# Site-directed mutagenesis of cysteine-195 in isocitrate lyase from *Escherichia coli* ML308

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Cysteine-195 was previously identified as a probable active site residue in isocitrate lyase (ICL) from *Escherichia coli* ML308 [Nimmo, Douglas, Kleanthous, Campbell and MacKintosh (1989) Biochem. J. **261**, 431–435]. This residue was replaced with serine and alanine residues by site-directed mutagenesis. The mutated genes expressed proteins with low but finite ICL activity, which co-migrated with wild-type ICL on both SDS/ and native PAGE. The mutant proteins were purified and characterized. Fluorimetry and c.d. in both the near- and the far-u.v. regions showed no differences between the mutants and wild-type ICL, indicating that the conformations of the three enzymes were very similar. ICL C195A (Cys-195→Ala) and C195S (Cys-195→Ser) showed 8.4-fold and 3.6-fold increases in the $K_m$ for isocitrate, while their $k_{cat.}$ values showed 30- and 100-fold decreases respectively. The effect of pH on the kinetic properties of the wild-type and mutant ICLs was investigated. The results showed that the response of the mutant enzymes to pH was simpler than that of the wild-type. For the mutants, ionisation of a group with a p$K_a$ of approx. 7.8 affected the $K_m$ for isocitrate and $k_{cat.}$. For the wild-type enzyme, these parameters were affected by the ionization of two or more groups, one of which is presumed to be cysteine-195. The results are consistent with the view that the previously identified group with a p$K_a$ of 7.1 whose ionization affects the reaction of ICL by iodoacetate is cysteine-195 itself.

## INTRODUCTION

The glyoxylate by-pass is essential for the growth of micro-organisms on acetate and other $C_2$ compounds, and for the germination of fatty seedlings. The first enzyme of this anaplerotic pathway is isocitrate lyase (ICL; EC 4.1.3.1). This enzyme catalyses the aldol cleavage of isocitrate to glyoxylate and succinate and therefore competes with the tricarboxylic-acid-cycle enzyme isocitrate dehydrogenase for the available isocitrate. The properties of both ICL and isocitrate dehydrogenase as well as the competition between them have recently been of considerable interest, especially in *Escherichia coli*, where it is regulated by the reversible phosphorylation of isocitrate dehydrogenase [1–6]. Isocitrate dehydrogenase is both phosphorylated and dephosphorylated by a bifunctional kinase/phosphatase (reviewed in [2]). Its phosphorylation state and activity respond to a number of effectors and control the partitioning of flux between the by-pass and the tricarboxylic acid cycle.

Although much is known about the structure and regulation of isocitrate dehydrogenase, there is relatively little information about the competing enzyme, ICL. The enzyme is a tetramer of identical subunits. The ICL of *E. coli* shows a random-order equilibrium mechanism [3], though a compulsory-order mechanism has been favoured for the enzyme from other sources (reviewed in [4]). Preliminary structural studies of ICL have been reported [5], and several ICL genes have been cloned and sequenced [6–12].

Several studies have identified possible active-site residues in ICL which may be important in catalysis and/or substrate binding. Inhibition of ICL from several organisms with diethyl pyrocarbonate (DEPC) [13–15] or Rose Bengal [16,17] implicated an active-site histidine residue. Ko et al. [18] identified two histidine residues in the *E. coli* enzyme which reacted with DEPC, namely histidine-266 and histidine-306. It seems unlikely that either of these residues is at the active site, since Rua et al. [19] reported that only histidine-306 was modified by DEPC and that the $K_s$ values for protection against DEPC inactivation by the substrates and products were much higher than the respective $K_m$ values. Itaconate epoxide, an analogue of the competitive inhibitor of succinate binding, itaconate, implicated the carboxylate group of either aspartate or glutamate at the active site of the *Citrullis vulgaris* enzyme [13]. More recently, chemical modification using vanadate as a substrate analogue indicated that two serine residues, namely those at positions 319 and 321 in the *E. coli* sequence, may form part of the active site [20].

Through studies with thiol-group-modifying reagents, ICL from many sources has been shown to have an essential cysteine residue [13,15,17,21–24]. Iodoacetate inhibited ICL from *E. coli* in a pseudo-first-order process, resulting in carboxymethylation of one cysteine residue per subunit. Studies of the effect of pH on this reaction, and the kinetic analysis of substrate protection, strongly suggested that the reactive group is in the active site [25]. The carboxymethylated peptide was isolated, and the modified residue was identified as cysteine-195. This and many of the surrounding residues are conserved in all ICL genes sequenced so far. This suggests that cysteine-195 is an active-site residue and as such may play an important role in either catalysis or substrate binding (or both) in ICL. We therefore decided to investigate the function of cysteine-195 further by site-directed mutagenesis.

## MATERIALS AND METHODS

### Media, enzymes and reagents

Luria–Bertani, 2 × TY, H-agar and M9 minimal medium were prepared as described in [26]. Restriction endonucleases were

Abbreviations used: ICL, isocitrate lyase; DEPC, diethyl pyrocarbonate; IPTG, isopropyl β-D-thiogalactopyranoside.

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obtained from GIBCO BRL (Life Technologies Inc.) and used as recommended. [$\alpha$-$^{35}$S]dATP (code SJ.304) and the Oligo-Nucleotide-Directed *In Vitro* Mutagenesis System Version 2 (code no. RPN.1523) were obtained from Amersham International. DNA sequencing was done using the Sequenase version 2.0 sequencing kit, obtained from United States Biochemical Corp. Iodoacetate was obtained from BDH Ltd. Other chemicals were obtained as described previously [3].

### Bacterial strains and plasmids

All bacterial strains used in the present study are derivatives of *E. coli* K-12. All plasmids are derivatives of pEM9 [27]. A 2.3 kb *Bam*HI–*Ava*I fragment of pEM9 was subcloned into an expression vector, pGLW11, in both orientations, by 'filling in' with Klenow DNA polymerase and blunt-end ligation with pGLW11 digested with *Sma*I. pAR9023 expressed ICL activity after induction from the *tac* promoter with 1 mM isopropyl $\beta$-D-thiogalactopyranoside (IPTG). The insert was then cloned into bacteriophage M13mp8 in both orientations (pAR23 and pAR24) and the nucleotide sequence determined on both strands [12].

### Site-directed mutagenesis

The site-directed mutagenesis was carried out using oligonucleotides 654 and 655 with the Amersham Oligonucleotide-Directed *In Vitro* Mutagenesis System Version 2 (code no. RPN.1523). The protocols described in the accompanying manual were strictly adhered to.

The strategy used was based on the method of Eckstein and coworkers [28–31] and involved annealing oligonucleotides carrying the specific mutations (oligo-655, cysteine-195→alanine: GTGAAGAAAGCCGGTCACATGGGCG; oligo-654, cysteine-195→serine: GTGAAGAAATCCGGTCACATGGGCG) to a single-stranded template, pAR24.

Single-stranded template DNA was prepared from six plaques of each of the two mutagenesis reactions, using oligo-654 and oligo-655. These were then sequenced using an oligonucleotide primer which anneals to the insert of pAR24 at a position that allowed sequencing of the region around the mutations. Five of the six templates of the reaction using oligo-654 had the cysteine-195 codon, TGC, changed to TCC, the codon for serine, at positions 847–849, while all six of the templates from the reaction with oligo-655 had GCC at the same position, changing cysteine-195 to alanine.

For each mutant (pAR654 and pAR655 encoding ICL C195S and ICL C195A respectively), one template showing the desired mutation was completely sequenced on one strand using oligonucleotides which anneal to various positions on the insert [12] to confirm that no other mutations had been introduced. The inserts were then cloned firstly into pUC19 by digesting pAR654 and pAR655 with *Bam*HI and *Eco*RI, 'filling in' as described above and ligating into pUC19 digested with *Sma*I. The orientation of the inserts was then checked by restriction mapping. Inserts obtained by digestion with *Bam*HI and *Eco*RI were introduced into pGLW11 to allow expression from the *tac* promoter.

### Purification of mutant enzymes, ICL C195S and ICL C195A

ICL, ICL C195A and ICL C195S were purified from *E. coli* strains KAT-1/pAR9023, pAR655 and pAR654 respectively, in which the ICL genes are under the control of the vector's *tac* promoter. The ICLs were expressed by addition of 1 mM IPTG when the $A_{420}$ of cultures was approx. 0.5. Preparation of crude extract and $(NH_4)_2SO_4$ fractionation were carried out as described by MacKintosh and Nimmo [3], and f.p.l.c. was performed on a Mono Q HR 10/10 column as described by Conder et al. [32], using a separate column for each of the three enzymes.

### Enzyme assays

ICL was assayed by coupling the production of glyoxylate to the oxidation of NADH by using lactate dehydrogenase as described by El-Mansi et al. [27]. The assay buffers were 50 mM Mops/NaOH, in the pH range 6.8–7.8 and 50 mM Taps/NaOH in the pH range 7.8–9.0. Protein concentrations were determined by the method of Bradford [33].

### Reaction of ICL with Iodoacetate

ICL (1 mg/ml) was incubated in 50 mM Mops/NaOH (pH 7.3)/1mM EDTA at 25 °C with 2.0 mM iodoacetate and other additions as indicated by Nimmo et al. [25]. Half-lives were calculated from semi-logarithmic plots of activity remaining against time.

### Fluorimetry

The analysis was carried out at the Department of Biological and Molecular Sciences, University of Stirling, Stirling, Scotland, U.K., by Dr. N. C. Price and one of us (H. G. N.). ICL at a concentration of 0.3 mg/ml in 50 mM Mops/NaOH (pH 7.3)/5mM $MgCl_2$/1mM EDTA was excited at 290 nm, and the intrinsic emission spectra was measured at 20 °C on a Perkin–Elmer fluorescence spectrophotometer, model L5 50, with slit widths of 2.5 nm.

### C.d.

C.d. analysis of wild-type ICL, ICL C195S and ICL C195A was carried out at 20 °C in 10 mM Mops/NaOH, pH 7.3, at a protein concentration of 0.3 mg/ml with slit widths of 0.02 cm and 0.5 cm for the far-u.v. (190–150 nm) and the near-u.v. (260–320 nm) respectively. The analysis was carried out at the Department of Biological and Molecular Sciences, University of Stirling, by Dr. N. C. Price, Dr. S. Kelly and one of us (H. G. N.) on a Jobin–Yvon dichrograph III equipped with a temperature-controlled cell holder.

### DNA sequencing

Dideoxy sequencing with [$\alpha$-$^{35}$S]ATP was performed using a modified T7 DNA polymerase, 'Sequenase', which carries no 3′,5′-exonuclease activity and gives a high rate of polymerization of nucleotides. Each sequencing reaction was carried out according to the manual 'Step By Step Protocols For Sequencing With Sequenase Version 2.0'. Single-stranded template was prepared by the method recommended by Amersham International (M13 Cloning and Sequencing Handbook).

### Oligonucleotides

The oligonucleotides for mutations and sequence determination [12] were synthesized on an Applied Biosystems model 280A DNA synthesizer using phosphoramidate chemistry by Dr. Veer Math (University of Glasgow).

## RESULTS AND DISCUSSION

### Site-directed mutagenesis of ICL

The *aceA* gene from *E. coli* ML308 has previously been cloned [27]. The nucleotide sequence was determined and was found to be identical with that deposited in the GenBank and EMBL databases by Byrne (accession number Pir:S05692). The residue modified by iodoacetate and 3-bromopyruvate [25,34] has been shown to be cysteine-195. We therefore designed oligonucleotides which would change cysteine-195 to an alanine and also to a serine residue. After mutagenesis, the nucleotide sequence was determined on one strand to ensure that no mutations had been introduced other than at position 195.

### Expression of mutant *aceA* gene products: ICL C195S and ICL C195A

In order to characterize the effects of replacing cysteine-195 with serine and alanine, the mutant enzymes were expressed and purified. Since the wild-type *aceA* gene had been cloned into pGLW11, this vector was chosen for expression of both of the mutant genes in the host, *E. coli* KAT-1, an *aceA*::Tn10 derivative of LE392 [35]. The activities of ICL C195S and ICL C195A could then be compared directly with that of the wild-type enzyme.

Wild-type ICL, ICL C195S and ICL C195A were expressed from pAR9023, pAR654 and pAR655 respectively, by addition of IPTG to cultures of *E. coli* KAT-1 containing these plasmids growing on L-broth with ampicillin. Samples were resolved by SDS/PAGE. In each case there was a time-dependent increase in intensity of a band which co-migrates with wild-type ICL after addition of IPTG. No similar band was observed with *E. coli* KAT-1/pGLW11 as a control (results not shown). Low but finite ICL activity was observed in cultures of KAT-1/pAR654 and KAT-1/pAR655, less than 3% of that seen with KAT-1/pAR9023 (results not shown). It therefore appears that mutation of cysteine-195 to either serine or alanine resulted in a dramatic decrease in the specific activity of ICL.

In order to study the effects of the mutations, wild-type ICL, ICL C195S and ICL C195A were purified using a combination of methods previously used to isolate the wild-type enzyme from overexpressing strains of *E. coli*, $(NH_4)_2SO_4$ fractionation followed by f.p.l.c. on Mono Q [3,32]. Each enzyme was purified using a different Mono Q column in order to avoid cross-contamination. Purification factors were in the range 2–10, and each enzyme was essentially homogeneous as judged by SDS/PAGE.

Both purified mutant ICLs had low but finite ICL activity. In order to rule out the possibility that this arose from the contamination of the purified ICL C195S and ICL C195A mutants by a subpopulation of wild-type enzyme, the thiol-group-modifying reagent iodoacetate was used to inactivate all three ICLs. Iodoacetate rapidly inactivates wild-type ICL in a pseudo-first-order process [25]. After 40 min incubation with iodoacetate, the activity of the wild-type ICL had been reduced to only 4.4% of the control activity, similar to previously reported data [25]. When the mutant ICLs were treated in the same manner, loss of activity was very slow and 54.8 and 64.8% of the initial activity remained after incubation of ICL C195A and ICL C195S respectively with iodoacetate for 40 min (results not shown). Isocitrate (5 mM) protected against the inactivation by iodoacetate for the wild-type enzyme, increasing the half life from 5 to 11 min, while 5 mM $Mg^{2+}$, an essential cofactor, increased the half-life to 12 min. With 5 mM $Mg^{2+}$ plus 5 mM isocitrate, the half-life was greater than 60 min. With the mutant

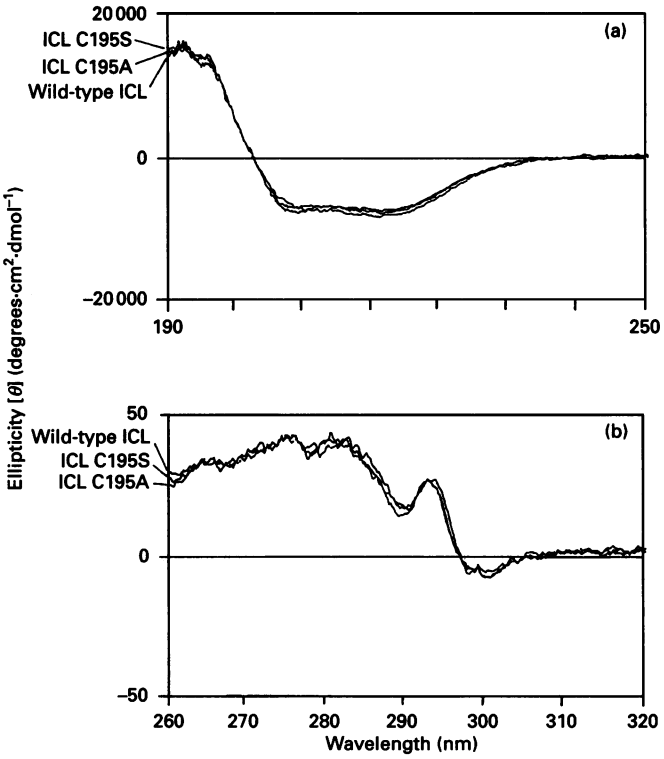


**Figure 1 C.d. of ICL, ICL C195A and ICL C195S**

C.d. was carried out according to the Materials and methods section, and results are plotted as ellipticity (degrees·$cm^2$·$dmol^{-1}$ per mean molar ellipticity) for (**a**) the far u.v. and (**b**) the near u.v.

enzymes, neither $Mg^{2+}$ nor isocitrate alone, or in combination, had a significant effect on the sensitivity of either enzyme to iodoacetate (results not shown). Thus the response of the two mutant ICLs to iodoacetate is quite different to that of the wild-type. It can be concluded that the activities of ICL C195S and ICL C195A are not due to the presence of a subpopulation of the wild-type ICL in the purified mutant enzymes caused by either homologous recombination or contamination during purification of the enzymes. It seems likely that the slow loss of activity of ICL C195S and C195A in the presence of iodoacetate is caused by reaction with cysteine residues other than those at the active site.

### Physical characterization of ICL C195S and ICL C195A

To investigate the possibility that replacing cysteine-195 with either alanine or serine reduced ICL activity by altering the conformation of the enzyme, ICL C195S, ICL C195A and the wild-type enzyme were compared using c.d. Figure 1(a) shows the c.d. spectra of ICL C195S, ICL C195A and wild-type ICL in the far-u.v. range (190–250 nm), while Figure 1(b) shows the c.d. spectra of all three enzymes in the near-u.v. range (260–320 nm). For both the near- and far-u.v. ranges there is very little difference in the c.d. spectra of the different enzymes, indicating that both the secondary and tertiary structures of all three ICL enzymes are very similar. Using the CONTIN procedure for determining the amount of each type of secondary structure from the c.d. spectra, the wild-type ICL was estimated to have $32 \pm 3\%$ $\alpha$-helix and $37 \pm 3\%$ $\beta$-sheet. In addition, the fluorescence-emission spectra of the enzymes were essentially identical, and both ICL

**Table 1 Kinetic properties of wild-type ICL, ICL C195S and ICL C195A**

The kinetic constants were measured for each enzyme at pH 7.3 and the data fitted to the Michaelis–Menten equation. Means ± S.D. (*n*) are shown.

| | $K_m$ (pM) | $k_{cat.}$ ($s^{-1}$) | $k_{cat.}/K_m$ ($s^{-1} \cdot M^{-1}$) |
|---|---|---|---|
| Wild-type ICL | 76 ± 6 (4) | 28.5 ± 2.1 (4) | $3.75 \times 10^5$ |
| ICL C195S | 272 ± 24 (3) | 0.296 ± 0.025 (3) | $1.089 \times 10^3$ |
| ICL C195A | 641 ± 37 (3) | 0.937 ± 0.072 (3) | $1.462 \times 10^3$ |

C195S and ICL C195A co-migrated with the wild-type enzyme on non-denaturing PAGE (results not shown). This evidence indicates that replacing cysteine-195 with either serine or alanine does not significantly affect the secondary, tertiary or quaternary structure of ICL.

### Kinetic properties of ICL C195S, ICL C195A and wild-type ICL

The $K_m$ and $k_{cat.}$ of ICL C195S and ICL C195A were compared with those of wild-type ICL (Table 1). The $k_{cat.}$ values of ICL C195S and ICL C195A are approx. 100-fold and 30-fold lower than that of the wild-type enzyme respectively. This implies that cysteine-195 plays an important, but not essential, role in catalysis by ICL. ICL C195S and ICL C195A also have higher $K_m$ values for the substrate, isocitrate, when compared with the $K_m$ of the wild-type enzyme, by factors of 3.6- and 8.4-fold respectively. Assuming that the mutants obey an equilibrium mechanism, as does the wild-type [8], it can be concluded that the side chain of cysteine-195 affects the binding of isocitrate. Replacement of the $CH_2$–SH of cysteine-195 with the $CH_2$–OH group of serine had less effect on the affinity of the enzyme than replacement by the –$CH_3$ group of alanine. The fact that the $K_m$ values of both mutant ICLs are different from that of the wild-type enzyme supports the conclusion that the activity of the mutant enzymes is not due to the presence of a subpopulation of wild-type ICL (see above). It appears that the side chain of cysteine-195 is important for both substrate binding and catalysis. Interestingly, ICL C195A has a higher $k_{cat.}$ than ICL C195S. One possible explanation is that a water molecule occupies the space created by replacing cysteine-195 with alanine, so providing an O–H group which is likely to be more reactive than that of –$CH_2$–OH in ICL C195S. A somewhat similar situation was reported by Lewendon et al. [36]. It was thought that serine-148 of chloramphenicol acyltransferase was involved in transition-state stabilization via a hydrogen bond to the oxyanion of the putative tetrahedral intermediate. Substitution of serine-148 with alanine resulted in an enzyme with $k_{cat.}$ reduced 53-fold and only minor changes in $K_m$, while substitution with glycine at position 148 resulted in an enzyme with only a 10-fold reduction in $k_{cat.}$. The three-dimensional structure of the alanine-148 enzyme was isosteric with the wild-type enzyme, and although the three-dimensional structure of the glycine-148 enzyme was not determined, it seemed likely that the structure of this enzyme was similar to that of the wild-type enzyme. Lewendon et al. [36] proposed that a water molecule may have partially replaced the hydrogen-bonding potential of serine-148 in the glycine mutant, but not the alanine mutant. A similar phenomenon may hold for ICL C195A, where the O–H group of a water molecule may interact with the substrate in a less deleterious way than that of serine-195. However, there are other possibilities, and more detailed analysis requires the determination of the crystal structure of ICL.

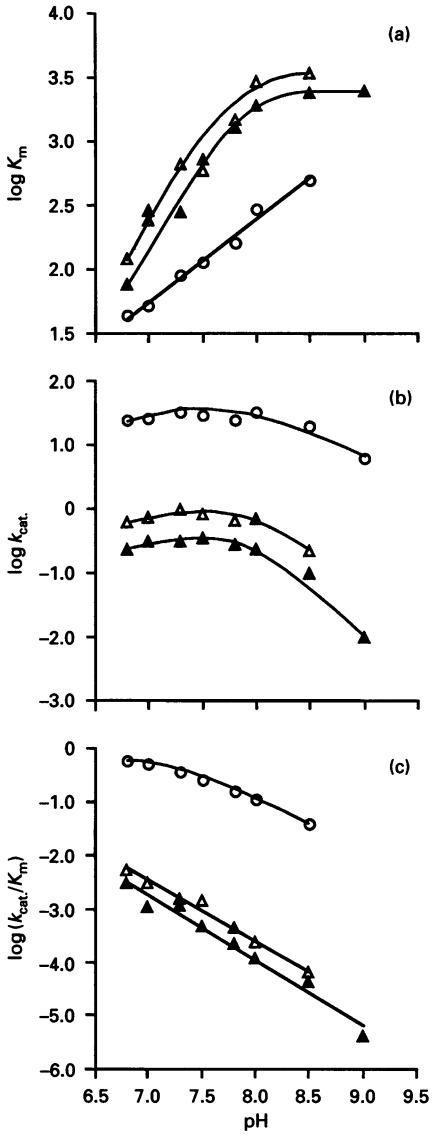


**Figure 2 Effect of pH on the kinetic parameters of ICL, ICL C195A and ICL C195S**

The $K_m$ and $k_{cat.}$ of ICL (○), ICL C195A (△) and ICL C195S (▲) were measured by assaying in 50 mM Mops/NaOH/5 mM $MgCl_2$/1 mM EDTA, pH 6.8–7.8, or 50 mM Taps/NaOH/5 mM $MgCl_2$/1 mM EDTA, pH 7.8–9.0, equilibrated to various pH values. (**a**) log $K_m$ versus pH; (**b**) log $k_{cat.}$ versus pH; (**c**) log ($k_{cat.}/K_m$) versus pH.

### Effect of pH on ICL C195S, ICL C195A and wild-type ICL

For the *E. coli* ICL, the $K_m$ for isocitrate decreases with pH over the pH range 6.0–8.5 [3,25]. Inactivation of ICL from *E. coli* ML308 by iodoacetate was shown to be dependent on a single ionizing group with p$K_a$ of 7.1 [25]. A similar result was obtained for the reaction of ICL with 3-bromopyruvate, which was affected by a single group with a p$K_a$ of 7.4 [34]. These values are lower than the expected p$K_a$ for ionization of a cysteine residue, by approx. 2 pH units [37], which suggests that the ionizing residue may not be cysteine-195 but another residue in the active site whose protonation affects the accessibility of cysteine-195 to iodoacetate, 3-bromopyruvate and isocitrate. We therefore decided to study the effects of pH on $K_m$ and $k_{cat.}$ for wild-type ICL and compare them with the effects on ICL C195S and ICL

C195A. Above pH 8.5 for wild-type ICL and ICL C195A, and above pH 9.0 for ICL C195S, the enzyme showed sigmoid kinetics, preventing meaningful comparisons of $K_m$ and $k_{cat.}$ values.

The $K_m$ of the wild-type ICL for isocitrate declined with pH over the range 6.8–8.5. A plot of log $K_m$ against pH yielded a straight line of slope approx. 0.5 (Figure 2a), indicating that the $K_m$ of wild-type ICL for isocitrate is dependent on more than one ionizing group on the enzyme–substrate complex [38,39]. A plot of log $k_{cat.}$ versus pH (Figure 2b) shows that there is very little change in this pH range, while the plot of log ($k_{cat.}/K_m$) versus pH (Figure 2c) shows a convex curve with a straight-line portion of gradient −0.5 in the range of pH 7.5–8.5. These results are difficult to interpret in terms of simple models.

In contrast, for both ICL C195S and ICL C195A, a plot of log $K_m$ versus pH (Figure 2a) gave straight lines with a slope of approx. 1 at low pH, levelling off above pH 8. This indicates that the $K_m$ is dependent on a single dissociating group on the enzyme–substrate complex with a p$K_a$ of approx. 7.8. Similarly, a plot of log $k_{cat.}$ versus pH for ICL C195S has linear segments with slope of −1 above pH 8.5 and 0 in the range pH 6.5–7.5 (Figure 2b). This indicates that $k_{cat.}$ is dependent on a single dissociating group with a p$K_a$ of approx. 7.8 on the enzyme–substrate complex. Unfortunately, the p$K_a$ of the group(s) affecting $k_{cat.}$ of ICL C195A could not be determined, since no data were obtained above pH 8.5. However, since the curve for ICL C195A is very similar to that of ICL C195S, it seems probable that the same group with p$K_a$ of approx. 7.8 also affects $k_{cat.}$ of ICL C195A. Thus the p$K_a$ of the group affecting $k_{cat.}$ and $K_m$ is the same. A plot of log ($k_{cat.}/K_m$) versus pH for both ICL C195S and ICL C195A showed a straight line with a slope of approx. −1 (Figure 2c), indicating that a single group with a p$K_a$ outside the pH range studied affects the catalytic efficiency. Since the p$K_a$ of the group affecting the $K_m$ of both ICL C195S and ICL C195A for isocitrate is approx. 7.8, it seems probable that the group is the same for both enzymes. Also, since the mutant enzymes have one dissociating group less than wild-type ICL, which affects $K_m$, it seems likely that one dissociating group on the wild-type enzyme has a p$K_a$ of approx. 7.8.

The main feature that emerges from this work is that the response of the mutant ICLs to pH is appreciably simpler than that of the wild-type enzyme. Thus it is clear that replacing cysteine-195 with either serine or alanine results in the loss of one dissociating group from the active site and allows the determination of the p$K_a$ of another dissociating group on which $K_m$ and $k_{cat.}$ depend. The data suggest that the p$K_a$ of the cysteine-195 −SH group must be within the pH range studied, namely 6.8–8.5. It is therefore likely that the p$K_a$ of 7.1 determined for the reaction between iodoacetate and cysteine-195 of ICL from *E. coli* ML308 [25] and 7.4 determined for the reaction of the same residue with 3-bromopyruvate from *E. coli* K12 [34] is the p$K_a$ of cysteine-195 itself rather than that of another residue whose protonation affects the accessibility of these reagents and the substrates to the active site.

## Conclusions

Although it has been shown by chemical modification that cysteine-195 is at or near the active site of ICL from *E. coli* ML308 [25], and is conserved between all the ICLs sequenced so far, previous work had not provided conclusive evidence that this residue is involved in catalysis. The present study indicates that cysteine-195 does indeed play an important role in the activity of ICL, since replacing this residue with either serine or alanine results in a radical decrease in $k_{cat.}$ and an increase in $K_m$. Both mutants have similar conformations to that of the wild-type enzyme, on the basis of c.d. and fluorimetric evidence. Therefore the changes in kinetic properties are probably directly due to replacement of $CH_2$–SH of cysteine-195 with $CH_2$–OH and $CH_3$ in ICL C195S and ICL C195A respectively.

The replacement of cysteine-195 by serine or alanine simplifies the pH-dependence of the enzyme. The data suggest that the ionization state of cysteine-195 does play a role in determining the activity of the wild-type enzyme. However, the pH studies of the wild-type ICL and both mutants throw little light on the mechanism of catalysis by ICL. Elucidation of the crystal structure of the wild-type ICL and also of any mutants is required to provide more information about the catalytic mechanism and interactions between isocitrate and residues on the enzyme [5].

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