 2005; termed pHis*CmWT* in the paper) and pHis*Cp*IDH (Maki et al. 2006; termed pHis*Cp*IDH-M in the paper), harboring the *Cm*IDH and *Cp*IDH 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 µl) 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^{o}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 $MgCl_2$, 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^{o}C until use.

Enzyme assay

Unless otherwise stated, the IDH activity was assayed at 20^{o}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 μmol of $NADP^+$ per min. To examine thermostability for IDH activity, all purified recombinant IDHs (1 mg/ml) were dialyzed overnight at 4^{o}C against 20 mM sodium phosphate buffer (pH 8.0) containing 2 mM $MgCl_2$, 100 mM 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^{o}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^{o}C against 0.1 M $NaHCO_3$ (pH 8.1). The enzyme samples (1 mg/ml) were incubated for various times at 25^{o}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×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 μg 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 *Cm*IDH and *Cp*IDH (Leu693, Leu724 or Phe 735 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 *Cm*IDH were designated as *Cm*L693F/L724Q (the two Leu693 and Leu724 residues of *Cm*IDH were substituted by the corresponding Phe693 and Gln724 residues of *Cp*IDH), *Cm*L693F/F735L, *Cm*L724Q/F735L and *Cm*L693F/L724Q/F735L, respectively. On the other hand, *Cp*F693L/Q724L, *Cp*F693L/L735F, *Cp*Q724L/L735F and *Cp*F693L/Q724L/L735F were three double and a triple mutants of *Cp*IDH.

pTrcHisB carrying these mutated genes, pHis*Cm*IDH and pHis*Cp*IDH 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 *Cm*IDH and *Cp*IDH 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^{o}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^{o}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^{o}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^{o}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 *Cp*IDH. Specific activity of the triple mutant, *Cp*F693L/Q724L/L735F, was 64 unit/mg protein at the optimum temperature for activity, 35^{o}C, at which wild-type *Cp*IDH 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 *Cp*IDH.

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 *Cm*IDH and *Cp*IDH were assayed at 20^{o}C (Figs. 3 and 4). All multiple *Cm*IDH mutants lost larger activities after incubation at 25 and 30^{o}C than wild-type enzyme, but the effects of the combinated mutations on the thermostability of *Cm*IDH activity were not necessarily additive (Fig. 3). In contrast, all double and triple *Cp*IDH mutants showed higher thermostability for activity than wild-type *Cp*IDH and its single mutants (Fig. 4). Even after incubation at 25^{o}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 *Cp*IDH 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 theramostability 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^{o}C while that of wild-type IDH was 25^{o}C (Fig. 1). Moreover, the specific activities of another multiple mutants of *Cm*IDH, *Cm*L693F/L724Q and

*Cm*L693F/L724Q/F735L, were much lower than wild-type *Cm*IDH and its single mutants. On the other hand, specific activities of *Cm*L693F/F735L at low temperatures below 20°C were higher than those of the single mutant, *Cm*F735L. In addition, after incubation at 25°C and 30°C, all double and triple mutants of *Cm*IDH maintained more activities than the single mutants (Fig. 3). These results indicated that the substitutions of these amino acid residues in *Cm*IDH 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 *Cp*IDH at the respective optimum temperatures were higher than those of wild-type *Cp*IDH and its single mutants (Fig. 2). Furthermore, at 35°C, the triple mutant, *Cp*F693L/Q724L/L735F, showed highest specific activity of wild-type and all mutated *Cp*IDH. In addition, thermostability of the *Cp*IDH 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 *Cm*IDH, the effects of the mutations in *Cp*IDH were additive. Furthermore, the multiple mutants of *Cp*IDH enhanced the activity, but wild-type *Cm*IDH 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 *Cm*IDH and *Cp*IDH and their catalytic activities, and further study is required to confirm this possibility. The molecular models of the wild-type *Cm*IDH and *Cp*IDH 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 *Cm*IDH and *Cp*IDH were lower and higher than those of their wild-type enzymes, respectively, and such changes

were found to result from the k_{cat} values altered by mutations (Table 1). Although structural bases of the altered k_{cat} values by the mutations could not be elucidated from their molecular models (Fig. 6), an increment of the k_{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 *Cm*IDH and *Cp*IDH showed k_{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 *Cm*IDH mutant was most sensitive to tryptic digestion of all wild-type and mutated *Cm*IDHs, but showed thermostability for activity similar to wild-type enzyme (Figs. 3 and 5). In addition, the triple *Cp*IDH mutant showed highest thermostability for activity of all wild-type and mutated *Cp*IDHs 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 *Cm*IDH and *Cp*IDH to tryptic digestion did not completely correspond to theramostability for their activities. The two double mutants, *Cm*L693F/L724Q and *Cp*F693L/Q724L, showed quite opposite sensitivity to tryptic digestion, that is, after incubation for 30 min with trypsin, *Cm*L693F/L724Q and *Cp*F693L/Q724L were much more sensitive and resistant than the respective wild-type enzymes, respectively. However, their single mutants, both *Cm*L724Q and *Cp*Q724L, 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 *Cm*IDH and *Cp*IDH proteins.

References

D'Aoust JY, Kushner DJ (1972) *Vibrio psychroerythrus* sp. N: Classification of the psychrophilic marine bacterium, NRC 1004. J Bacteriol 111:340-342

Ishii A, Imagawa S, Fukunaga N, Sasaki A, Minowa O, Mizuno Y, Shiokawa H (1987) Isozymes of isocitrate dehydrogenase from an obligately psychrophilic bacterium, *Vibrio sp*. strain ABE-1: purification and modulation of activities by growth conditions. J Biochem 102:1489-1498

Kurihara T, Takada Y (2012) Analysis of amino acid residues involved in thermal properties of monomeric isocitrate dehydrogenases of psychrophilic bacterium, *Colwellia maris*, and mesophilic acterium, *Azotobacter vinelandii*. Biosci Biotechol Biochem 76:2242-2248

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275

Maki S, Yoneta M, Takada Y (2006) Two isocitrate dehydrogenases from a psychrophilic bacterium, *Colwellia psychrerythraea*. Extremophiles 10:237-249

Matsuo S, Shirai H, Takada Y (2010) Isocitratet dehydrogenase isozymes from a psychrotrophic bacterium, *Pseudomonas psychrophila*. Arch Microbiol 192:639-650

Ochiai T, Fukunaga N, Sasaki S (1979) Purification and some properties of two $NADP^+$-specific isocitrate dehydrogenases from an obligately psychrophilic marine bacterium, *Vibrio* sp., strain ABE-1. J Biochem 86:377-384

Ochiai T, Fukunaga N, Sasaki S (1984) Two structurally different $NADP^+$-specific isocitrate dehydrogenases in an obligately psychrophilic

bacterium, *Vibrio* sp. strain ABE-1. J Gen Appl Microbiol 30:479-487

Sahara T, Takada Y, Takeuchi Y, Yamaoka N, Fukunaga N (2002) Cloning, sequencing, and expression of a gene encoding the monomeric isocitrate dehydrogenase of the nitrogen-fixing bacterium, *Azotobacter vinelandii*. Biosci Biotechol Biochem 66:489-500

Sambrook J, Russell D (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold spring Harbor Laboratory, Cold spring Harbor, N. Y.

Takada Y, Ochiai T, Okuyama H, Nishi K, Sasaki S (1979) An obligately psychrophilic bacterium isolated on the Hokkaido coast. J Gen Appl Microbiol 25:11-19

Thorsness PE, Koshland DE, Jr (1987) Inactivation of isocitrate dehydrogenase by phosphorylation is mediated by the negative charge of the phosphate. J Biol Chem 262:10422-10425

Wang ZX, Brämer C, Steinbüchel A (2003) Two phenotypically compensating isocitrate dehydrogenases in *Ralstonia eutropha*. FEMS Microbiol Lett 227:9-16

Watanabe S, Yasutake Y, Tanaka I, Takada Y (2005) Elucidation of stability determinants of cold-adapted monomeric isocitrate dehydrogenase from a psychrophilic bacterium, *Colwellia maris*, by construction of chimeric enzymes. Microbiology 151:1083-1094

Yasuda W, Kobayashi M, Takada Y (2013) Analysis of amino acid residues involved in cold activity of monomeric isocitrate dehydrogenase from psychrophilic bacteria, *Colwelia maris* and *Colwellia psychrerythraea*. J Biosci Bioeng 116:567-572

Yasutake Y, Watanabe S, Yao M, Takada Y, Fukunaga N, Tanaka I (2002) Structure of the monomeric isocitrate dehydrogenase: evidence of a protein monomerization by a domain duplication. Structure 10:1637-1648

Yasutake Y, Watanabe S, Yao M, Takada Y, Fukunaga N, Tanaka I (2003) Crystal structure of the monomeric isocitrate dehydrogenase in the presence of $NADP^+$. J Biol Chem 278:36897-36904

Yoneta M, Sahara T, Nitta K, Takada Y (2004) Characterizatiion of chimeric isocitrate dehydrogenases of a mesophilic nitrogen-fixing bacterium, *Azotobacter vinelandii*, and a psychrophilic bacterium, *Colwellia maris*. Curr Microbiol 48:383-388

Yumoto I, Kawasaki K, Iwata H, Matsuyama H, Okuyama H (1998) Assignment of *Vibrio* sp. strain ABE-1 to *Colwellia maris* sp. Nov., a new psychrophilic bacterium. Int J Syst Bacteriol 48:1357-1362

Zecchinon L, Claverie P, Collins T, D'Amico S, Delille D, Feller G, Georlette D, Gratia E, Hoyoux A, Meuwis M, Sonan G, Gerday C (2001) Did psychrophilic enzymes really win the challenge? Extremophiles 5:313-321

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 C*m*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 C*p*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 trypin. Each enzyme was incubated for various times at 25^{o}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.

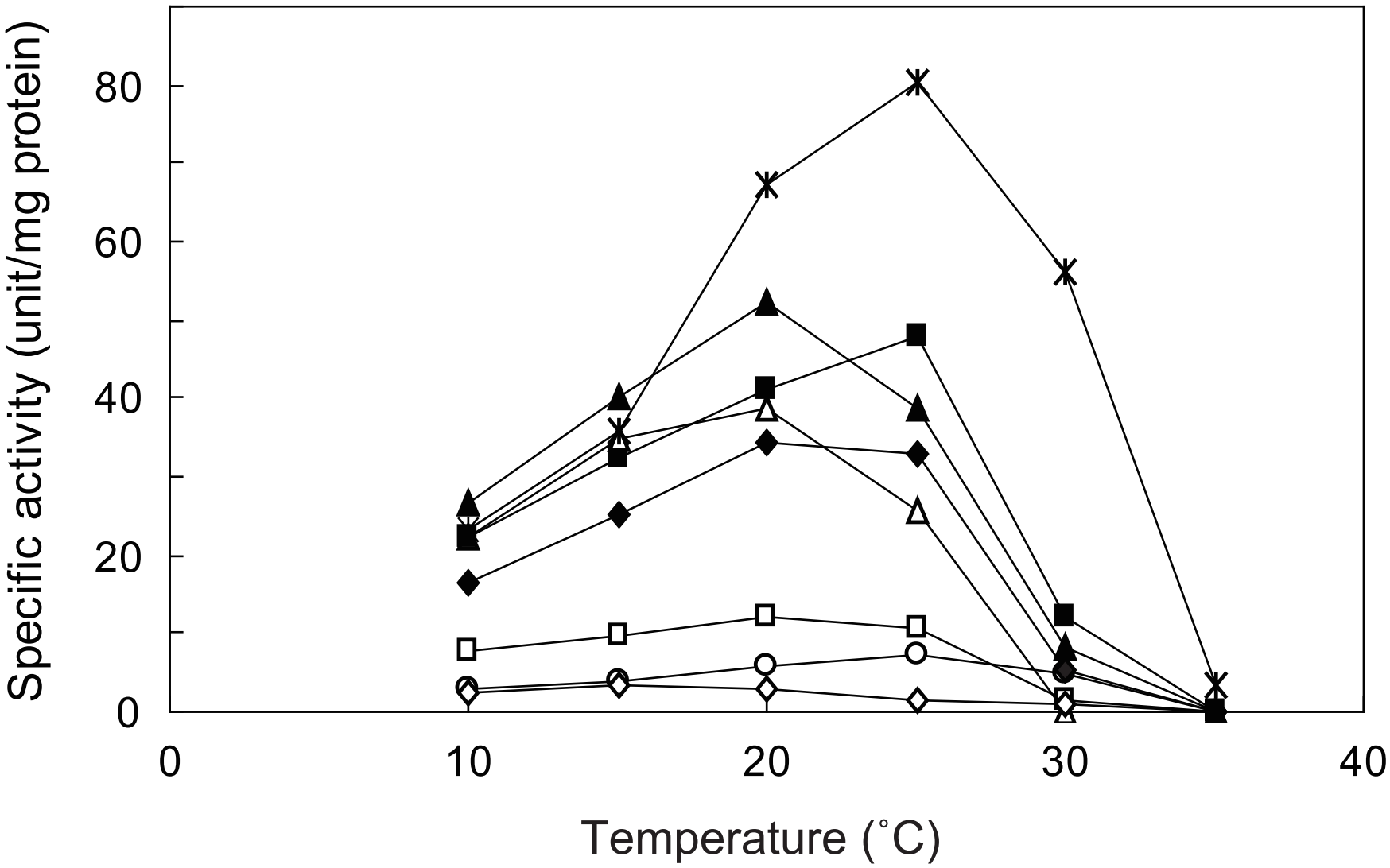
Specific activity (unit/mg protein)
80
60
40
20
0
0
10
20
30
40
Temperature (˚C)

Fig. 1

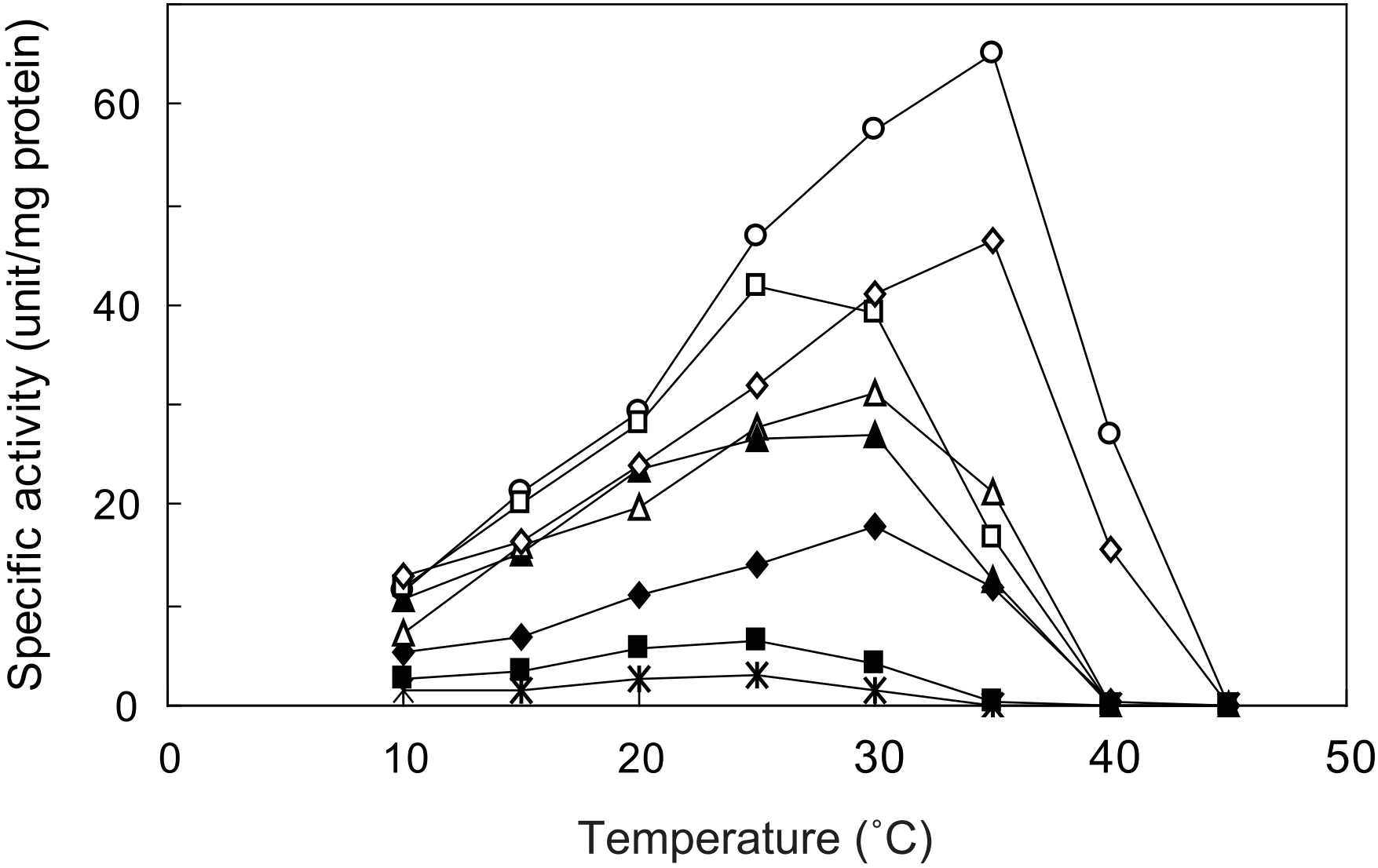
Specific activity (unit/mg protein)
60
40
20
0
0
10
20
30
40
50
Temperature (˚C)

Fig. 2

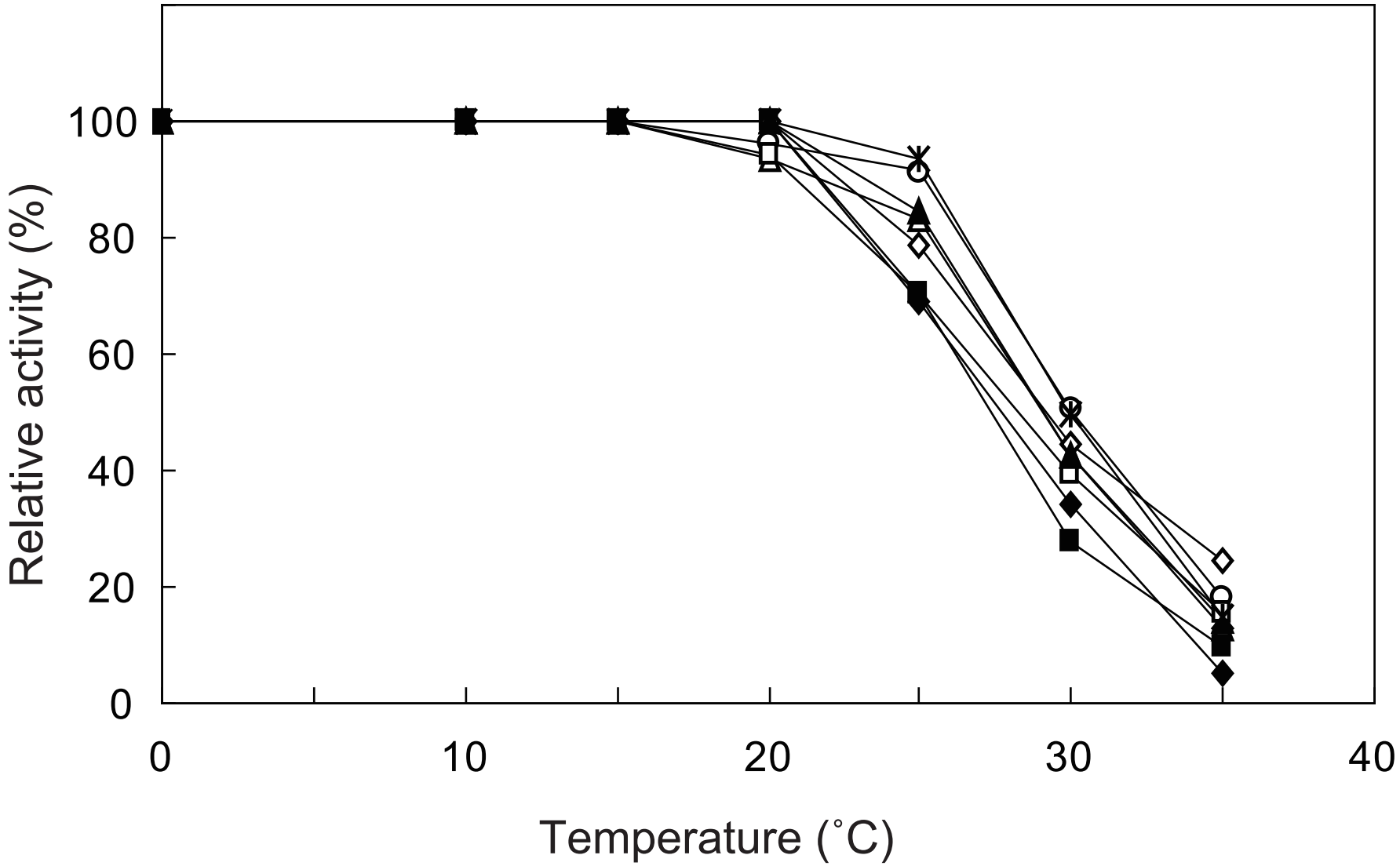

Fig. 3

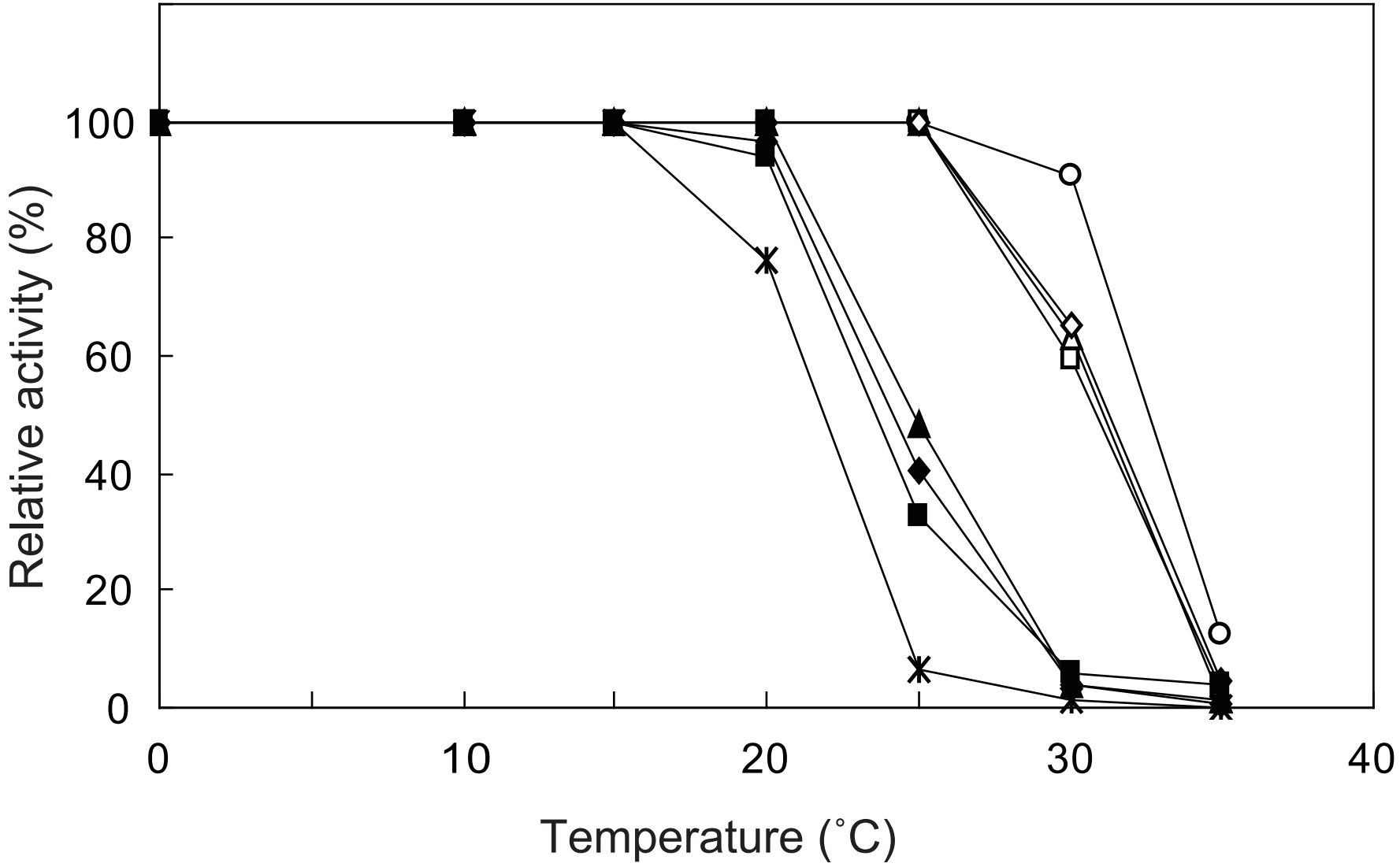

Fig. 4

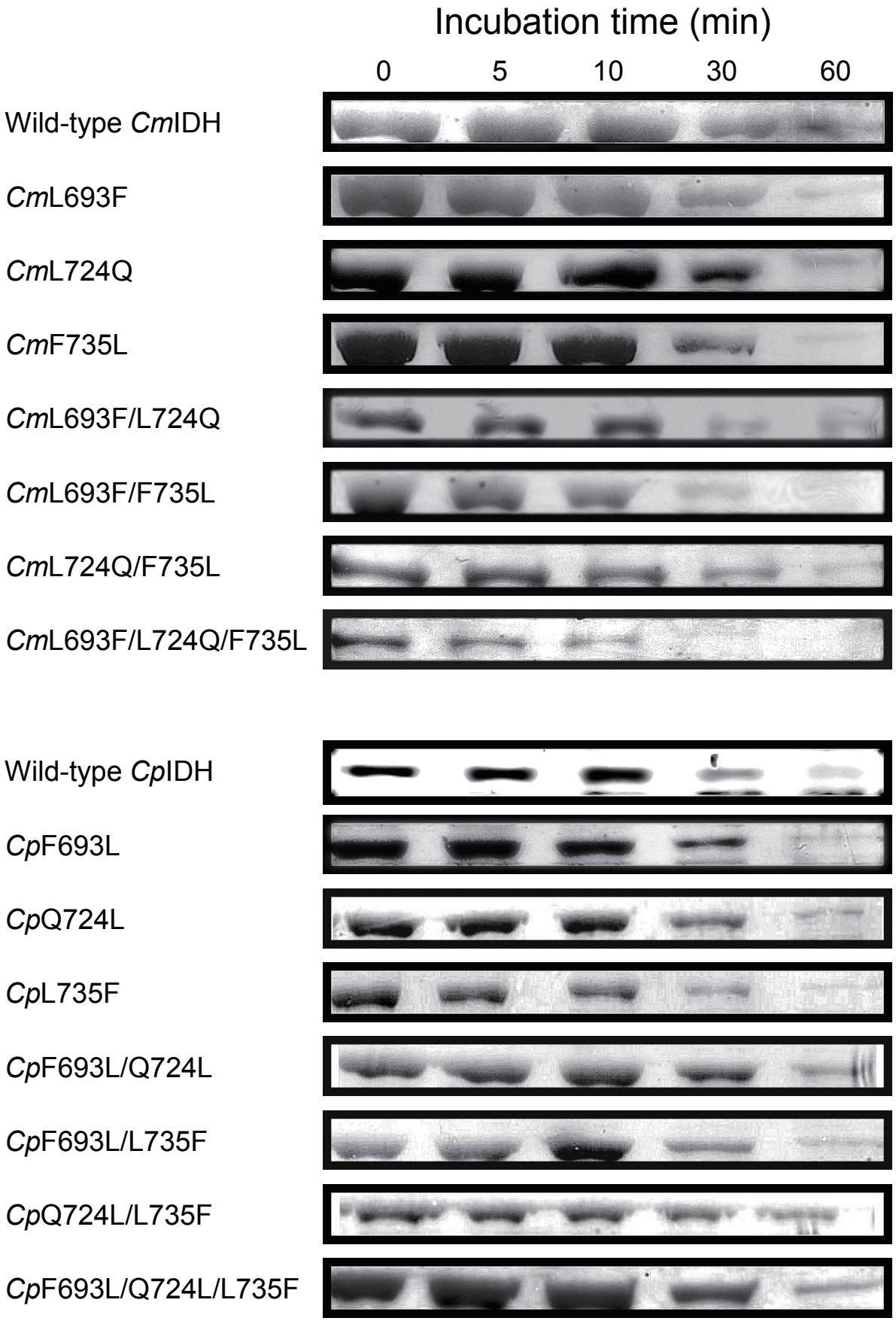
Incubation time (min)
0
5
10
30
60
Wild-type *Cm*IDH
*Cm*L693F
*Cm*L724Q
*Cm*F735L
*Cm*L693F/L724Q
*Cm*L693F/F735L
*Cm*L724Q/F735L
*Cm*L693F/L724Q/F735L
Wild-type *Cp*IDH
*Cp*F693L
*Cp*Q724L
*Cp*L735F
*Cp*F693L/Q724L
*Cp*F693L/L735F
*Cp*Q724L/L735F
*Cp*F693L/Q724L/L735F

Fig. 5

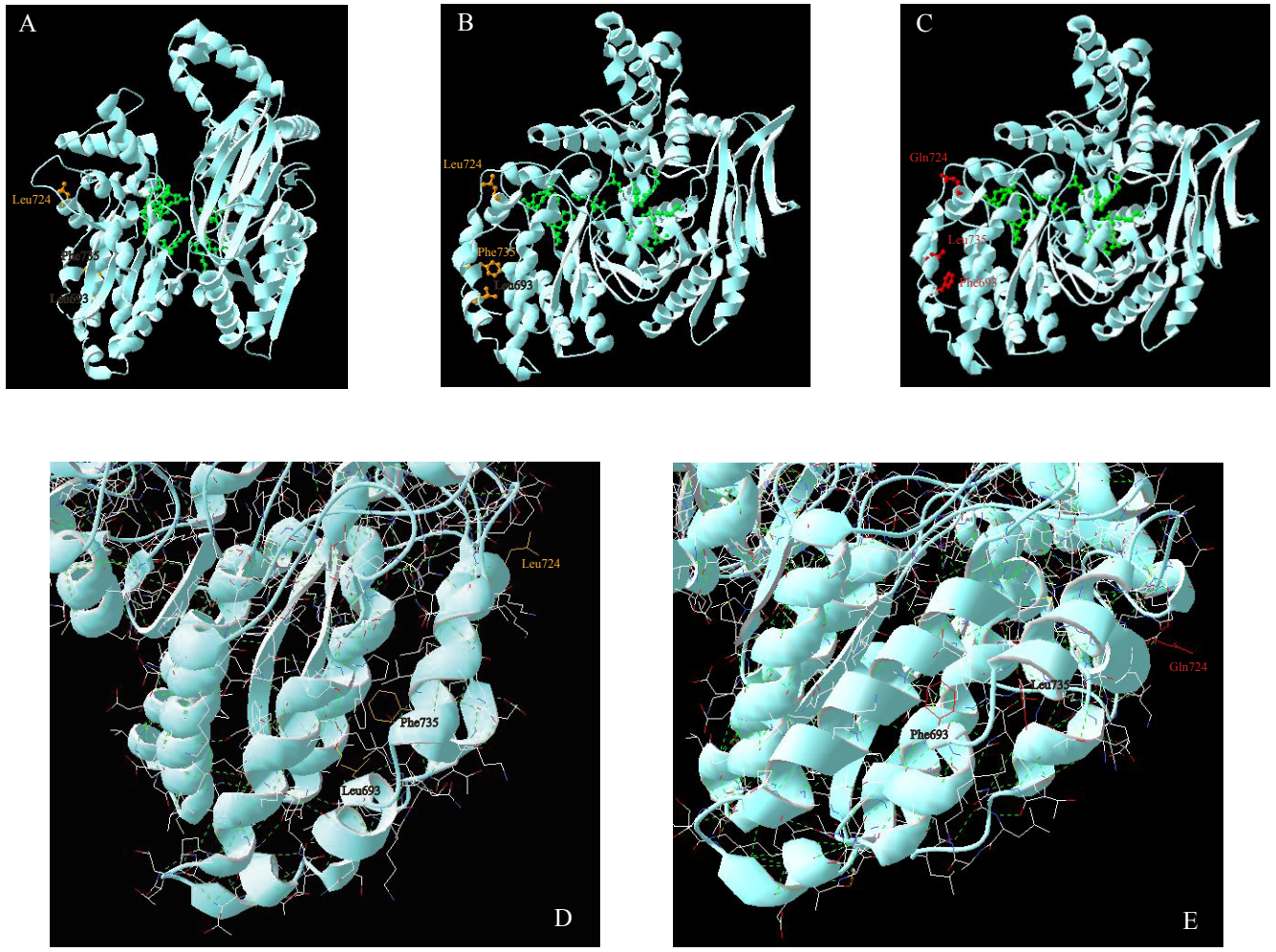
A
Leu724
B
Leu724
Phe735
Leu693
C
Gln724
D
Leu724
Phe735
Leu693
E
Gln724
Phe693

Fig. 6

Table 1 Kinetic parameters at 20°C

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

* K_m values for isocitrate are indicated.