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Amino acid residues involved in cold adaptation of isocitrate lyase from a psychrophilic bacterium, *Colwellia maris*

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To investigate the mechanism of cold adaptation of isocitrate lyase (ICL; EC 4.1.3.1) from the psychrophilic bacterium *Colwellia maris*, Gln207 and Gln217 of this enzyme were substituted by His and Lys, respectively, by site-directed mutagenesis. His184 and Lys194 of ICL from *Escherichia coli*, corresponding to the two Gln residues of *C. maris* ICL, are highly conserved in the ICLs of many organisms and are known to be essential for catalytic function. The mutated ICLs (*Cm*-Q207H and *Cm*-Q217K, respectively) and wild-type enzymes of *C. maris* and *E. coli* (*Cm*-WT and *Ec*-WT) with His-tagged peptides were overexpressed in *E. coli* cells and purified to homogeneity. Thermolabile *Cm*-WT and mutated ICLs were susceptible to digestion with trypsin, while relatively thermostable *Ec*-WT was resistant to trypsin digestion, suggesting that the thermostability and resistance to tryptic digestion of the ICLs are related. *Cm*-Q207H and *Cm*-Q217K showed specific activities similar to *Cm*-WT at temperatures between 30 °C and 40 °C, but their activities between 10 °C and 25 °C were decreased, indicating that the two Gln residues of the *C. maris* ICL play important roles in its cold adaptation. Phylogenetic analysis of ICLs from various organisms revealed that the *C. maris* ICL can be categorized in a novel group, subfamily 3, together with several eubacterial ICLs.

INTRODUCTION

Enzymes capable of functioning efficiently at low temperature, termed cold-adapted enzymes, represent one of the strategies for environmental adaptation adopted by psychrophiles, bacteria adapted to cold habitats, because the catalytic rates of enzyme reactions are strongly dependent on temperature. The cold-adapted enzymes display higher specific activities at low temperature and pronounced thermolability compared to their mesophilic and thermophilic counterparts; these properties are probably accomplished by the enhanced conformational flexibility of the enzyme proteins, which is regarded as the main adaptive feature to low temperature (Gerday *et al.*, 1997; Fields & Somero, 1998). The enhanced flexibility allows the cold-adapted enzymes to bring about rapid conformational changes without energy loss for the catalysis. Although some features of the cold-adapted enzymes have been proposed on the basis of comparisons between the primary structures of the corresponding enzymes from various organisms with different ranges of growth temperature, it is difficult to distinguish exactly whether they are attributable to cold adaptation or to phylogenetic differences between the organisms (Sheridan *et al.*, 2000). On the other hand, analyses of the crystal structures of cold-adapted enzymes has revealed the diversity of their mechanisms for cold adaptation (Fields & Somero, 1998; Russell *et al.*, 1998; Kim *et al.*, 1999; Alvarez *et al.*, 1998; Bentahir *et al.*, 2000).

Directed modification of the enzyme proteins by random PCR mutagenesis is a useful approach to examine their adaptation mechanism. By comparing the amino acid sequences of mutated enzyme proteins that exhibit thermodynamic properties different from those of the wild-type enzyme, the cause of the alteration can be specified. Such studies for thermophilic (Suzuki *et al.*, 2001; Lönn *et al.*, 2002), mesophilic (Taguchi *et al.*, 1999; Wintrode *et al.*, 2000) and psychrophilic enzymes (Miyazaki *et al.*, 2000) have been reported. Even among mutated enzymes with similar characteristics to each other, various patterns of amino acid substitution have been observed (Suzuki *et al.*, 2001). Furthermore, the substituted amino acid residues in a cold-adapted mutant (P3C9) of mesophilic subtilisin SSII were not necessarily identical to the corresponding residues of the naturally cold-adapted subtilisin S41 (Wintrode *et al.*, 2000), supporting the view that nature has not tested all possibilities for the adaptation of proteins to low temperature (Gerday *et al.*, 1997).

Cold adaptation of enzymes in central metabolism should

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Abbreviations: CD, circular dichroism; ICL, isocitrate lyase.

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be essential for the survival and growth of psychrophiles at low temperatures. Isocitrate lyase (ICL; EC 4.1.3.1) catalyses the cleavage of isocitrate to glyoxylate and succinate, and plays important roles in the metabolism of acetate and fatty acids in micro-organisms and higher plants as a key enzyme of the glyoxylate cycle (Kornberg, 1966; Vanni *et al.*, 1990; Cozzone, 1998). We previously reported that the ICLs of two psychrophilic bacteria, *Colwellia maris* and *Colwellia psychrerythraea*, are homotetrameric, as are their counterparts in other organisms, including *Escherichia coli*, that they are typical cold-adapted enzymes, and that the expression of the genes encoding these enzymes is cold-inducible (Watanabe *et al.*, 2001, 2002a, 2002b). Furthermore, the recently resolved crystal structures of ICLs and their complexes with ligands from several organisms can provide us with useful information to understand their catalytic function (Britton *et al.*, 2000, 2001; Sharma *et al.*, 2000). We previously found that about 20 amino acid residues among those strictly conserved in ICLs from various organisms are substituted in the corresponding enzyme of *C. maris* (*Cm*ICL) (Watanabe *et al.*, 2002a). Furthermore, some of the substitutions were found in amino acid residues essential for the catalytic function of *E. coli* ICL (Ko & McFadden, 1990; Ko *et al.*, 1991, 1992; Diehl & McFadden, 1993, 1994; Rehman & McFadden, 1996, 1997a, b, c) or the adjacent residues. Similar substitutions of conserved amino acid residues have also been observed in other cold-adapted enzymes (Davail *et al.*, 1994). In this study, to examine whether such substituted residues are related to the cold adaptation of *Cm*ICL, they were exchanged for amino acid residues homologous to mesophilic counterparts by site-directed mutagenesis, and the properties of the mutated ICLs were investigated.

METHODS

Construction of expression vectors for *C. maris* and *E. coli* ICLs. The genes encoding the ICLs of *C. maris* and *E. coli* (*Cm*ICL and *Ec*ICL, respectively) were amplified by PCR so as to introduce restriction sites for *Bam*HI and *Sac*I at the 5′ and 3′ terminals of the ORFs, respectively. Therefore, the following primers were synthesized: for *Cm*ICL, sense primer 5′-CGATAAGGATCCGTCTAACTATCAAAGTGCAATAGAAGCAGTTAAAGC-3′ (48-mer) and antisense primer 5′-GATCTCGAGCTCTTAGCTAAACTGATTAGACGTATTGCGCCGCC-3′ (48-mer); for *Ec*ICL, sense primer 5′-ATAAGGATCCGAAAACCCGTACACAACAAATTGAAGAATTACAGAAAG-3′ (48-mer) and antisense primer 5′-TCTCGAGCTCTTAGAACTGCGATTCTTCAGTGGAGCCGGTCAGCGCGG-3′ (48-mer). Cleavage sites for restriction enzymes are underlined. The amplification was carried out for 30 cycles in a DNA thermal cycler 2400 (Perkin-Elmer) in a 50 µl reaction mixture containing 100 ng plasmid pCM477 (Watanabe *et al.*, 2002a) or pICL1 (Matsuoka & McFadden, 1988), in turn containing the *C. maris icl* and *E. coli aceA* genes, respectively, as template DNA. The reaction mixture also contained 1·5 pmol of each sense and antisense primer, and 1 U of KOD-plus DNA polymerase (Toyobo) in a buffer system provided by the manufacturer. Cycling conditions were as follows: denaturation at 94 °C for 2 min, annealing at 50 °C for 0·5 min and extension at 68 °C for 2·5 min, for 30 cycles. Each amplified PCR fragment was ligated to the *Bam*HI–*Sac*I site of pTrcHisB (Invitrogen), which is a plasmid vector for conferring an N-terminal $(His)_6$-tag on the expressed proteins, to obtain plasmids pHis-*CmWT* and pHis-*EcWT*, respectively. The plasmids were transformed into competent cells of *E. coli* strain TOP10 by a conventional heat-shock method. Then, the amplified plasmids of pHis-*CmWT* and pHis-*EcWT* were purified by the Wizard plus SV minipreps DNA purification system (Promega). To check precise insertion of the *Cm*ICL and *Ec*ICL genes into pTrcHisB, the insert DNAs in the resulting plasmids were sequenced in both directions by using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) with an ABI 373A DNA sequencer (Applied Biosystems). The sequence was analysed with the Genetyx computer program (Software Development Co.).

Site-directed mutagenesis. Site-directed mutagenesis was carried out by a standard PCR method. The following primers were used to introduce the mutations into *Cm*ICL: sense primer^{207} and antisense primer^{207} for the substitution of His for Gln207 were 5′-GGTGCTTGTGCTCTTCATATTGAAAACC-3′ (28-mer) and 5′-GGTTTTCAATATGAAGAGCACAAGCACC-3′ (28-mer), respectively; sense primer^{217} and antisense primer^{217} for the substitution of Lys for Gln217 were 5′-GCTGATGAAAAAAAATGTGGACATCAAGACG-3′ (31-mer) and 5′-CGTCTTGATGTCCACATTTTTTTTCATCAGC-3′ (31-mer), respectively. The mutated regions are underlined. Except for the use of 100 pmol of each primer, the PCR was performed as described above. Both the PCR products were ligated into pTrcHisB to obtain plasmids pHis-*Cm207* (the Q207H mutation) and pHis-*Cm217* (the Q217K mutation). A plasmid carrying both of the mutations (pHis-*Cm207/217*) was obtained by PCR with the plasmid pHis-*Cm207* and a set of sense and antisense primers^{217}. The mutated plasmids were transformed into *E. coli* TOP10 and purified. To verify the introduced mutations, the relevant regions of the plasmids were sequenced in both directions, as described above.

Overexpression and purification of His-tagged ICLs. The *E. coli* TOP10 cells carrying one of the expression vectors pHis-*CmWT*, pHis-*Cm207*, pHis-*Cm217*, pHis-*Cm207/217* or pHis-*EcWT* were grown at 37 °C in Super broth medium (12 g tryptone, 24 g yeast extract, 5 ml glycerol, 3·81 g KH_2PO_4 and 12·5 g K_2HPO_4 per litre, pH 7·0) containing 50 mg ampicillin l^{-1} until OD_{600} of the culture reached 0·6. The cultures were rapidly cooled on ice and were further incubated for 18–24 h at 15 °C after the addition of 0·1 M IPTG. Cells were then collected and resuspended in Buffer A (50 mM sodium phosphate, pH 6·85, containing 2 mM $MgCl_2$, 0·5 M NaCl, 10 mM 2-mercaptoethanol and 10 mM imidazole), employing 20 ml of buffer per litre of the original culture medium. Hen-egg lysozyme (2 mg ml^{-1}) was added to the cell suspension, and the mixture was gently shaken overnight at 4 °C. The cells were then disrupted by ultrasonic oscillation. After the centrifugation of the cell lysate at 39 120 ***g*** for 30 min at 4 °C to remove cell debris, the supernatant was centrifuged at 65 600 ***g*** for 6 h at 4 °C. The resultant supernatant was loaded onto a Ni-NTA column (25 ml; Qiagen) equilibrated with Buffer A. After thorough washing with the same buffer, the column was further washed with 50 ml Buffer B (Buffer A containing 10 %, v/v, glycerol and 20 mM imidazole instead of 10 mM imidazole) and next with 50 ml Buffer C (Buffer B containing 50 mM imidazole instead of 20 mM imidazole). In each case the enzyme was then eluted with 50 ml of Buffer D (Buffer B containing 250 mM imidazole instead of 20 mM imidazole), and the elutant was concentrated with polyethylene glycol #20 000 and dialysed against Buffer E (20 mM potassium phosphate, pH 6·85, containing 2 mM $MgCl_2$, 0·5 M NaCl, 1 mM DTT and 0·01 % NaN_3). All His-tagged recombinant ICLs were stored at 4 °C and used in further experiments within a week.

PAGE. SDS-PAGE was carried out by the method of Laemmli (1970) with a 10 % gel at 25 mV.

Western blot analysis. After SDS-PAGE of the purified ICLs and the sonicated extracts of the *E. coli* transformant cells, the proteins on the gels were transferred onto a nitrocellulose membrane (Hybond-C; Amersham Pharmacia Biotech). Western blot analysis was carried out with the ECL Western blotting detection system (Amersham Pharmacia Biotech) and either rabbit antibody against the *C. maris* ICL (Watanabe *et al*., 2002a) or mouse antibody against the additional $(His)_6$–Gly sequence at the N-terminal of the overexpressed ICLs (Invitrogen). Native ICL of *C. maris* was purified as described previously (Watanabe *et al*., 2001).

Enzyme assay. Unless otherwise stated, the ICL activity was assayed at pH 6·85 as described previously (Watanabe *et al*., 2001). Optimum pH for activity was determined with the following buffers: potassium phosphate at pH 6·0–7·6 and Tris/HCl at pH 7·5–8·0. Protein was measured by the method of Lowry *et al*. (1951) with BSA as a standard.

Measurement of kinetic and thermodynamic parameters. The kinetic parameters, K_m and V_{max}, of ICL were calculated by Lineweaver–Burk analysis. Molecular masses of the wild-type and the mutants of *Cm*ICL (*Cm*-Q207H and *Cm*-Q217K) and *Ec*ICL with the additional N-terminal His-tags were calculated as 246 641, 246 677, 246 641 and 204 124 Da, respectively, from the gene sequences. The activation energies (E_a) of the enzymes were calculated by Arrhenius plotting. The thermodynamic activation parameters were calculated according to the following equations.

$$\Delta G\# = RT \times (23{\cdot}76 + \ln T - \ln k_{cat})$$

$$\Delta H\# = E_a - RT$$

$$\Delta S\# = (\Delta H\# - \Delta G\#)/T$$

Digestion with trypsin. The purified recombinant ICLs were dialysed overnight at 4 °C against 0·1 M $NaHCO_3$ (pH 8·1). The ICL enzymes (100 μg) were then digested at 4 °C with 0·1 μg trypsin in the same buffer. At appropriate times after the addition of trypsin into the reaction mixture, the mixture was withdrawn and immediately analysed by SDS-PAGE.

Circular dichroism (CD) spectra of recombinant ICLs. All purified recombinant ICLs were dialysed overnight at 4 °C against 20 mM potassium phosphate buffer (pH 6·85) containing 2 mM $MgCl_2$ and 1 mM DTT. UV-CD of the ICLs was measured at 20 °C with a J-725 spectropolarimeter (Jasco) by using a cuvette with a 0·1 cm path length. The protein concentrations were determined by the absorbance at 280 nm from the extinction coefficients 53056·5 and 71946·0 M^{-1} cm^{-1} for three *Cm*ICLs (wild-type and two mutants) and *Ec*ICL, respectively, which were calculated as reported previously (Wetlaufer, 1962). The contents of the secondary structures in the ICLs were estimated with program K2D (Andrade *et al*., 1993).

Phylogenetic analysis. Phylogenetic analysis was done with a CLUSTAL-W program with the unrooted neighbour-joining method. The phylogenetic tree was produced with the program TreeView 1.6.1 distributed by the Bioinformatics Center of Kyoto University, Japan (www.genome.ad.jp/Japanese/).

Molecular modelling of *Cm*ICL. The three-dimensional structural model of *Cm*ICL was built on the basis of its homology to *Ec*ICL (PDB No. 1IGW) with the program SWISSPDB VIEWER.

RESULTS

Site-directed mutagenesis of *Cm*ICL

*Ec*ICL has been studied more extensively than its counterparts in other organisms with regard to the catalytic mechanism and crystal structure (Britton *et al*., 2001; Ko & McFadden, 1990; Ko *et al*., 1991, 1992; Diehl & McFadden, 1993, 1994; Rehman & McFadden, 1996, 1997a, b, c). We previously reported that, in the cold-adapted *Cm*ICL, several amino acid residues strictly conserved in the ICLs of many organisms, including *E. coli*, are substituted, and some unique insertions of 3–36 amino acid residues are present (Watanabe *et al*., 2002a). Among these substitutions, we noted Gln207 and Gln217 of *Cm*ICL (stars in Fig. 1a) for the following two reasons: (1) from studies on site-directed mutagenesis, His184 and Lys194 of *Ec*ICL, corresponding to the two Gln residues of *Cm*ICL, were reported to be essential for catalytic function of the enzyme (Diehl & McFadden, 1994; Rehman & McFadden, 1997b), and (2), in spite of low homology between the amino acid sequences of *Cm*ICL and *Ec*ICL (less than 30 %), this region containing the two Gln residues is more highly conserved than other regions of the enzyme proteins, probably due to the existence of amino acid residues constituting the active site (asterisks in Fig. 1a). Thus, the Q207H and Q217K mutations were introduced into the *Cm*ICL by site-directed mutagenesis.

Expression of recombinant ICLs in *E. coli*

Wild-types of *Cm*ICL and *Ec*ICL (*Cm*-WT and *Ec*-WT) and three *Cm*ICL mutants (*Cm*-Q207H, *Cm*-Q217K and *Cm*-Q207H/Q217K) with the N-terminal His-tags were expressed at 15 °C in *E. coli* cells because of the marked thermolability of *Cm*ICL (Watanabe *et al*., 2001). In spite of the induction by IPTG, all recombinants of *Cm*ICL were expressed at lower levels than *Ec*-WT (Fig. 2a). The yields of the purified *Cm*-WT, *Cm*-Q207H, *Cm*-Q217K and *Ec*-WT were estimated to be 3·60, 0·55, 3·64 and 17·0 mg protein per litre of culture, respectively (mean of three independent experiments). All recombinant ICLs, except for *Cm*-Q207H/Q217K, were purified to homogeneity by single chromatography on a Ni^{2+}-chelating affinity column (Fig. 2b). The purification of the *Cm*-Q207H/Q217K double mutant was unsuccessful since its expression in the *E. coli* cells was too poor (Fig. 2a). Mobilities on the gel of both wild-type and mutated *Cm*ICL recombinants were slightly slower than that of the native *Cm*ICL purified from *C. maris* cells (estimated as 63 and 65 kDa, respectively) due to the addition of 33 amino acid residues including His-tags at their N-terminals (Fig. 2b). *Cm*-WT, *Cm*-Q207H and *Cm*-Q217K cross-reacted with anti-*Cm*ICL antibody, whereas *Ec*-WT did not at all (Fig. 2c). These were consistent with the previous results for the native *Cm*ICL and *Ec*ICL (Watanabe *et al*., 2002a). Although *Ec*-WT as well as *Cm*-WT had the His-tag attached at the N-terminal, the molecular mass of the *Ec*-WT subunit (47 kDa) was much smaller than that of *Cm*-WT (Fig. 2b) because *Cm*ICL has several insertions of amino acid residues (Watanabe *et al*., 2002a). On the other hand, all recombinant ICLs cross-reacted at similar intensities with the anti-His-tag antibody (Fig. 2d).

Temperature dependence of ICL activity

The activities of recombinant *Cm*ICLs and *Ec*-WT at various temperatures are shown in Fig. 3(a). The optimum

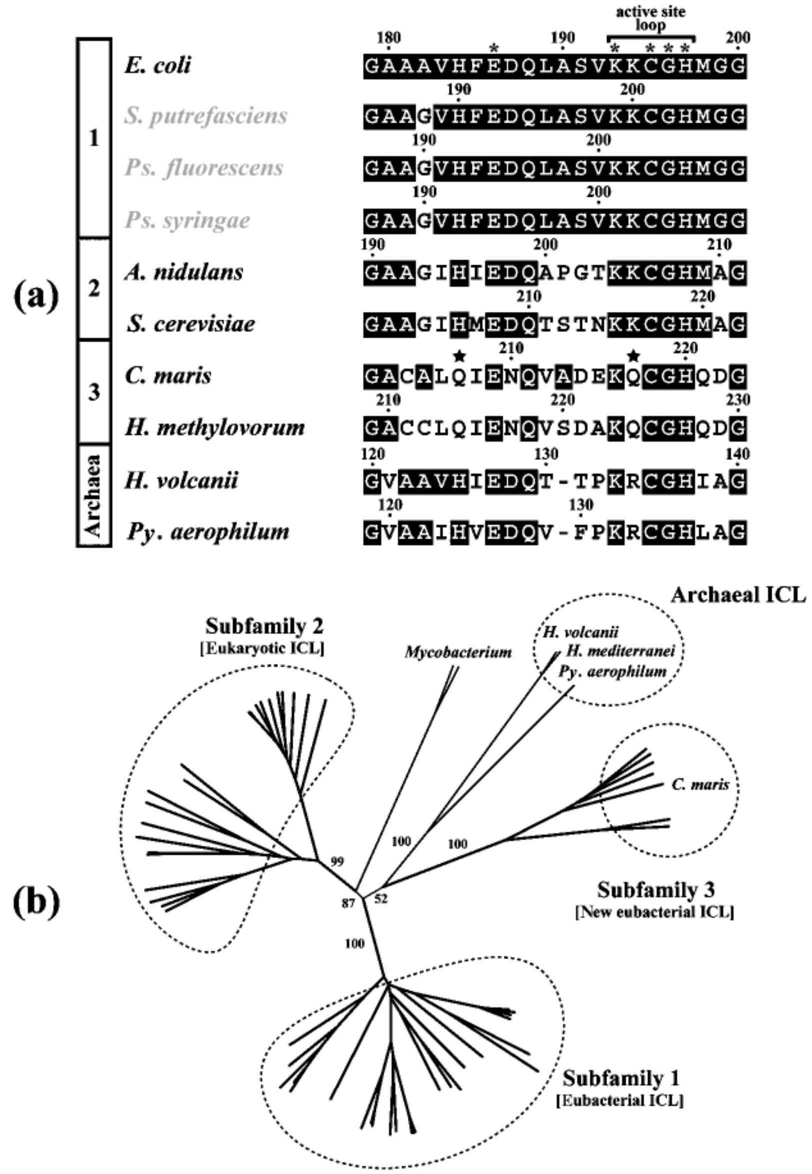

Fig. 1. Alignment of partial amino acid sequences of ICLs (a) and phylogenetic tree of ICLs (b). (a) Numbers in boxes indicate each subfamily in phylogenetic tree. Black stars indicate positions of amino acid residues of *Cm*ICL substituted by site-directed mutagenesis in this study. Amino acid residues identical to those of *Ec*ICL are shown as white letters in black boxes. Asterisks indicate amino acid residues constituting the active site. Organism names in grey type represent psychrophilic and psychrotrophic bacteria in which putative ICL genes are found in the genome sequence. (b) Number on each branch indicates bootstrap value. Accession numbers for the protein database of ICLs used for the analysis are as follows. Prokaryotic ICLs: NP_251324 (*Ps. aeruginosa*); BAB62107 (*C. maris*); BAA23678 (*H. methylovorum*); NP_420572 (*C. crescentus*); AAF02533, NP_230385, AAC43109 (*E. coli*); AAL23008, NP_407175, P41554, AAK97785, P42449, NP_214981, CAC44332, CAD13354, NP_519479, NP_294552, NP_243543, NP_384818, NP_531308, NP_107017, NP_539326, NP_503306, AAF00535, AAB97828, T08046, NP_342781, AAK46238, CAC30940. Eukaryotic ICLs: S26857 (*Aspergillus nidulans*); AAK72548, AAK54240, S26858, BAA00611, AAF34690, CAC34630, P41555, NP_010987 (*Saccharomyces cerevisiae*); O94198, CAA67367, AAL56614, P25248, NP_188809, CAA84632, P93110, CAA36381, P15479, P49297, AAG44479, P45457, AAF04598, AAC49686, AAC64373. Archaeal ICLs: NP_559494 (*Pyrobaculum aerophilum*); CAD23069, CAC48388 (*Haloferax volcanii*). Amino acid sequences of putative ICLs from *Ra. metallidurans*, *N. aromaticivorans*, *Rh. palustris*, *Pseudomonas fluorescens*, *Pseudomonas syringae* and *Shewanella putrefasciens* were obtained through the BLAST search of NCBI (www.ncbi.nlm.nih.gov).

pH values for the recombinant *Cm*ICLs and *Ec*-WT were 6·85, almost the same as that of the native *Cm*ICL (pH 6·8; Watanabe *et al.*, 2001). Specific activities of the ICLs at optimum temperature are summarized in Table 1. The optimum temperature for activity (20–25 °C) and specific activity at the optimum temperature ($14{\cdot}3 \pm 0{\cdot}3$ s^{-1}; two independent experiments) of *Cm*-WT were similar to those of the native *Cm*ICL (20 °C and 18·1 s^{-1}) (Watanabe *et al.*, 2001). In addition, rapid reduction of the activity above 30 °C was found in *Cm*-WT as well as the native *Cm*ICL. These results suggest that the additional His-tag at the N-terminal did not affect the activity and thermal characteristics of the enzyme. *Cm*-Q207H and *Cm*-Q217K possessed specific activities comparable to that of *Cm*-WT, but their optimum temperatures increased by about 5 °C and 7·5 °C, respectively, from that of *Cm*-WT (Fig. 3a and Table 1). Specific activity of the mesophilic *Ec*-WT increased markedly with the elevation of assay temperature at least up to 40 °C and, above 30 °C, was much higher than the maximum activity of *Cm*-WT. On the other hand, the specific activity of *Cm*-WT between 10 °C and 20 °C was 1·3–1·6-fold higher than that of *Ec*-WT. Interestingly, the activity of both *Cm*-Q207H and *Cm*-Q217K between 10 °C and 25 °C was clearly lower than that of *Cm*-WT. Relative

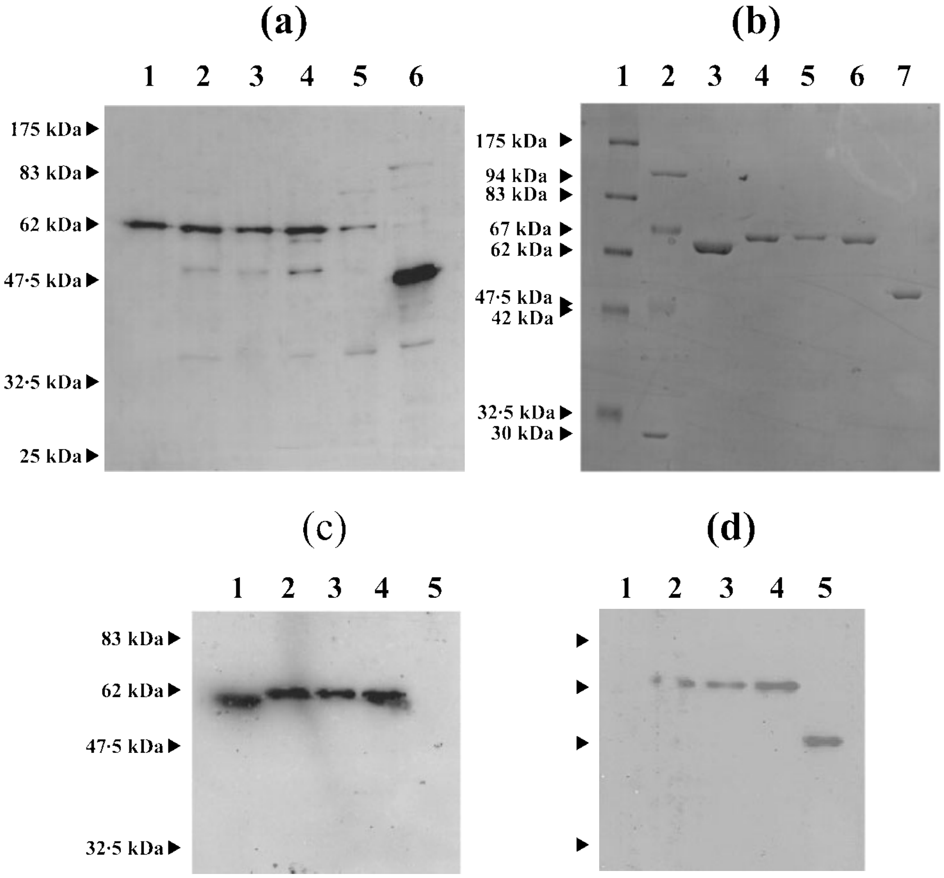

Fig. 2. SDS-PAGE and immunoblot analysis. (a) Expression levels of recombinant *Cm*ICLs and *Ec*ICL in *E. coli* cells. Lane 1, purified *Cm*-WT (0·1 μg protein); lanes 2–6, sonication extracts of *E. coli* TOP10 cells transformed with pHis-*CmWT* (lane 2), pHis-*Cm207* (lane 3), pHis-*Cm217* (lane 4), pHis-*Cm207/217* (lane 5) and pHis-*EcWT* (lane 6) (10 μg protein each). For immunoblot analysis, anti-His-tag antibody was used. The antibody (20 mg ml^{-1}) was diluted to 1/2500. (b) SDS-PAGE of purified *Cm*ICLs and *Ec*ICL. Lanes 1 and 2, molecular marker proteins; lane 3, native *Cm*ICL; lane 4, *Cm*-WT; lane 5, *Cm*-Q207H; lane 6, *Cm*-Q217K; lane 7, *Ec*-WT. In lanes 3–7, 10 μg of protein was applied. After electrophoresis, proteins on the gel were stained by Coomassie Brilliant Blue. (c) and (d) Immunoblot analyses of purified *Cm*ICLs and *Ec*ICL. Lane 1, native *Cm*ICL; lane 2, *Cm*-WT; lane 3, *Cm*-Q207H; lane 4, *Cm*-Q217K; lane 5, *Ec*-WT. In each lane, 0·1 μg of protein was subjected to electrophoresis. Anti-*Cm*ICL antibody (c) and anti-His-tag antibody (d) were used for immunoblotting.

activities of *Cm*-Q207H and *Cm*-Q217K at 10 °C were $8{\cdot}1\pm1{\cdot}3$ and $9{\cdot}1\pm0{\cdot}4$ % (two independent experiments), respectively, and these values were almost the same as that of *Ec*-WT ($8{\cdot}2\pm1{\cdot}1$ %; two independent experiments) (Fig. 3b). This result suggests the possibility that Gln207 and Gln217 of *Cm*ICL are responsible for cold adaptation.

Thermostability of the recombinant *Cm*ICLs and *Ec*ICL

To estimate whether the mutations of *Cm*ICL affect the thermostability of its activity, residual activities were assayed after heat treatment for various times at 30 °C for *Cm*ICLs and 40 °C for *Ec*-WT (Fig. 3c and Table 1). Similarly to the native *Cm*ICL, irreversible inactivation of recombinant *Cm*ICLs proceeded during incubation at this temperature. The half time of inactivation of *Cm*-Q217K was greater than that of *Cm*-WT. On the other hand, the half time of *Cm*-Q207H was shorter than that of *Cm*-WT, in spite of its higher optimum temperature for activity. Although *Ec*-WT was incubated at 40 °C, the half time of inactivation of this enzyme was longer than that of all the *Cm*ICLs, and it was hardly inactivated even after incubation for 1 h at 30 °C (data not shown).

Kinetic and thermodynamic parameters of the ICLs

The effects of temperature on the kinetic parameters of recombinant *Cm*ICLs and *Ec*-WT were examined (Fig. 4). K_m values for isocitrate ($K_m^{\text{isocitrate}}$) of all ICL enzymes tested were increased by elevating temperature. Whereas the $K_m^{\text{isocitrate}}$ values of *Cm*-Q207H were 2·2–3·5-fold larger than those of *Cm*-WT, the values of *Cm*-Q217K were

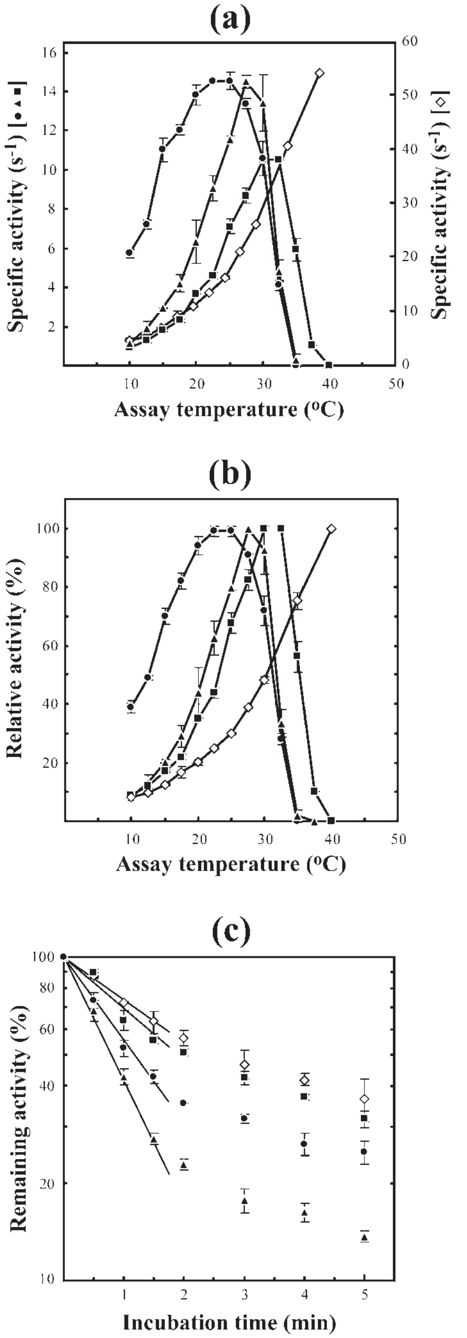

Fig. 3. Effect of temperature on specific activity (a) and relative activity (b) of ICL, and thermal inactivation of *Cm*ICLs and *Ec*-WT (c). Symbols: *Cm*-WT (●), *Cm*-Q207H (▲), *Cm*-Q217K (■) and *Ec*-WT (◇). Error bars indicate values from two separate experiments. In (a) and (b), the specific activities of *Cm*-WT, *Cm*-Q217K and *Ec*-WT were measured by a standard assay method containing 1·7 mM isocitrate. Since *Cm*-Q207H showed a high $K_m^{isocitrate}$ value (see Fig. 4a), 17 mM isocitrate was used for the assay. Relative activities were expressed as percentages of the maximum activities. (c) The purified enzymes were dialysed at 4 °C against 20 mM potassium phosphate (pH 6·85) containing 2 mM $MgCl_2$ and 1 mM DTT. After incubation for the indicated time periods, at 30 °C for *Cm*ICLs or 40 °C for *Ec*-WT, the dialysed enzymes were withdrawn and immediately cooled for 10 min on ice. The ICL activity was then measured at each optimum temperature as described in Methods.

0·6–0·8 times those of *Cm*-WT, and were almost the same as those of *Ec*-WT. On the other hand, catalytic efficiencies ($k_{cat}/K_m^{isocitrate}$) of these ICLs were maximal near the optimum temperature for activity of each enzyme (Fig. 4b). The catalytic efficiency of *Cm*-WT at 10 °C was 1·2-fold that of *Ec*-WT and was 41·3 % of its maximum value at 20 °C, while the relative efficiency values of *Cm*-Q207H, *Cm*-Q217K and *Ec*-WT at the same temperature were only 13·5, 23·3 and 15 %, respectively. These results indicate that the catalytic function of both *Cm*-Q207H and *Cm*-Q217K was impaired at low temperature.

Sensitivities of recombinant ICLs to trypsin

Partial digestion with proteases has been utilized to detect structural changes in proteins and identify their exposed and flexible regions (Aghajanian *et al.*, 2003). Therefore, the susceptibility of the recombinant *Cm*ICLs and *Ec*-WT to tryptic digestion was examined (Fig. 5). All *Cm*ICL recombinants were more sensitive to trypsin than *Ec*-WT. These results indicate that thermolabile *Cm*ICLs are more susceptible to tryptic digestion than *Ec*-WT.

CD spectrometric analysis of the ICLs

CD spectra of the ICLs were measured at 20 °C both to estimate the effect of these mutations on the secondary structure of the enzyme proteins and to compare the secondary structures of recombinant *Cm*ICLs and *Ec*-WT with each other (Fig. 6). The contents of α-helix and β-sheet calculated from the CD spectra were 42 and 17 % in *Cm*-WT and 61 and 7 % in *Ec*-WT, respectively, and the latter enzyme contained more secondary structure than the former. Little difference in the CD spectra between *Cm*-WT and *Cm*-Q207H was observed, while *Cm*-Q217K resembled *Ec*-WT rather than *Cm*-WT. For all ICLs, the CD spectra measured at 10 °C were almost the same as those at 20 °C (data not shown).

Phylogenetic relationship of ICLs from various organisms

Serrano & Bonete (2001) have reported that ICLs from various organisms can be classified into two groups: eubacterial and eukaryotic (termed subfamilies 1 and 2, respectively, in Fig. 1b), although some archaeal ICLs belong to neither subfamily. On the other hand, phylogenetic analysis with the CLUSTAL-W program of ICL amino acid sequences from 29 species of eubacteria, 24 eukaryotes, 3 Archaea and several putative eubacterial ICLs revealed that *Cm*ICL is different from these three groups and, together with the ICL of *Hyphomicrobium methylovorum* and five putative eubacterial ICLs from *Pseudomonas*

Table 1. Properties of *Cm*-WT, *Cm*-Q207H, *Cm*-Q217K and *Ec*-WT

Property	Protein							
	***Cm*-WT**		***Cm*-Q207H**		***Cm*-Q217K**		***Ec*-WT**	
Optimum temperature (°C)	20–25		27·5–30		30–32·5		Above 40	
Specific activity (s^{-1})*	14·6		14·5		10·5		54·0	
Half time of thermal inactivation (s)†	88·2		58·4		120·3		161·8	
Assay temperature	10 °C	20 °C‡	10 °C	30 °C‡	10 °C	30 °C‡	10 °C	40 °C‡
$K_m^{isocitrate}$ (mM)	0·33	0·47	0·76	2·07	0·26	0·50	0·25	0·65
$V_{max}^{isocitrate}$ (s^{-1})	5·76	13·9	0·62	14·3	1·51	12·5	3·70	54·0
k_{cat}/K_m (s^{-1} M^{-1})	1·77	2·65	0·08	0·54	0·58	2·50	1·50	8·31
E_a (kJ mol^{-1})§	46·8		107·8		89·2		63·5	
$\Delta G\#$ (kJ mol^{-1})‖	64·5		68·8		69·3		65·7	
$\Delta H\#$ (kJ mol^{-1})‖	44·4		105·5		86·9		61·1	
$T\Delta S\#$ (kJ mol^{-1})‖	−20·1		36·7		17·6		−4·52	

*Activities were assayed at each optimum temperature as described in Methods, except that activity of *Cm*-Q207H was assayed by using 17 mM isocitrate.

†The three *Cm*ICLs and *Ec*-WT were incubated at 30 and 40 °C, respectively.

‡Optimum temperature for activity.

§E_a values were calculated by Arrhenius plotting.

‖Values at 10 °C.

aeruginosa, *Ralstonia metallidurans*, *Novosphingobium aromaticivorans*, *Rhodopseudonomas palustris* and *Caulobacter crescentus*, can be categorized to a novel group, termed subfamily 3 in Fig. 1(b). Partial sequences of typical ICLs of these subfamilies are shown in Fig. 1(a). Gln207 of *Cm*ICL was conserved in subfamily 3 ICLs, but the corresponding residue in all other ICL subfamilies was His. Furthermore, Gln217 of *Cm*ICL was substituted by different amino acid residues in ICLs other than subfamily 3: Arg in Archaea and Lys in other ICLs, respectively. Although no ICL gene has been cloned from psychrophilic and psychrotrophic bacteria except *Cm*ICL, putative ICL genes belonging to subfamily 1 could be found in the genome sequences of several psychrophilic and psychrotrophic bacteria (organisms in grey type in Fig. 1a).

DISCUSSION

The wild-type and mutants of *Cm*ICL together with *Ec*ICL were expressed as recombinant forms in *E. coli* cells, but the expression levels of the *Cm*ICLs were much less than that of *Ec*ICL (Fig. 2a). A similar result has been found when elongation factor 2 of an Antarctic methanogenic archaeon, *Methanococcoides burtonii*, is expressed in *E. coli* cells, and is attributed to the existence of many Arg codons (AGA and AGG) which are used rarely in *E. coli* in the *aef2* gene

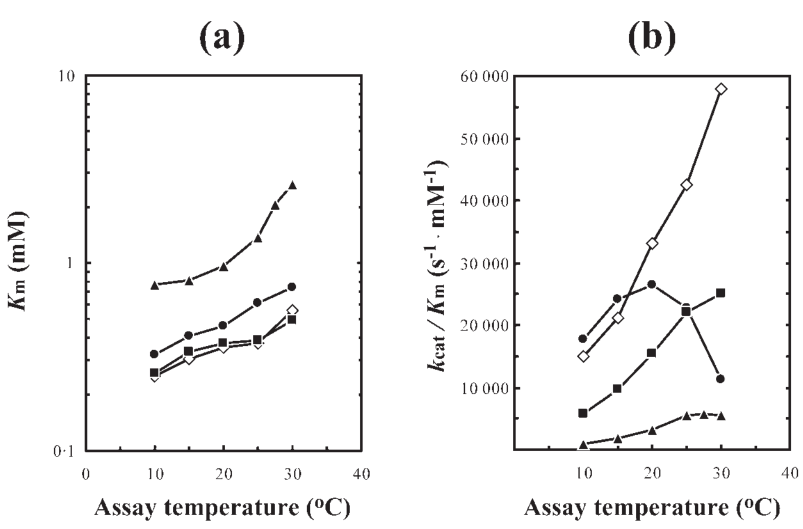

Fig. 4. Effect of temperature on $K_m^{isocitrate}$ (a) and $k_{cat}/K_m^{isocitrate}$ (b). Symbols are the same as Fig. 3.

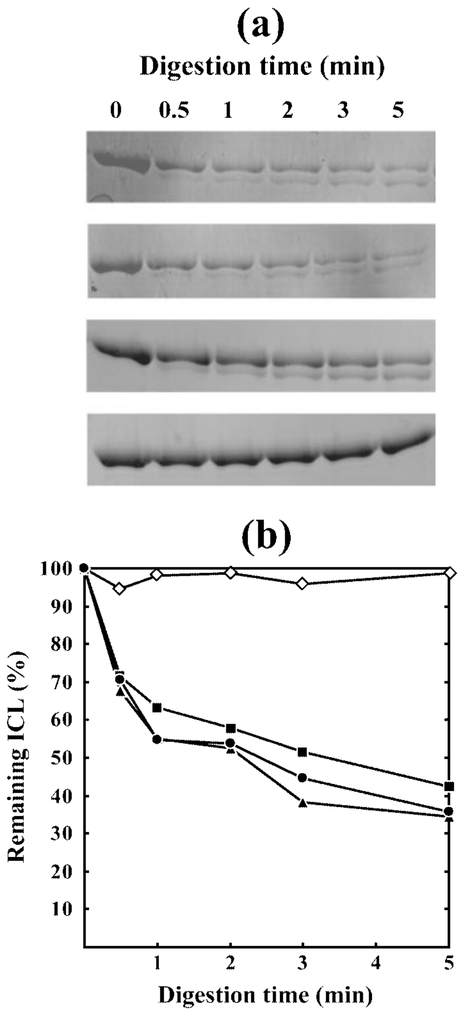

Fig. 5. Digestion of recombinant ICLs with trypsin. (a) SDS-PAGE: all recombinant ICLs (100 μg) were digested at 4 °C with 0·1 μg trypsin. At indicated times, 10 μg enzyme was withdrawn and subjected immediately to electrophoresis. After electrophoresis, proteins on the gel were stained by Coomassie Brilliant Blue. (b) Densitometric analysis of the amounts of ICL proteins after digestion. Symbols are the same as Fig. 3. Values are expressed as a percentage of the amount of ICL protein at $t=0$.

encoding this protein (Thomas & Cavicchioli, 2000). However, this is not the case for the *C. maris icl* gene. Since the recombinants of *Cm*ICL were more sensitive to tryptic digestion than *Ec*ICL (Fig. 5), the poor expression of the *Cm*ICLs might be ascribed to their post-translational proteolysis in *E. coli* cells. On the other hand, *Cm*-WT showed an obviously lower optimum temperature for activity and thermostability than the mesophilic *Ec*-WT (Fig. 3), indicating that the sensitivities of *Cm*-WT, *Cm*-Q207H and *Cm*-Q217K to trypsin are related to their thermolability. Therefore, the more flexible structures of

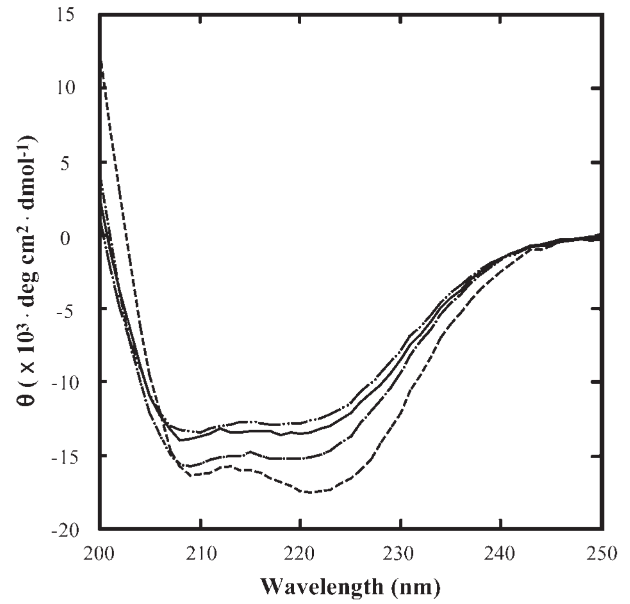

Fig. 6. UV-CD spectra of *Cm*-WT (solid line), *Cm*-Q207H (—··—), *Cm*-Q217K (—·—) and *Ec*-WT (– –). Based on the tetrameric configurations, the protein concentrations of *Cm*-WT, *Cm*-Q207H, *Cm*-Q217K and *Ec*-WT in assay mixtures were calculated as 17·2, 20·1, 20·1 and 16·1 μM, respectively. The ellipticity of each sample was measured by scanning five times between 200 and 250 nm at 1 nm increments.

cold-adapted enzymes, compared to those of their mesophilic counterparts, which are known to be responsible for their thermolability (Gerday *et al.*, 1997; Fields & Somero, 1998), may bring about their marked sensitivity to trypsin. It has been proposed that the low stability of cold-adapted enzyme proteins could contribute to the regulation of metabolic flux at low temperature by the maintenance of an adequate level of proteolytic degradation, in spite of the diminished rate of intracellular proteolysis under such conditions (Wintrode *et al.*, 2000; Somero, 1995; Feller & Gerday, 1997). The susceptibility of *Cm*ICL to trypsin supports this possibility.

Two amino acid residues, His184 and Lys194, of *Ec*ICL are known to be conserved in many prokaryotic and eukaryotic ICLs, but both of the corresponding residues in *Cm*ICL are Gln (Fig. 1a). Lys194 of *Ec*ICL should be essential for the catalytic function, because an 'active-site loop' including this residue moves flexibly when the enzyme binds to the substrate and releases the reaction products (Britton *et al.*, 2000, 2001; Sharma *et al.*, 2000; cf. Fig. 7). Between 30 °C and 40 °C, *Cm*-Q217K has a specific activity similar to that of *Cm*-WT (Fig. 3a, b) and the $K_m^{\text{isocitrate}}$ value of the former was lower than that of the latter (Table 1 and Fig. 4a). These results suggest that, in this temperature range, this mutation strengthens the affinity for isocitrate

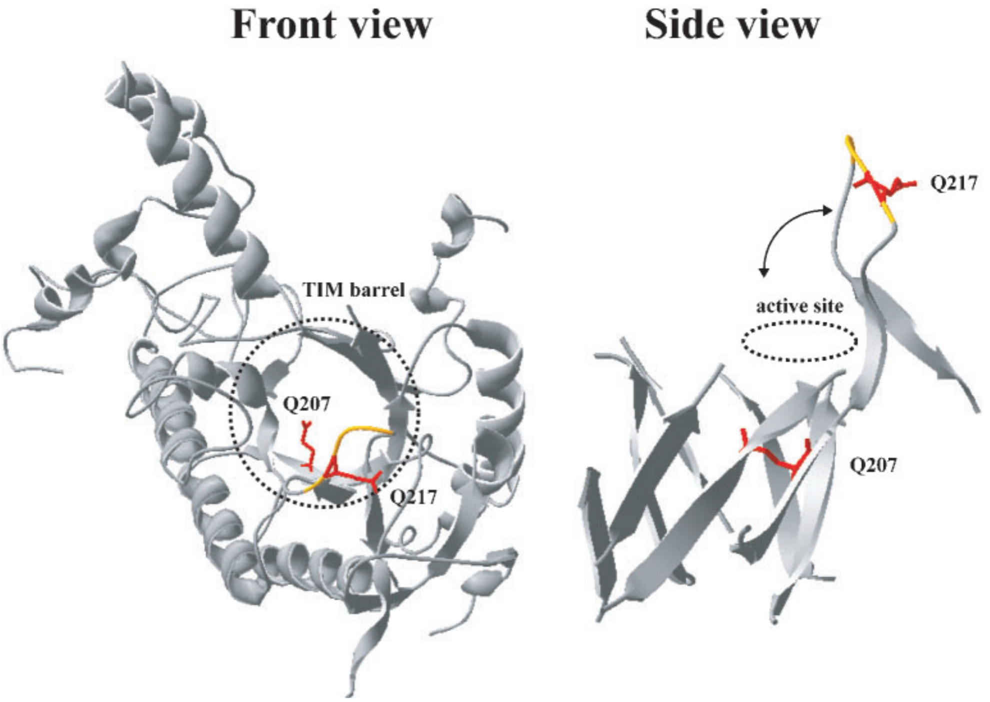

Fig. 7. Molecular model of *Cm*ICL. A loop region containing the active site is indicated by orange. The side chains of Gln207 and Gln217 are indicated by red.

without diminishing the catalytic function. As reported for *Ec*ICL (Rehman & McFadden, 1997b), the substitution of the cationic Lys residue for Gln should facilitate the binding to the Mg^{2+}–isocitrate complex as substrate. On the other hand, the catalytic rates of *Cm*-Q217K between 10 °C and 25 °C were obviously decreased (Figs 3a and 4b), indicating that the Gln residue is important for cold adaptation of *Cm*ICL. This may be due to a decrease in the structural flexibility of the mutated enzyme, revealed as an increase in thermostability and a change in the CD spectrum (Figs 3c and 6, Table 1). The high flexibility enhances the accommodation of enzyme with substrate at low temperature, but is also responsible for poor binding to ligand. It has been reported that the K_m values of cold-adapted enzymes are often higher than those of mesophilic and/or thermophilic counterparts (Fields & Somero, 1998). In fact, between 10 °C and 30 °C, the $K_m{}^{isocitrate}$ of *Cm*-WT was larger than those of *Ec*-WT and *Cm*-Q217K (Fig. 4a).

Eubacterial and eukaryotic ICLs contain the triose phosphate isomerase (TIM) barrel fold consisting of $(\beta/\alpha)_7\beta$. The molecular modelling of *Cm*ICL revealed that the Gln207 of *Cm*ICL is present in the barrel fold, and its side chain is directed toward the inner space of the barrel (Fig. 7), in the same way as the His184 of *Ec*ICL (Britton *et al.*, 2000, 2001; Sharma *et al.*, 2000). It has been reported that four *Ec*ICL mutants, in which His184 was replaced by Leu, Lys, Arg or Gln, are not able to form the tertiary structure, and that only the H184Q mutant shows activity (0·28 % of the wild-type *Ec*ICL activity; Diehl & McFadden, 1994). On the other hand, *Cm*-Q207H showed significant enzyme activity at moderate temperatures between 20 °C and 30 °C, while its activities at low temperatures were diminished (Fig. 3a, b), indicating that Gln207 of *Cm*ICL is also involved in the cold adaptation of the enzyme. Although this mutation resulted in only a small conformational change of the enzyme protein (Fig. 6), the thermostability and affinity for isocitrate as a substrate were lower than those of *Cm*-WT (Figs 3c and 4a). These results suggest that Gln207 of *Cm*ICL contributes to cold adaptation by a different mechanism from that of the Gln217 residue.

A decrease of $\Delta H\#$ (or E_a) is one of the common strategies for psychrophilic enzymes to weaken the temperature dependence of k_{cat} (Lonhienne *et al.*, 2000). Furthermore, as seen in the comparison of *Cm*-WT and *Ec*-WT, the $T\Delta S\#$ values of psychrophilic enzymes are also generally known to be smaller than those of their mesophilic counterparts, because the enhanced flexibility of psychrophilic enzyme proteins allows them to diversify the transition states of intermediate enzyme–substrate complexes during the reaction more than mesophilic ones (Fields & Somero, 1998; Lonhienne *et al.*, 2000). Conversely, such a relationship was not observed in the comparison of *Cm*-Q207H and *Cm*-Q217H with *Ec*-WT (Table 1). Therefore, with regard to their thermodynamic parameters also, both the mutated *Cm*ICLs are judged to be inferior to *Cm*-WT as a cold-adapted enzyme, although they still exhibit a lower optimum temperature for activity and higher thermolability than *Ec*-WT.

The phylogenetic analysis revealed that, among the subfamily 3 ICLs from a limited number of eubacteria including *C. maris*, Gln207 and Gln217 of *Cm*ICL are conserved, while the putative ICLs of other psychrophilic and psychrotrophic bacteria are classified into subfamily 1, separate from *Cm*ICL, and possess His and Lys, identical to *Ec*ICL, at the corresponding positions (Fig. 1). This implies that the importance of the two Gln residues to cold adaptation is specific to *Cm*ICL, and agrees with the finding that more than one pattern of amino acid substitution contributes to the cold and/or thermal adaptation of proteins (Fields & Somero, 1998; Sheridan *et al.*, 2000; Suzuki *et al.*, 2001; Lönn *et al.*, 2002; Gerike *et al.*, 2001). On the other hand, the possibility remains that additional amino acid residue(s), interacting and/or cooperating with the two Gln residues, are involved in the cold adaptation of *Cm*ICL. Therefore, further study is in progress to confirm this possibility.

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