Analysis of amino acid residues involved in cold activity of monomeric isocitrate dehydrogenase from psychrophilic bacteria, *Colwellia maris* and *Colwellia psychrerythraea*

Running title: Mutations in cold-adapted isocitrate dehydrogenases

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

Abstract

Monomeric isocitrate dehydrogenases from psychrophilic bacteria, *Colwellia maris* and *Colwellia psychrerythraea* (*Cm*IDH-II and *Cp*IDH-M, respectively) are cold-adapted enzymes and show a high degree of amino acid sequential identity to each other (77%). However, maximum activity of *Cp*IDH-M at optimum temperature is much less than that of *Cm*IDH-II. In the C-terminal Region 3 of these enzymes, which was suggested from previous study to be responsible for their distinct catalytic ability, several sequential differences of amino acid residue are present. Among them, ten amino acid residues were exchanged between them by site-directed mutagenesis and several properties of the mutated enzymes were examined in this study. The mutated enzymes of *Cm*IDH-II substituted its Gln671, Leu724 and Phe735 residues with the corresponding residues of *Cp*IDH-M (termed Q671K, L724Q and F735L, respectively) showed lower specific activity and thermostability for activity than the wild-type enzyme. Furthermore, the decreased specific activity was also observed in L693F. In contrast, the corresponding mutants of *Cp*IDH-M, F693L, Q724L and L735F, showed the increased specific activity and thermostability for activity. The catalytic efficiency (k_{cat}/K_m) values of these mutated *Cm*IDH-II and *Cp*IDH-M were lower and higher than those of their wild-type IDHs, respectively. These results suggest that the Gln671, Leu693, Leu724 and Phe735 residues of *Cm*IDH-II are important for exerting its high catalytic ability.

Introduction

$NADP^+$-specific isocitrate dehydrogenase (IDH; EC 1.1.1.42) is a key enzyme of the TCA cycle in many bacteria and catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate and CO_2 coupled with the reduction of $NADP^+$. Based on the subunit structure, bacterial IDHs can be categorized into two types, namely homodimer consisting of about 40-45 kDa subunits and single polypeptide with a molecular mass of 80-100 kDa. Many bacteria possess only one of either type IDH. *Escherichia coli* (1), *Thermus thermophilus* (2), *Bacillus stearothermophilus* (3) and *Rhodopseudomonas spheroides* (4) possess dimeric IDH, while those of *Azotobacter vinelandii* (5,6), *Corynebacterium glutamicum* (7) and *Vibrio parahaemolyticus* (8) are monomeric. On the other hand, both dimeric and monomeric IDHs are known to be present in several bacteria, including *Colwellia maris* (9,10) and *Ralstonia eutropha* (11).

In a psychrophilic bacterium *C. maris* (12,13), dimeric IDH (*Cm*IDH-I) is a typical mesophilic enzyme and the optimum temperature for activity is 45°C. On the other hand, monomeric IDH (*Cm*IDH-II) shows the maximum activity at 20°C, and about a half of the activity is lost even by incubation for 10 min at 30°C (9,14). The low optimum temperature and thermolability for activity indicate that it is a cold-adapted enzyme.

Monomeric IDH of a nitrogen-fixing mesophilic bacterium, *Azotobacter vinelandii*, (*Av*IDH) shows a high degree of amino acid sequential identity to *Cm*IDH-II (69.5%) (6), indicating that their three-dimensional structures are similar to each other. However, *Av*IDH is mesophilic (the optimum temperature for activity of 45°C) and completely retains its activity after incubation for 20 min at 40°C (15). Therefore, the properties of the two enzymes are quite different. In addition, *Av*IDH has very high catalytic ability over a wide range of temperature, and its activity at low temperatures was comparable to that of *Cm*IDH-II (16). The determined crystal structure of *Av*IDH (17,18) reveals that it contains domain I, consisting of N- and C-terminal segments (Region 1 and 3, respectively), and

domain II, which corresponds to the intermediate segment (Region 2). From previous study on chimeric enzymes exchanging each region between *Cm*IDH-II and *Av*IDH, the C-terminal Region 3 of *Av*IDH and *Cm*IDH-II was found to be involved in their thermal properties such as optimum temperature and thermostability for activity (16).

As well as *C. maris*, *Colwellia psychrerythraea* is also a psychrophilic bacterium (19) and has both of the two type IDHs (20). From the optimum temperature for activity of 25°C and the marked thermolability, monomeric IDH of this bacterium (*Cp*IDH-M) was found to be a typical cold-adapted enzyme similarly to *Cm*IDH-II. This enzyme showed high degrees of amino acid sequential identities to *Cm*IDH-II and *Av*IDH (77 and 68%, respectively). However, catalytic activity of *Cp*IDH-M was much less than that of *Cm*IDH-II (about 15% by the comparison of the maximum activities at respective optimum temperatures). Furthermore, from preliminary analysis of chimeric enzymes that exchanged each region between *Cp*IDH-M and *Cm*IDH-II, the C-terminal Region 3 of these enzymes was suggested to be responsible for the difference between their catalytic abilities (20).

From alignment of amino acid sequences of *Cm*IDH-II and *Av*IDH with high catalytic activities and *Cp*IDH-M with low activity, several amino acid residues, which are common to the former two enzymes but different from the latter one, are found in the Region 3 (Fig. 1). In this study, to identify amino acid residue(s) of the Region 3 involved in the catalytic activity at low temperatures and the thermostability for activity, the genes for the enzymes exchanged such amino acid residues between *Cm*IDH-II and *Cp*IDH-M were constructed by site-directed mutagenesis, and several properties of the mutated IDHs were investigated.

MATERIALS AND METHODS

Bacteria, plasmids and growth media *E. coli* DEK2004 (21), a mutant defective in

IDH, was used as a host for the expression of wild-type and mutated genes for *Cm*IDH-II and *Cp*IDH-M. For growth of the *E. coli* transformants, Luria-Bertani (LB) medium (22) or Super broth medium (16) was used. If necessary, ampicillin was added to the culture media at a concentration of 100 μg/ml. Plasmid pTrcHisB (Invitrogen) was used for overexpression of the genes encoding recombinant IDHs conferred His-tags at the N-terminals. Plasmids pHis*Cm*IDH-II (16; termed pHis*CmWT* in the paper) and pHis*Cp*IDH-M (20), harboring the *Cm*IDH-II and *Cp*IDH-M genes in the *Bam*HI-*Sac*I site of pTrcHisB, respectively, were used as templates for PCR in site-directed mutagenesis.

Construction of the mutated IDH genes by site-directed mutagenesis Mutated IDH genes were constructed by three PCRs as previously reported (23). At first, a 5'-terminal fragment of the IDH gene containing the mutated site was amplified in a reaction mixture (50 μl) containing 100 ng pHis*Cm*IDH-II or pHis*Cp*IDH-M as a template, 100 pmol forward primer (primer A or E in Tables S1 and S2, respectively), 100 pmol reverse primer to introduce each substitution of amino acid residue (primer B or F in Tables S1 and S2, respectively) and 1 U KOD-plus DNA polymerase (TOYOBO) in the buffer system prepared by the manufacturer. Next, a 3'-terminal fragment of the IDH gene containing the mutated site was amplified in the same reaction mixture as the first PCR, except that 100 pmol primer C or G and 100 pmol primer D or H (Tables S1 and S2) were used as forward primer for the introduction of each substitution and reverse primer, respectively. Then, by third PCR, the full lengths of mutated IDH genes were amplified in the reaction mixture same as the first PCR, except for using both products of the first and second PCRs as templates, and primer A or E as forward primer and primer D or H as reverse primer, respectively. Since the codons for Asp713, Leu724 and Phe735 of *Cm*IDH-II and Asn713, Gln724 and Leu735 of *Cp*IDH-M are located at 3'-terminals of the respective IDH genes, an oligonucleotide, pTrcHisB-R (Tables S1 and S2), which was designed from the nucleotide sequence of the pTrcHisB

located about 100-bp downstream of the IDH genes, was used as the reverse primer for the second and third PCRs instead of primer D or H. These PCRs were carried out for 30 cycles under the cycling conditions shown in Table S3 in a DNA thermal cycler 2400 (Perkin-Elmer). The final PCR products were digested with *Bam*HI and *Sac*I and then ligated to the *Bam*HI-*Sac*I site of pTrcHisB with DNA Ligation Kit Ver. 2.1 (TaKaRa). Introduction of the mutation were certified by nucleotide sequencing of the relevant regions of the plasmids in both directions using appropriate primers and a Big Dye Terminator V1.1 Cycle Sequencing Reaction Kit (Applied Biosystems) with an ABI PRISM 310 Genetic Analyzer or 3130 Genetic Analyzer.

Overexpression and purification of His-tagged IDHs *E. coli* DEK2004 transformed with pHis*Cm*IDH-II, pHis*Cp*IDH-M or pTrcHisB carrying the mutated IDH genes was grown at 37^{o}C with vigorous shaking in Super broth medium until OD_{600} of the culture reached 1.2. Then, the cultures were rapidly cooled on ice and further incubated for 18-24 h at 15^{o}C (because of the thermolability of *Cm*IDH-II and *Cp*IDH-M (9,20)) after the addition of 1 mM isopropyl-β-D-thiogalactopyranoside to induce the expression of the His-tagged IDH proteins. Cells were harvested at 4^{o}C and suspended in 30 ml of buffer A (50 mM sodium phosphate buffer (pH 8.0), containing 2 mM $MgCl_2$, 0.5 M NaCl, 10 mM imidazole and 10 mM 2-mercaptoethanol) for the wild-type and mutated *Cm*IDH-II or buffer B (buffer A containing 0.3 M NaCl instead of 0.5 M NaCl) for the wild-type and mutated *Cp*IDH-M per liter of the culture. Hen-egg lysozyme was added to the cell suspension at a concentration of 2 mg/ml, and the mixture was gently shaken for 2 h at 4^{o}C. Then, the cells were disrupted by an ultrasonic oscillation. After cell debris was removed by centrifugation of the cell lysate at 16,000 ×*g* for 20 min at 4^{o}C, the supernatant was further centrifuged at 105,000 ×*g* for 1 h at 4^{o}C. The resultant supernatant was loaded onto a Ni-NTA column (bed volume of 25 ml; Qiagen). The purification of the wild-type and mutated *Cm*IDH-II by Ni-NTA column

chromatography was carried out as reported previously (16). For the purification of the wild-type and mutated *Cp*IDH-M, the column was equilibrated with buffer B. After thorough washing with buffer B, the column was further washed with 50 ml of buffer C (buffer B containing 10% (v/v) glycerol and 20 mM imidazole instead of 10 mM imidazole) and next with 50 ml of buffer D (buffer C containing 30 mM imidazole instead of 20 mM imidazole). The enzymes were then eluted with 50 ml of buffer E (buffer C containing 250 mM imidazole instead of 20 mM imidazole). Each elutant was concentrated with polyethylene glycol #20,000 and dialyzed against 20 mM sodium phosphate buffer (pH 8.0), containing 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 stocked at -30^{o}C until use.

Enzyme assay Unless otherwise noted, the IDH activity was assayed at 20^{o}C as described previously (9). For *Cm*IDH-II and its mutants, the reaction mixture (2 ml) contained 33 mM Tris-HCl (pH 8.0), 0.67 mM $MnCl_2$, 0.12 mM $NADP^+$, 0.25 M NaCl, 2 mM isocitrate and an appropriate amount of enzyme. For *Cp*IDH-M and its mutants, 33 mM Tris-HCl (pH 7.0) was added to the reaction mixture instead of 33 mM Tris-HCl (pH 8.0). To examine the thermostability of IDH activity, all purified IDHs were dialyzed overnight at 4^{o}C against 20 mM sodium phosphate buffer (pH 8.0) containing 2 mM $MgCl_2$, 0.1 M 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. One unit of enzyme activity was defined as the amount capable of catalyzing the reduction of 1 μmol of $NADP^+$ per min. Protein was measured by the method of Lowry et al. (24). All data for activity and kinetic parameters are the mean values of at least two independent experiments.

RESULTS

Construction and purification of mutated IDHs Several sequential differences of the amino acid residues are found in Region 3 of *Av*IDH, *Cp*IDH-M and *Cm*IDH-II (Fig. 1). Among them, amino acid residues, common to the two cold-adapted *Cp*IDH-M and *Cm*IDH-II but different from the mesophilic *Av*IDH, are expected to be involved in psychrophilic properties of the former two enzymes, such as lower optimum temperature for activity and more thermolabile than the mesophilic *Av*IDH. On the other hand, the catalytic ability of *Cp*IDH-M is considerably less than that of *Cm*IDH-II (20). Furthermore, *Av*IDH showed very high catalytic ability over a wide range of temperature, and its activity at low temperatures was comparable to that of the cold-adapted *Cm*IDH-II (16). Therefore, in this study, ten amino acid residues, which are shared by the two IDHs with high activity at low temperatures (*Cm*IDH-II and *Av*IDH) but are distinct from *Cp*IDH-M with markedly low activity, were selected to identify amino acid residue involved in high catalytic function at low temperatures (Stars in Fig. 1).

The Lys641, Lys652, Gln671, Ala675, Gln683, Leu693, Thr694, Asp713, Leu724 and Phe735 residues of *Cm*IDH-II were substituted with the corresponding amino acid residues of *Cp*IDH-M (Glu, Arg, Lys, Gln, Lys, Phe, Ala, Asn, Gln and Leu, respectively) by site-directed mutagenesis to obtain mutated enzymes of *Cm*IDH-II termed K641E, K652R, Q671K, A675Q, Q683K, L693F, T694A, D713N, L724Q and F735L, respectively. In contrast, the corresponding mutants of *Cp*IDH-M substituted with amino acid residue of *Cm*IDH-II were also produced (designated as E641K, R652K, K671Q, Q675A, K683Q, F693L, A694T, N713D, Q724L and L735F, respectively). These His-tagged wild-type and mutated IDH proteins were overexpressed in the *E. coli* DEK2004 cells and were then purified. SDS-PAGE of the final elutants of Ni-NTA column chromatography revealed that the purified mutant enzymes contain a major protein with a molecular mass of about 80 kDa, similar to

wild-type *Cm*IDH-II and *Cp*IDH-M (data not shown). It has been reported that the His-tagging to the N-terminals of *Cm*IDH-II and *Cp*IDH-M proteins has no significant effect on their thermal properties (15,20).

Properties of the mutated IDHs of *C. maris* To examine the effect of the substituted amino acid residues on the catalytic function of *Cm*IDH-II, activities of the wild-type and mutated *Cm*IDH-IIs were assayed at various temperatures (Fig. 2). The His-tagged wild-type *Cm*IDH-II showed the maximum activity (37 unit/mg protein) at 25°C in this assay, and its optimum temperature for activity was estimated to be between 20 and 25°C. Optimum temperature for activity of A675Q was similar to that of wild-type enzyme, but the other mutated enzymes, in particular L724Q and F735L, appeared to lower their optimum temperature. Furthermore, all mutated enzymes showed the decreased specific activities at respective optimum temperatures compared to the wild-type *Cm*IDH-II. The largest loss of specific activity was observed in Q671K and was decreased to 34% of that of the wild-type *Cm*IDH-II. Furthermore, L693F, L724Q and F735L showed only 48, 38 and 43% of the wild-type enzyme activity, respectively. In contrast, T694A and D713N retained 80 and 76% of the wild-type enzyme activity, respectively.

After incubation for 10 min at the indicated temperatures, remaining activities of the wild-type and mutated *Cm*IDH-IIs were assayed at 20°C (Fig. 3). Even after incubation at 20°C, 7 and 9% of the Q671K and F735L activities were lost, respectively, while the other mutated and wild-type enzymes retained completely their activities under the same condition. In addition, the two mutated enzymes showed the lower activities than wild-type *Cm*IDH-II after incubation at 25 and 30°C. These results indicate that the two mutants are more thermolabile than the wild-type enzyme. Furthermore, since remaining activities of L724Q after incubation at 25 and 30°C (72 and 30%, respectively) were lower than those of wild-type enzyme (89 and 35%, respectively), L724Q also exhibited a lowered thermostability above

25°C. On the other hand, K652R and A675Q were more thermostable rather than the wild-type enzyme because of higher remaining activities of the two mutated enzyme after incubation at 25 and 30°C (94 and 43% in K652R, and 93 and 41% in A675Q, respectively) compared with those of wild-type one (shown above). Therefore, Q671K, L724Q and F735L showed both the decreased specific activity and the lowered thermostability for their activities, suggesting that the three amino acid residues of *Cm*IDH-II are involved in its higher catalytic ability and thermostability than *Cp*IDH-M.

Properties of the mutated IDHs of *C. psychrerythraea* Since six *Cm*IDH-II mutants showed different thermal properties from the wild-type enzyme as described above, similar experiments were carried out in the corresponding mutants of *Cp*IDH-M, E641K, K671Q, F693L, A694T, Q724L and L735F (Figs. 4 and 5). Wild-type *Cp*IDH-M showed the maximum activity (1.2 unit/mg protein) at 20°C (Fig. 4), and its activity was considerably less than that of *Cm*IDH-II as reported previously (20). In contrast to the corresponding *Cm*IDH-II mutants (L724Q and F735L), optimum temperatures for activities of the two *Cp*IDH-M mutants, Q724L and L735F, were about 5°C higher than that of the wild-type enzyme. In addition, specific activities of these mutants at temperatures between 10 and 30°C were markedly increased and their activities at respective optimum temperatures (11.3 and 7.2 unit/mg protein, respectively) were about 9- and 6-folds higher than that of the wild-type *Cp*IDH-M, respectively. Furthermore, F693L also showed a somewhat enhanced activity at 20°C (about 3-fold of the wild-type enzyme activity).

Although the activity of the wild-type *Cp*IDH-M after incubation at 20°C was decreased to 78%, F693L and L735F maintained 91 and 96% of their activities, respectively, and no loss of activity was observed in Q724L (Fig. 5). Therefore, F693L, Q724L and L735F appeared to acquire the increased thermostability. On the other hand, E641K, K671Q and A694T were somewhat more thermolabile rather than the wild-type enzyme because the three

mutated enzyme showed 61, 73 and 70% of remaining activities after the same incubation, respectively. Thus, the results for the three *Cp*IDH-M mutants, F693L, Q724L and L735F, were opposite to those of the corresponding mutants of *Cm*IDH-II (L693F, L724Q and F735L).

Kinetic parameters of the mutated IDHs As described above, the three *Cm*IDH-II mutants (L693F, L724Q and F735L) and the corresponding *Cp*IDH-M mutants (F693L, Q724L and L735F) showed the altered thermal properties, compared with the respective wild-type IDHs, and the changes of thermal properties in the former mutants were in contrast with those of the latter ones. Therefore, the values of K_m for isocitrate, k_{cat} and k_{cat}/K_m of the wild-type and these mutated IDHs at 20°C were examined (Table 1). In the case of *Cm*IDH-II, the values of catalytic efficiency, k_{cat}/K_m, of all mutated enzymes (L693F, L724Q and F735L) were diminished to 50, 20 and 33% of that of the wild-type enzyme, respectively. This diminished k_{cat}/K_m values was found to result from the decrease of k_{cat} values in all mutated enzymes, in addition to the increase of K_m value in L724Q. By contraries, in the case of *Cp*IDH-M, Q724L and L735F were found to acquire markedly high k_{cat}/K_m values (about 5-7-folds of that of the wild-type enzyme) by their mutations. In addition, F693L also showed 2.5-fold higher k_{cat}/K_m value than the wild-type enzyme. In contrast to the case of *Cm*IDH-II, the increased k_{cat}/K_m values of all *Cp*IDH-M mutants were confirmed to be ascribable to the increase of k_{cat} values.

DISCUSSION

In this study, the *Cm*IDH-II mutants, L724Q and F735L, which are substituted the Leu724 and Phe735 with the corresponding Gln and Leu residues of *Cp*IDH-M, respectively, showed

lower specific activity and less thermostability than the wild-type enzyme (Figs. 2 and 3). Although L693F also decreased its specific activity, its thermostability for activity decreased only slightly. In contrast, the respective exchanges of Phe693, Gln724 and Leu735 in *Cp*IDH-M for the corresponding Leu, Leu and Phe residues of *Cm*IDH-II (F693L, Q724L and L735F) brought about the opposite results to the three *Cm*IDH-II mutants, L693F, L724Q and F735L (Figs. 4 and 5). Therefore, it is concluded that these three residues of *Cm*IDH-II are involved in its high catalytic activity and thermostability for activity, and can improve the inferior properties of *Cp*IDH-M. The substitution of Gln671 in *Cm*IDH-II with the corresponding Lys residue of *Cp*IDH-M and its counter mutation of *Cp*IDH-M brought interesting results. Loss of specific activity in Q671K was the largest of all *Cm*IDH-II mutants, and this mutated enzyme was more thermolabile than the wild-type *Cm*IDH-II. From these results, it was expected that the counter mutation of *Cp*IDH-M (K671Q) cause the increase of specific activity and/or thermostability. However, conversely, K671Q showed the decreased specific activity and thermostability rather than the wild-type enzyme. Thus, the Gln671 residue of *Cm*IDH-II seems to be important for its high catalytic activity but, at least by itself, to be ineffective for improving the *Cp*IDH-M activity. On the other hand, additional substitution(s) of other amino acid residue(s) in the vicinity of Gln671, for example Ala675, Gln683 or Thr694, might be required for the increase of *Cp*IDH-M activity (Fig. 6). Since single mutations were merely introduced in *Cm*IDH-II and *Cp*IDH-M in this study, further studies for their multiple mutants are needed to confirm this possibility. From the comparison between molecular models of the wild-type *Cm*IDH-II and Q671K built with a program SWISSPDB VIEWER (Fig. 7), it was suggested that the Gln671 residue of the wild-type enzyme can form four hydrogen bonds between side chains of Met667, Ala674, Ala675 and Thr694, but the hydrogen bond between Thr694 is lost in Q671K because of its substitution with Lys. Such a decreased hydrogen bond may be responsible for the lowered thermostability of the mutated enzyme. However, no change for hydrogen bond was

detected in the other mutated enzymes of *Cm*IDH-II and *Cp*IDH-M, implying that the other structural factors are involved in the altered thermostability of these mutated enzymes. On the other hand, the other six mutations in *Cm*IDH-II also resulted in the decrease of specific activity but their influences were smaller than the above four mutations. In addition, the thermostability of these six mutant was similar to, or even more than, the wild-type enzyme.

Analysis of kinetic parameters revealed that the exchanges of the 693rd, 724th and 735th amino acid residues between *Cm*IDH-II and *Cp*IDH-M result in the decrease and increase of k_{cat}/K_m values, respectively, and such changes are ascribable to the altered k_{cat} values of the mutated enzymes. These results imply significant effect(s) of these substituted mutations on their catalytic ability. In cold-adapted enzymes, an increase of the k_{cat} values is known to be a strategy to improve the catalytic efficiency (25). Furthermore, catalytic activity of cold-adapted enzymes at low temperatures is thought to be achieved by local flexibility in regions close to the catalytic site (26,27). On the other hand, local rigidity in regions far from catalytic site was reported to be involved in catalytic activity of cold-adapted elastase (28). Similarly, in this study, Leu693, Leu724 and Phe735 of *Cm*IDH-II, which are far from the catalytic site (Fig. 6), were found to be involved its high catalytic activity. However, no difference in the formation of hydrogen bond was present between the three residues of wild-type *Cm*IDH-II and the substituted ones of mutated enzymes by the comparison of molecular models. Therefore, further study is required to understand the mechanism for the effects of the mutations on the enzyme activity, and experiments for the combined mutations of the three substituted amino acid residues in *Cm*IDH-II and *Cp*IDH-M are in progress.

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Figure legends

FIG. 1 Alignment of amino acid sequences of Region 3 in *Cp*IDH-M (C.p; database accession no. AB174851), *Cm*IDH-II (C.m; D14047) and *Av*IDH (A.v; D73443) and secondary structure of *Av*IDH. The area surrounded by line represents Region 3 of the IDH proteins. Identical and similar amino acid residues among these three IDHs are indicated in black and white boxes, respectively. The secondary structure, β-sheet and α-helix, of *Av*IDH are depicted above the alignment by arrow and coil, respectively. The stars indicate the positions of amino acid residues substituted in this study. This figure, including the secondary structure of *Av*IDH (PDB No. 1ITW), was made with a program ESPript Ver.2.3 (URL http://espript.ibcp.fr/ESPript/ESPript/).

FIG. 2 Effect of temperature on activities of wild-type and mutated *Cm*IDH-IIs.

FIG. 3 Thermostability of wild-type and mutated *Cm*IDH-IIs. Remaining activity assayed at 20^{o}C after incubation for 10 min at the indicated temperatures is represented as a percentage of that without the incubation.

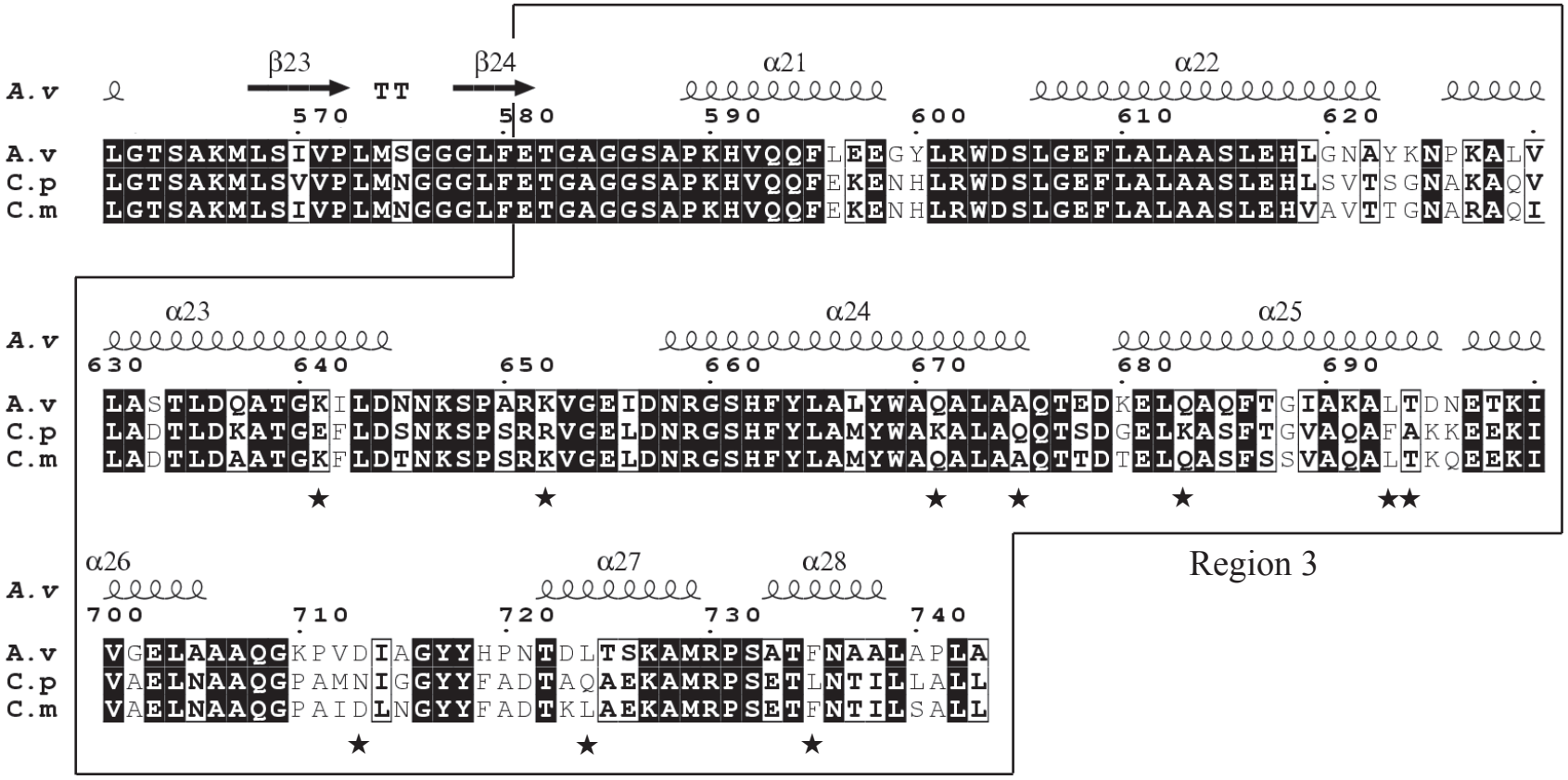
FIG. 4 Effect of temperature on activities of wild-type and mutated *Cp*IDH-Ms.

FIG. 5 Thermostability of wild-type and mutated *Cp*IDH-Ms. Remaining activity assayed at 20^{o}C after incubation for 10 min at the indicated temperatures is represented as a percentage of that without the incubation.

FIG. 6 Positions of substituted amino acid residues in molecular models of *Cm*IDH-II. The models of *Cm*IDH-II were built with a program SWISSPDB VIEWER (URL

http://br.expasy.org/spdbv/), using the *Av*IDH (PDB No. 1ITW) as a homology model. (a), Front view. (b), Left view. The amino acid residues involved in the binding of isocitrate and metal ion are indicated by green (gray in print version). The amino acid residues substituted in this study are indicated by blue (black in print version), and three residues having significant effects on the catalytic function (Leu693, Leu724 and Phe735) among them are shown by red (dark gray in print version).

FIG. 7 Hydrogen bonds in Gln671 of wild-type *Cm*IDH-II and in Lys 671 of Q671K. The models of *Cm*IDH-II and Q671K were built as described in the legend for Fig. 6. Oxygen, hydrogen and sulfur in the side chains of amino acid residues are indicated by red, blue and yellow, respectively. Hydrogen bonds are shown by green dashes.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β24
α21
α22
A.v
TT
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580
590
600
610
620
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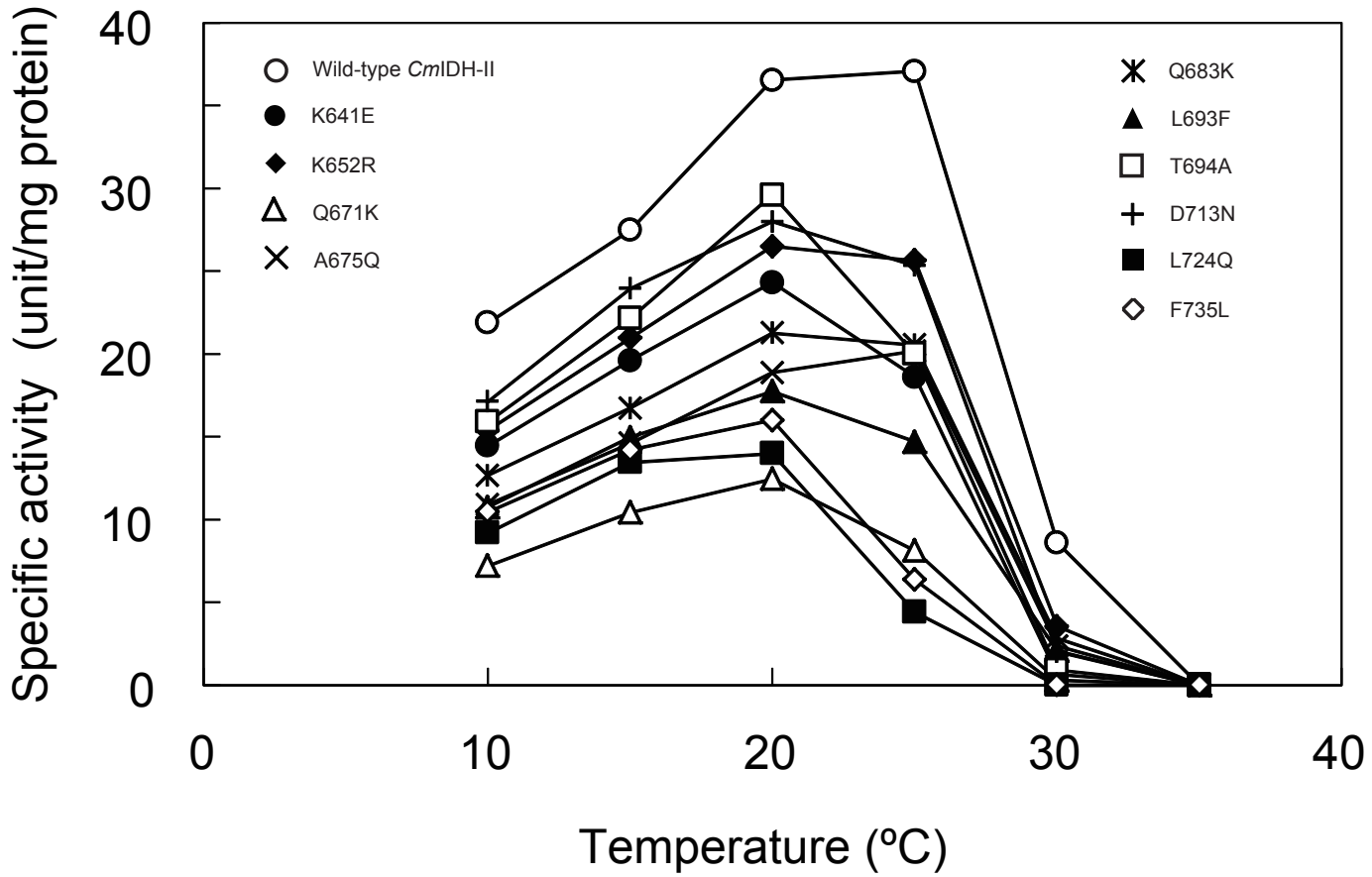
C.p LGTSAKMLSVVPLMNGGGLFETGAGGSAPKHVQQFEKENHLRWDSLGEFLALAASLEHLSVTSGNAKAQV
C.m LGTSAKMLSIVPLMNGGGLFETGAGGSAPKHVQQFEKENHLRWDSLGEFLALAASLEHVAVTTGNARAQI
α23
α24
α25
A.v
630
640
650
660
670
680
690
A.v LASTLDQATGKILDNNKSPARKVGEIDNRGSHFYLALYWAQALAAQTEDKELQAQFTGIAKALTDNETKI
C.p LADTLDKATGEFLDSNKSPSRRVGELDNRGSHFYLAMYWAKALAQQTSDGELKASFTGVAQAFAKKEEKI
C.m LADTLDAATGKFLDTNKSPSRKVGELDNRGSHFYLAMYWAQALAAQTTDTELQASFSSVAQALTKQEEKI
α26
α27
α28
A.v
700
710
720
730
740
A.v VGELAAAQGKPVDIAGYYHPNTDLTSKAMRPSATFNAALAPLA
C.p VAELNAAQGPAMNIGGYYFADTAQAEKAMRPSETLNTILLALL
C.m VAELNAAQGPAIDLNGYYFADTKLAEKAMRPSETFNTILSALL
Region 3

Fig. 1


Specific activity (unit/mg protein)
40
30
20
10
0
0
10
20
30
40
Temperature (ºC)
Wild-type CmIDH-II
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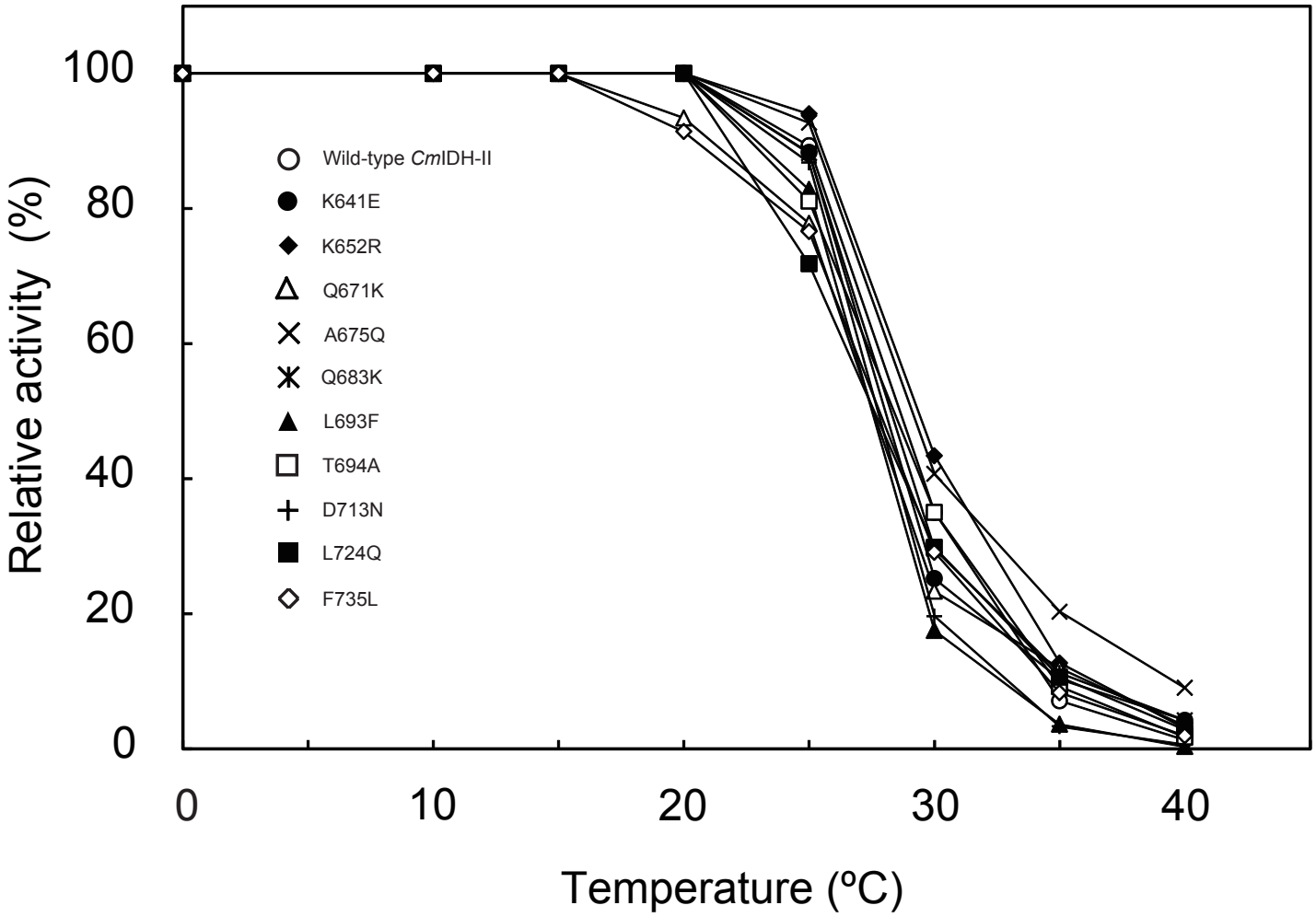
K652R
Q671K
A675Q
Q683K
L693F
T694A
D713N
L724Q
F735L

Fig. 2


Relative activity (%)
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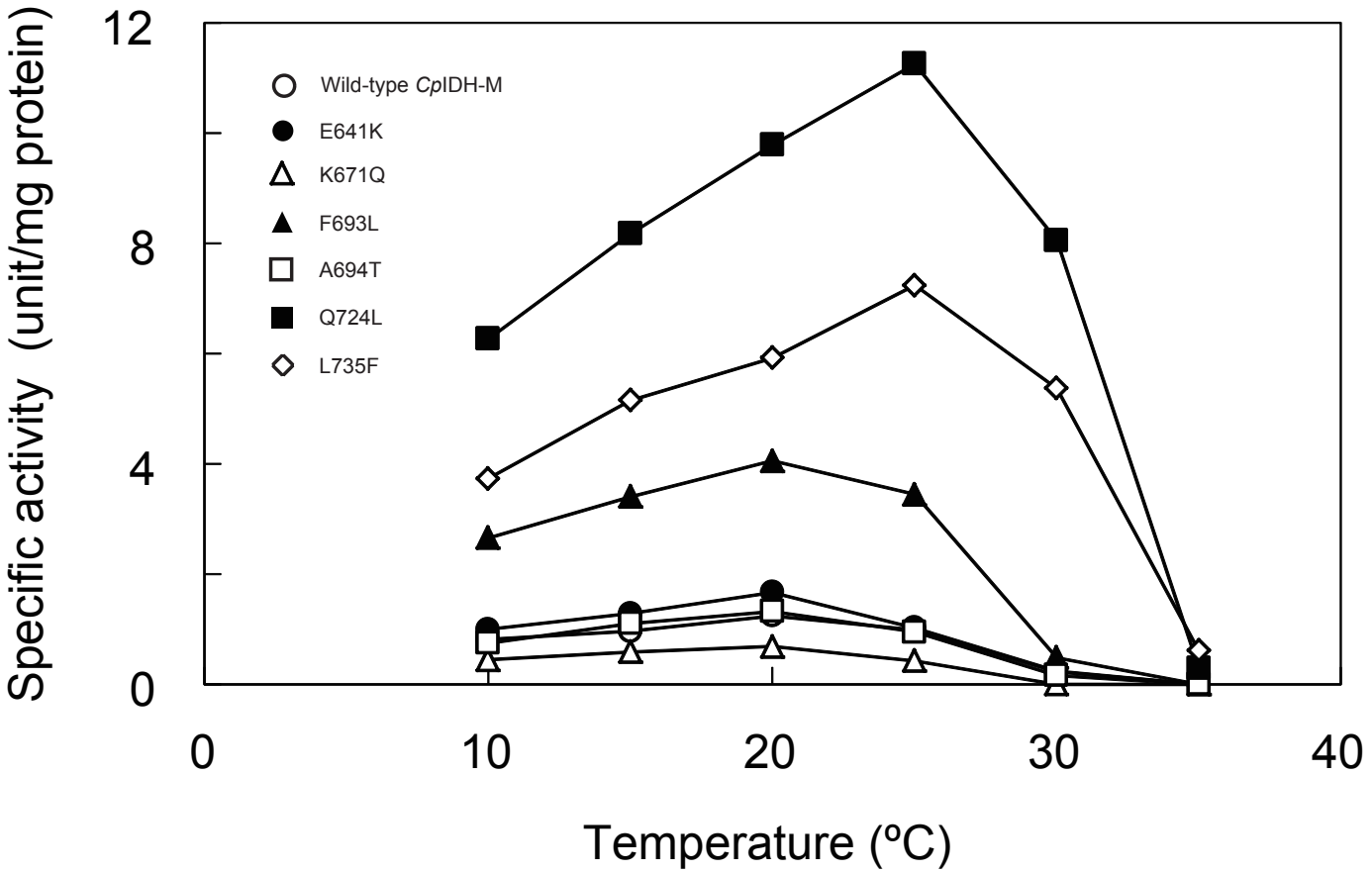
80
60
40
20
0
0
10
20
30
40
Temperature (ºC)
Wild-type *Cm*IDH-II
K641E
K652R
Q671K
A675Q
Q683K
L693F
T694A
D713N
L724Q
F735L

Fig. 3


Specific activity (unit/mg protein)
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8
4
0
0
10
20
30
40
Temperature (ºC)
Wild-type CpIDH-M
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F693L
A694T
Q724L
L735F

Fig. 4

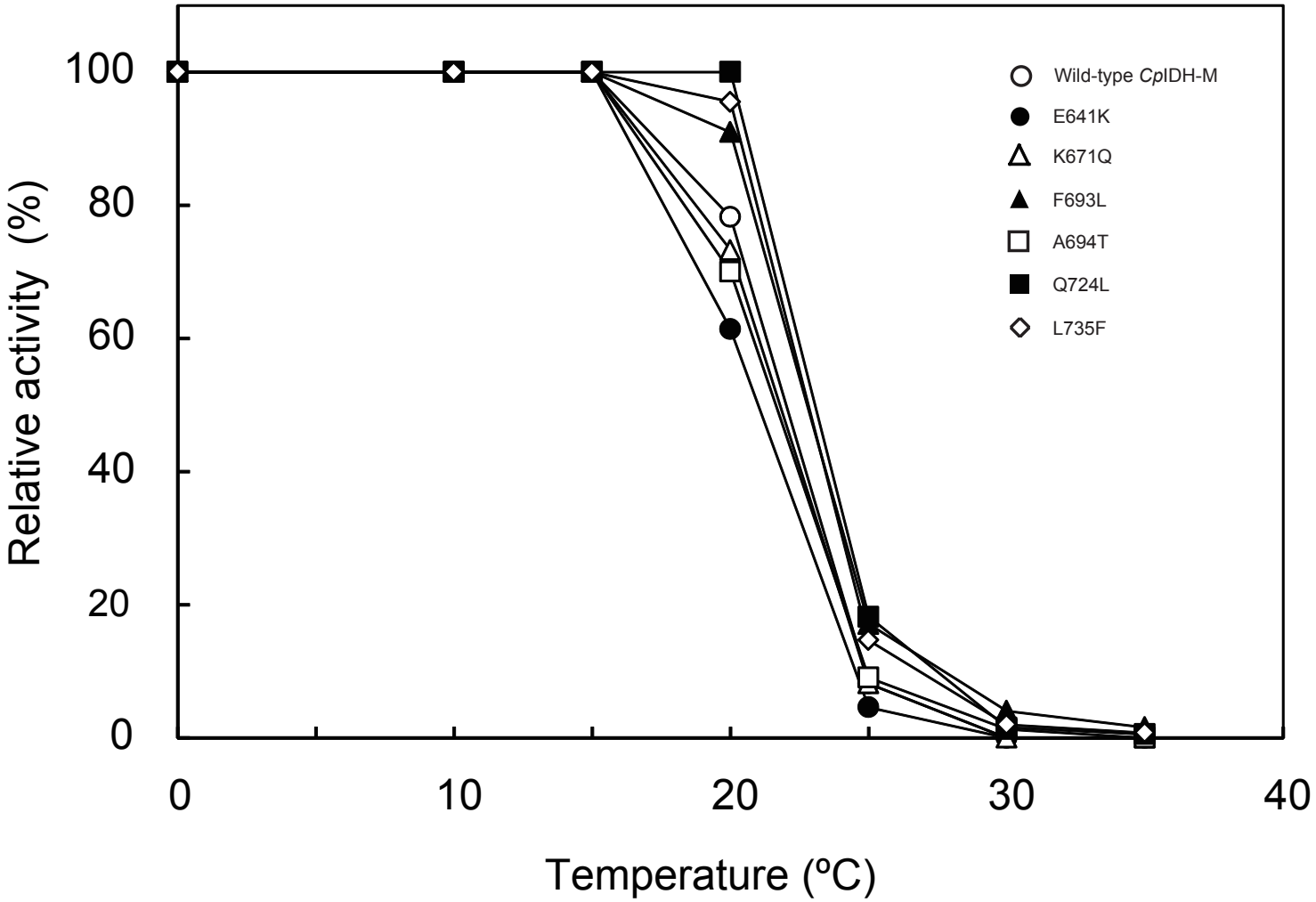

Relative activity (%)
100
80
60
40
20
0
0
10
20
30
40
Temperature (ºC)
Wild-type CpIDH-M
E641K
K671Q
F693L
A694T
Q724L
L735F

Fig. 5

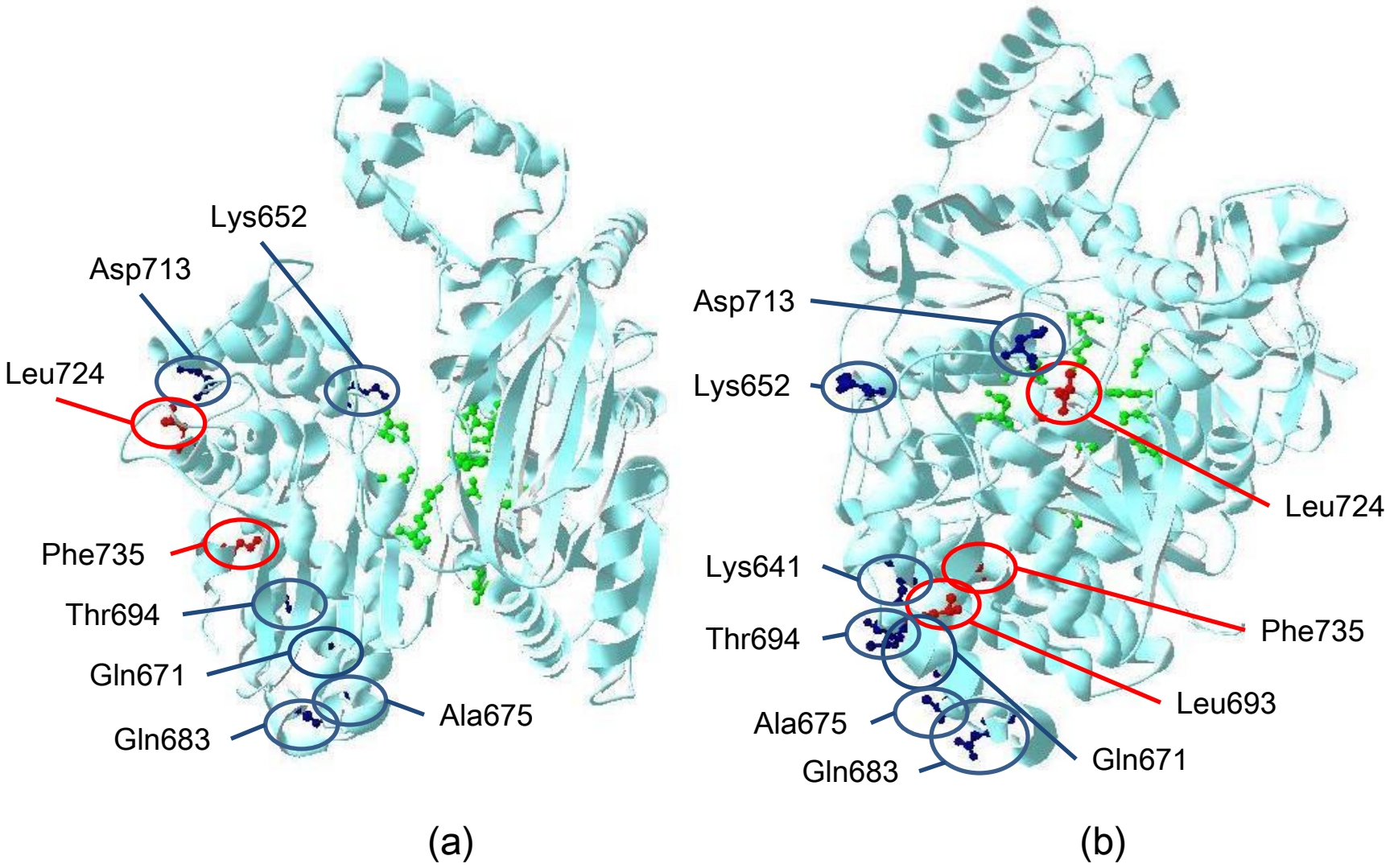
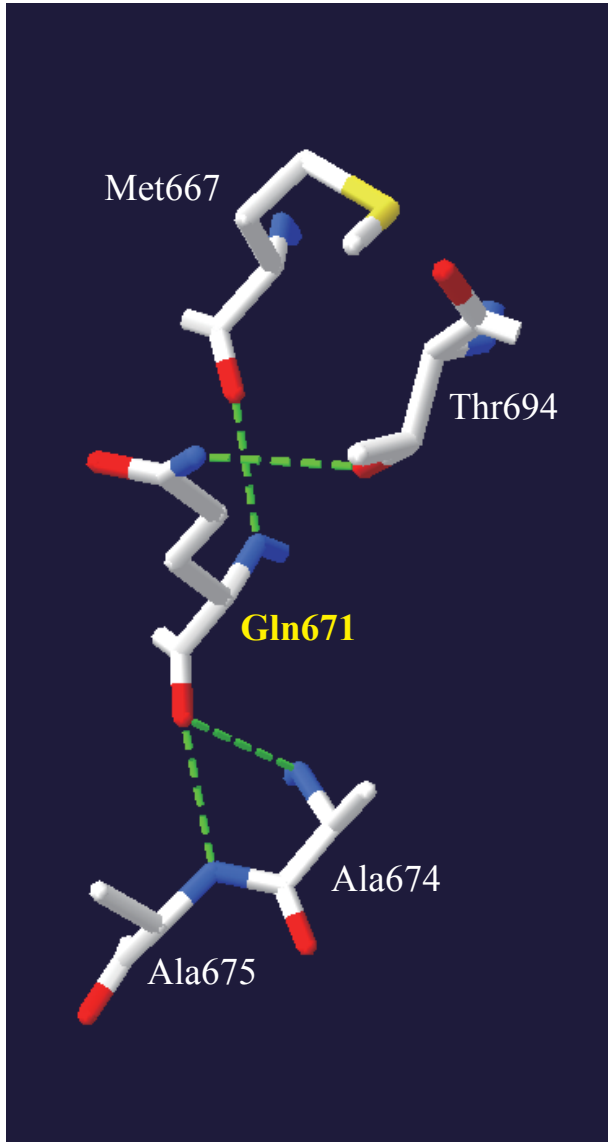
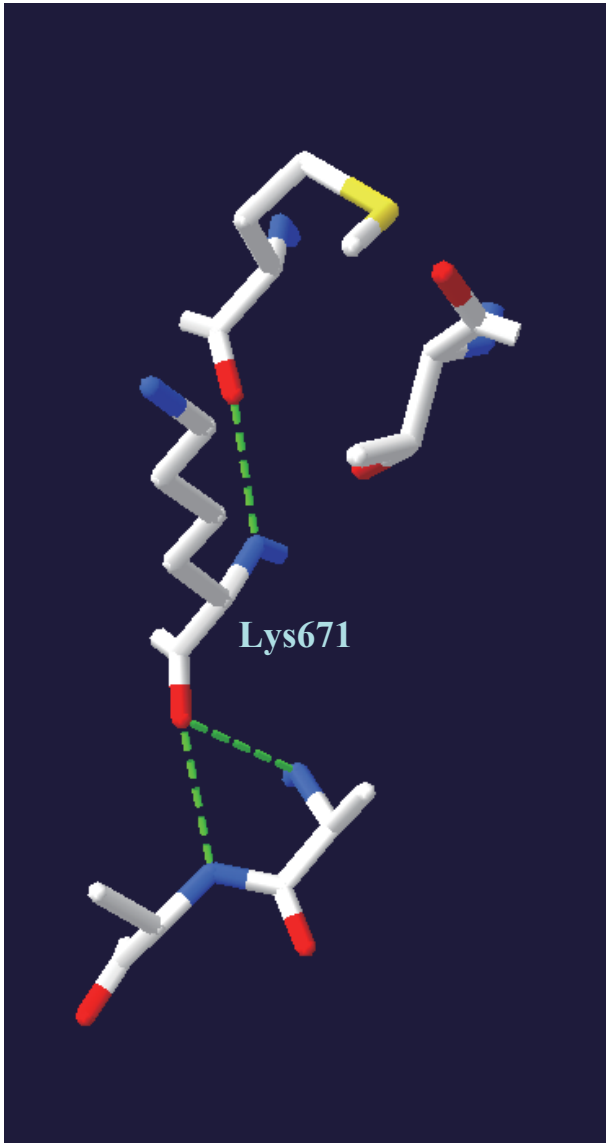

Lys652
Asp713
Leu724
Phe735
Thr694
Gln671
Gln683
Ala675
(a)
Asp713
Lys652
Leu724
Lys641
Phe735
Thr694
Leu693
Ala675
Gln671
Gln683
(b)

Fig. 6



Wild-type *Cm*IDH-II



Q671K

Fig. 7

TABLE S1. Oligonucleotides used in site-directed mutagenesis of *Cm*IDH-II.

	Primer name	Nucleotide sequence (5' to 3') [a]
Primer A	CF0	gcgcggatccgAGCACTGATAACTCAAAAATC
Primer B	K641E-R	CGTATCTAAGAACTCACCTGTCGC
	K652R-R	GTCTAGCTCACCCACTCTACGAGAAGGTG
	Q671K-R	GCAGCAAGGGCTTTCGCCCAATACATTGC
	A675Q-R	CTGTTGTTTGCTGAGCAAGGGCTTGCGC
	Q683K-R	CTAAAACTTGCTTTCAGTTCAGTATC
	L693F-R	CTTCTTGCTTAGTAAACGCTTGAGC
	T694A-R	CTTCTTGCTTAGCTAGCGCTTGAGCAAC
	D713N-R	CCATTAAGATTAATAGCAGGACC
	L724Q-R	GCATTGCTTTTTCTGCTTGTTTAGTATCG
	F735L-R	GATAAAATGGTATTTAATGTTTCGCTTGG
Primer C	K641E-F	GCGACAGGTGAGTTCTTAGATACG
	K652R-F	CACCTTCTCGTAGAGTGGGTGAGCTAGAC
	Q671K-F	GCAATGTATTGGGCGAAAGCCCTTGCTGC
	A675Q-F	GCGCAAGCCCTTGCTCAGCAAACAACAG
	Q683K-F	GATACTGAACTGAAAGCAAGTTTTAG
	L693F-F	GTTGCTCAAGCGTTTACTAAGCAAGAAG
	T694A-F	GTTGCTCAAGCGCTAGCTAAGCAAGAAG
	D713N-F	GCTCAAGGTCCTGCTATTAATCTTAATGG
	L724Q-F	CGATACTAAACAAGCAGAAAAAGCAATGC
	F735L-F	CCAAGCGAAACATTAAATACCATTTTATC
Primer D	CR0	gcgcgagctcTTAAAGTAATGCAGATAAAATGG
	pTrcHisB-R	GGCAAATTCTGTTTTATCAGACCGC

[a]Small letters indicate additional bases for introducing the digestion sites for *Bam*HI and *Sac*I (single and dotted underlines, respectively).

TABLE S2. Oligonucleotides used in site-directed mutagenesis of *Cp*IDH-M.

	Primer name	Nucleotide sequence (5' to 3') [a]
Primer E	CPMF0	gataaggatccgAGCACTGATAACTCAAA
Primer F	E641K-R	GCTATCTAAAAACTTACCGGTGGC
	R652K-R	GCTCACCCACTTTACGTGATGGTGAC
	K671Q-R	CCAATGCTTGTGCCCAATACATAGC
	Q675A-R	CACTCGTTTGCGCCGCCAATGCTTTTGC
	K683Q-R	GTAAAGCTGGCTTGAAGCTCTCCATC
	F693L-R	CTTCTTTCTTAGCTAGCGCTTGAGC
	A694T-R	CTTCTTTCTTAGTAAACGCTTGAGC
	N713D-R	GTAGCCGCCGATATCCATTGC
	Q724L-R	CGCTTTTTCTGCAAGGGCTGTATC
	L735F-R	CAAAATAGTATTAAACGTTTC
Primer G	E641K-F	GCCACCGGTAAGTTTTTAGATAGC
	R652K-F	GTCACCATCACGTAAAGTGGGTGAGC
	K671Q-F	GCTATGTATTGGGCACAAGCATTGG
	Q675A-F	GCAAAAGCATTGGCGGCGCAAACGAGTG
	K683Q-F	GATGGAGAGCTTCAAGCCAGCTTTAC
	F693L-F	GCTCAAGCGCTAGCTAAGAAAGAAG
	A694T-F	GCTCAAGCGTTTACTAAGAAAGAAG
	N713D-F	GCAATGGATATCGGCGGCTAC
	Q724L-F	GATACAGCCCTTGCAGAAAAAGCG
	L735F-F	CCAAGTGAAACGTTTAATACTATTTTG
Primer H	CPMR0	atctcgagctcTTATAACAATGCTAACAA
	pTrcHisB-R	GGCAAATTCTGTTTTATCAGACCGC

[a]Small letters indicate additional bases for introducing the digestion sites for *Bam*HI and *Sac*I (single and dotted underlines, respectively).

TABLE S3. Cycle conditions of PCR in site-directed mutagenesis of *Cm*IDH-II and *Cp*IDH-M.[a]

	Mutation	Annealing temperature (^{o}C)	Extension time
First PCR	K641E, E641K	55	2 min
	K652R, R652K	55	
	Q671K, K671Q	55	
	A675Q, Q675A	57	
	Q683K, K683Q	52	
	L693F, F693L	48	
	T694A, A694T	60	
	D713N, N713D	52	
	L724Q, Q724L	55	
	F735L, L735F	53	
Second PCR	K641E, E641K	55	25 s
	K652R, R652K	55	
	Q671K, K671Q	55	
	A675Q, Q675A	55	
	Q683K, K683Q	52	
	L693F, F693L	55	
	T694A, A694T	55	
	D713N, N713D	51	
	L724Q, Q724L	50	
	F735L, L735F	53	
Third PCR	All samples	56	2 min 25 s

[a]Mutations of *Cp*IDH-M are indicated by the underlined letters. Each PCR condition was denaturation at 94^{o}C for 15 s, annealing at the indicated temperatures for 30 s and extension at 68^{o}C for the indicated times. Amplification was carried out for 30 cycles.

TABLE 1. Kinetic parameters at 20°C.

		K_m (μM) [a]	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)[a]
*Cm*IDH-II	Wild-type	34.4	46.2	13.4×10^5
	L693F	42.1	28.3	6.7×10^5
	L724Q	91.7	24.6	2.7×10^5
	F735L	48.2	21.6	4.5×10^5
*Cp*IDH-M	Wild-type	30.4	2.1	0.7×10^5
	F693L	37.3	6.3	1.7×10^5
	Q724L	40.2	18.1	4.5×10^5
	L735F	28.1	9.5	3.4×10^5

[a] K_m values for isocitrate are indicated.