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The Importance of Four Histidine Residues in Isocitrate Lyase from *Escherichia coli*

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By site-directed mutagenesis, substitutions were made for His-184 (H-184), H-197, H-266, and H-306 in *Escherichia coli* isocitrate lyase. Of these changes, only mutations of H-184 and H-197 appreciably reduced enzyme activity. Mutation of H-184 to Lys, Arg, or Leu resulted in an inactive isocitrate lyase, and mutation of H-184 to Gln resulted in an enzyme with 0.28% activity. Nondenaturing polyacrylamide gel electrophoresis demonstrated that isocitrate lyase containing the Lys, Arg, Gln, and Leu substitutions at H-184 was assembled poorly into the tetrameric subunit complex. Mutation of H-197 to Lys, Arg, Leu, and Gln resulted in an assembled enzyme with less than 0.25% wild-type activity. Five substitutions for H-266 (Asp, Glu, Val, Ser, and Lys), four substitutions for H-306 (Asp, Glu, Val, and Ser), and a variant in which both H-266 and H-306 were substituted for showed little or no effect on enzyme activity. All the H-197, H-266, and H-306 mutants supported the growth of isocitrate lyase-deficient *E. coli* JE10 on acetate as the sole carbon source; however, the H-184 mutants did not.

Isocitrate lyase catalyzes the diversion of isocitrate to the glyoxylate cycle at the metabolic branch point that alternatively leads to the tricarboxylic acid (TCA) cycle. The glyoxylate cycle enables an organism to bypass the two oxidative CO_2-forming steps of the TCA cycle when converting isocitrate to malate and succinate (11, 12). Thus, it conserves carbon while enabling an organism to convert fatty acids to carbohydrates and maintain an adequate supply of TCA cycle intermediates for biosynthetic purposes. For organisms whose only source of carbon is fatty acids, an active glyoxylate cycle is necessary for growth. This cycle is present in bacteria, fungi, oil-rich higher-plant seedlings, parasitic invertebrates, and some insects (for a review, see reference 22).

Isocitrate lyase has been characterized from a number of sources and usually is a tetrameric enzyme which requires a divalent metal, preferably magnesium, and a thiol, such as dithiothreitol, for activity in vitro. Isocitrate lyase from *Escherichia coli* has been shown to be phosphorylated on a histidine residue in the active form (19). The proposed catalytic mechanism for this enzyme involves acid-base catalysis in which D_S-isocitrate is deprotonated by isocitrate lyase, leading to glyoxylate and the succinate anion, which is then protonated to complete the reaction (22).

The amino acid sequence for isocitrate lyase has been derived from eight different organisms. An inspection of the alignment and a comparison of these derived sequences reveal several features (6). Particularily evident is a 112- to 137-amino-acid stretch which is present only in the eukaryotic sequences and which may function in targeting isocitrate lyase to perioxisomes, in which it is found in eukaryotes. In addition, four highly conserved regions (*E. coli* isocitrate lyase residues 177 to 201, 231 to 241, 310 to 324, and 348 to 356) are evident in the aligned sequences. The first region contains two completely conserved lysines, 193 and 194, and two completely conserved histidines, 184 and 197 (Fig. 1). Considering the highly anionic character of the substrate, it is plausible to postulate an important binding and/or catalytic function for this first conserved region. Indeed, substitution of Lys-193 with Arg or His greatly reduces the catalytic activity of the enzyme, and substitution with Leu or Glu inactivates the enzyme (6). In addition, affinity labeling studies with bromopyruvate have chemically modified the conserved Cys-195 residue, indicating that this residue is located in the active-site domain of the enzyme (9). Accordingly, substitution of this residue with Ser also inactivates the enzyme (6). Other chemical studies with diethyl pyrocarbonate have indicated that His-266 and His-306 may be active-site residues. Although these residues are not highly conserved, saturation kinetics with respect to inactivation were observed, and the substrate protected these residues from modification (10). The present study investigates the role of some of the identified histidines present in the *E. coli* enzyme by site-directed mutagenesis. In particular, the two highly conserved histidine residues present in the first conserved region of the enzyme, His-184 and His-197, and the two diethyl pyrocarbonate-modified residues, His-266 and His-306, have been changed.

Histidines selected for mutation. The histidines at positions 184 (H-184) and 197 (H-197) were selected because they are present in the first highly conserved region of the aligned isocitrate lyase enzymes. These two residues were changed to lysine (K), arginine (R), leucine (L), and glutamine (Q). Two other histidine residues, H-266 and H-306, were selected for mutation because diethyl pyrocarbonate modification indicated that they are in the active-site domain of the enzyme (10). In addition, on the basis of the similiarity of the flanking sequences to histidine phosphorylation sites in other *E. coli* proteins, Matsuoka and McFadden (15) had postulated that H-266 might be the phosphorylation site for *E. coli* isocitrate lyase (19). These two residues were changed to aspartate (D), glutamate (E), valine (V), serine (S) and, in the case of H-266, additionally to lysine (K). If either of these residues, H-266 or H-306, were, in fact, phosphorylated in vivo, it was hoped that the Asp and/or Glu substitutions would mimic the negative charge introduced by phosphorylation. In addition, a double mutant in which glutamate was substituted for both H-266 and H-306 (H266E/H306E) was constructed.

Mutagenesis of *aceA*. In total, four histidine residues in *E. coli* isocitrate lyase, at positions 184, 197, 266, and 306, were altered by site-directed mutagenesis. Mutagenesis of the

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castor bean     ErGAAgvHiE DQssvtKKCG HMaGk
cotton          ErGAAgvHiE DQssvtKKCG HMaGk
rape seed       ErGAAgvHiE DQssvtKKCG HMaGk
E. nidulans     ErGAAgiHiE DQapgtKKCG HMaGk
N. crassa       EkGAAgiHiE DQapgtKKCG HMaGk
C. tropicalis   ErGAAgiHiE DQapgtKKCG HMaGk
S. cerevisiae   ErGAAgiHmE DQtstnKKCG HMaGr
E. coli         EaGAAavHfE DQlasvKKCG HMgGk
                     ↑          ↑ ↑  ↑
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FIG. 1. First of four conserved regions in aligned isocitrate lyase sequences (6). Isocitrate lyase sequences are derived from the organisms identified on the left (*Emericella nidulans*, *Neurospora crassa*, *Candida tropicalis*, *Saccharomyces cerevisiae*, and *E. coli*). This first conserved region corresponds to amino acids 177 to 201 of the *E. coli* sequence. Residues that are conserved in all eight sequences are shown in uppercase letters, and nonconserved residues are shown in lower case letters. Arrows point to H-184, K-193, C-195, and H-197 of the *E. coli* sequence.

H-184, H-197, and H-306 residues was done by unique restriction enzyme site elimination (5) with a Transformer mutagenesis kit (Clontech Laboratories) as described by Diehl and McFadden (6). This is a two-primer technique which enables the mutagenesis of double-stranded DNA. The H-266 residue was mutated by *dut-ung* mutagenesis with a Muta-Gene M13 mutagenesis kit (Bio-Rad Laboratories) (13). For this technique, a section of the *aceA* gene from 787 to 1,307 bp and containing the codon for H-266 was excised from plasmid pICL1 by digestion with *Hae*III. This fragment was ligated into the *Sma*I site of M13mp18 to create M13mp18/H266-*aceA*. *E. coli* CJ236 (*dut ung thi-1 relA1*; containing pCJ106 [Cm^r]) was used to produce phage containing single-stranded M13mp18/H266-*aceA* DNA, which was isolated and used for in vitro mutagenesis. Plaques resulting after transformation of *E. coli* MV1190 [Δ(*lac-proAB*) *thi supE* Δ(*srl-recA*)*306*::Tn*10* (F′ *traD36 proAB lacI*^{q}ZΔM15)] by the in vitro mutagenesis reaction were used to isolate mutated DNA. After identification, double-stranded (replicative-form) M13mp18/H266-*aceA* DNA and pICL1 DNA were digested with restriction enzyme *Dsa*I. The *Dsa*I fragment from M13 containing the mutated H-266 codon was transferred back into plasmid pICL1. The resulting mutated pICL1 DNA was used to transform *E. coli* JE10 (Δ*aceA recA*::Tn*10*) cells (15). Finally, plasmid pICL1 with the double mutant, H266E/H306E, was created by swapping the *Dsa*I fragment containing the H-266 mutation for the wild-type fragment in plasmid pICL1 containing the H-306 mutation. All mutations were verified by dideoxynucleotide sequencing.

Isocitrate lyase assay. High-speed supernatant fractions of cell lysates prepared from cultures of isocitrate lyase-deficient *E. coli* JE10 expressing the wild-type enzyme, the mutated enzyme, or an inactive form (because of a 400-bp *aceA* deletion) of isocitrate lyase were used for isocitrate lyase assays as described by Diehl and McFadden (6). In brief, after induction of expression with isopropyl-β-D-thiogalactopyranoside (IPTG), JE10 cells expressing plasmid pICL1 containing either wild-type *aceA* or a mutant form of *aceA* in 200-ml cultures in Luria-Bertani medium (21) with 150 μg of ampicillin per ml were lysed by freezing-thawing in 3 ml of lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl [pH 8.0]) containing 0.08 mg of lysozyme. After thawing and centrifugation for 1 h at 220,000 × *g* in isocitrate lyase assay buffer (0.05 M morpholine propanesulfonic acid [MOPS], 5 mM $MgCl_2$, 1 mM dithiothreitol [pH 7.3]), the supernatant from this high-speed spin was either assayed immediately or stored at −70°C until needed. The protein concentration of this supernatant was determined by the Bradford method (3) with a Bio-Rad protein assay kit.

TABLE 1. Kinetic values for isocitrate lyase variants

Enzyme	K_m (mM)	Relative sp act[a]	Relative sp act/K_m[b]
Wild type	0.13 ± 0.03	100	769 (100)
H184L	ND[c]	<0.07	ND
H184K	ND	<0.07	ND
H184Q	0.11 ± 0.05	0.28 ± 0.09	2.5 (0.3)
H184R	ND	<0.07	ND
H197L	0.17 ± 0.11	0.23 ± 0.10	1.4 (0.2)
H197K	0.10 ± 0.07	0.20 ± 0.10	2.0 (0.3)
H197Q	0.12 ± 0.10	0.20 ± 0.10	1.7 (0.2)
H197R	0.13 ± 0.17	0.20 ± 0.09	1.5 (0.2)
H266D	0.16 ± 0.03	80.5 ± 4.2	503 (65)
H266E	0.16 ± 0.04	81.4 ± 5.4	509 (66)
H266V	0.15 ± 0.03	75.2 ± 14.7	501 (65)
H266S	0.15 ± 0.03	78.5 ± 11.8	523 (68)
H266K	0.13 ± 0.04	89.0 ± 3.4	685 (89)
H306D	0.14 ± 0.03	69.5 ± 8.7	496 (65)
H306E	0.11 ± 0.02	54.9 ± 8.1	499 (65)
H306V	0.12 ± 0.05	58.1 ± 9.2	484 (63)
H306S	0.18 ± 0.06	83.6 ± 4.1	464 (60)
H266E/H306E	0.16 ± 0.06	39.8 ± 11.4	249 (32)

[a] Percent specific activity, in micromoles of glyoxylate produced per minute per milligram of protein, relative to that of the wild-type enzyme. Wild-type enzyme activity varied depending on the preparation. The average specific activity for the wild-type enzyme was 10.6 ± 6 μmol/min/mg. This figure can be contrasted with the specific activities of 1.1 μmol/min/mg obtained for *E. coli* JE10 expressing wild-type *aceA*-containing pICL1 after growth in minimal acetate medium without induction by IPTG and 0.7 μmol/min/mg obtained for *E. coli* JM105 grown in minimal acetate medium. An extract of cells containing a 400-bp deletion in the *aceA* gene contained no enzyme activity, confirming earlier work (6).

[b] Units are liters per milligram of protein per minute. Ratios were normalized with respect to that (100) for the wild type. Since equivalent subunit concentrations were expressed for the variants listed, the numbers in parentheses represent relative k_{cat}/K_m values (i.e., relative catalytic efficiencies).

[c] ND, not determinable because of little or no activity of the protein.

The discontinuous method described by Matsuoka and McFadden (15) and Ko and McFadden (9), with a few modifications, was used to measure the conversion of D$_S$-isocitrate to succinate and glyoxylate. This method exploits a colorimetric reaction between the phenylhydrazine of glyoxylate and ferricyanide (16). In brief, at 37°C, 0.05 ml of DL-isocitrate was added to 0.95 ml of isocitrate lyase assay buffer with 0.1% phenylhydrazone and an aliquot of the high-speed supernatant. After incubation of the reaction mixture at 37°C, 0.5 ml of concentrated HCl was added to stop the reaction, and 2 ml of a 0.25% solution of potassium ferricyanide in H_2O was added to initiate color development. After incubation at room temperature for 12 to 15 min, the A_{520} of the reaction was determined. A molar absorption coefficient of 2.6×10^4 for glyoxylate was typically obtained.

Specific activities of isocitrate lyase variants. Of the four histidines altered, only changes in H-184 and H-197 appreciably reduced the specific activity of isocitrate lyase in high-speed supernatant fractions from IPTG-induced cells (Table 1). Substitution of these residues resulted in either complete or

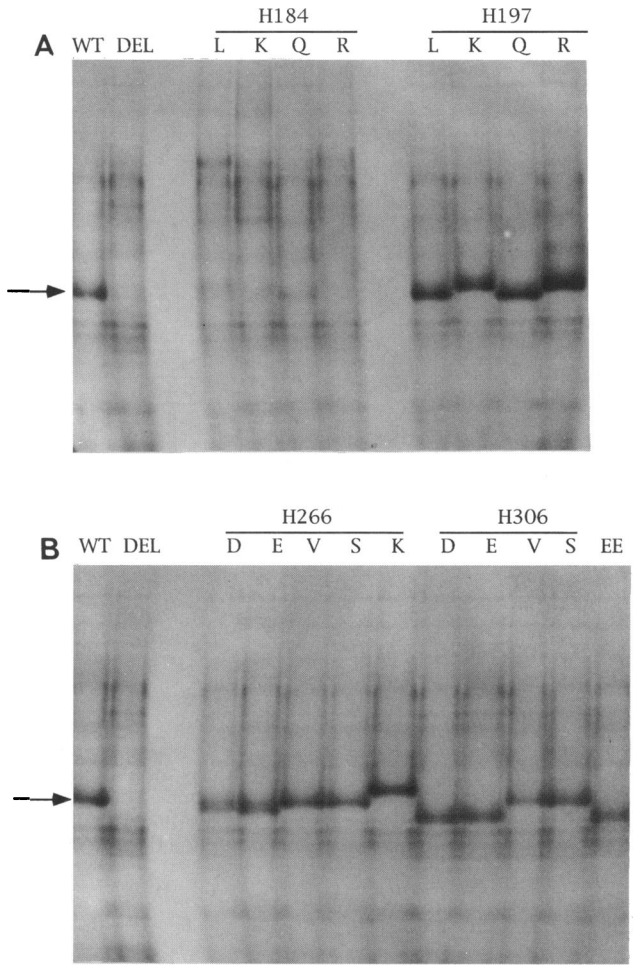

FIG. 2. Nondenaturing PAGE of high-speed supernatants. Coomassie-blue stained, 7% acrylamide gels of high-speed supernatants from cultures expressing H-184 and H-197 mutants (A) and H-266 and H-306 mutants and H266E/H306E (EE) (B). Supernatants from cultures of cells harboring wild-type *aceA*-containing pICL1 (WT) and pICL1 containing an approximately 400-bp deletion in the *aceA* gene (DEL) were also run (15). The band corresponding to the wild-type isocitrate lyase tetramer is identified by the arrow. The slight differences in the mobilities of the mutant proteins seem to correspond to the charge of the substituted residue.

almost complete inactivation of the enzyme. For three of the four H-184 mutant enzymes (H184L, H184K, and H184R), no activity was detected. For H184Q and the four H-197 mutant enzymes, a specific activity of 0.3% or less was detected. Although sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) of all fractions assayed (6, 14) for enzyme activity revealed marked and equivalent expression of a 48-kDa protein corresponding to the subunit of isocitrate lyase (data not shown), analysis by nondenaturing 7% PAGE (17, 18) showed that assembly into the tetrameric complex characteristic of the wild-type enzyme either did not occur or was extremely limited for the H-184 mutant proteins, including the H184Q variant with low activity (Fig. 2). Hence, changes in this residue interfere with the assembly of the monomeric subunits (15) into the tetrameric enzyme. All the other mutant proteins, including the marginally active H-197 series, migrated like the wild-type tetramer in nondenaturing PAGE (Fig. 2). Even though low activity made the K_m values difficult to measure in some cases, they differed little from that for the wild-type enzyme (Table 1). This result indicates that little or no pertubation of substrate binding occurred with the active mutants. Therefore, the extremely low specific activity in the H-197 mutant enzymes results from a disruption of catalysis reflected in the k_{cat}. For convenience, catalytic efficiencies (k_{cat}/K_m values) are also compared in Table 1.

These results for the H-184 and H-197 substitutions are not too surprising, since both of these histidines are completely conserved cationic residues in the first highly conserved region of the enzyme whose substrate is an anionic salt of a TCA. However, for these two sites, only changes in H-197 directly interfere with catalysis, since changes in H-184 prevent proper assembly. Thus, H-197 is probably a critical residue in the catalytic mechanism, whereas the role of H-184 in catalysis, if any, cannot be determined. The present results can be compared with previous mutagenic changes of another cationic residue in the same region, K-193. Isocitrate lyase which had Arg and His substituted for K-193 retained 7 and 2% activities and had K_m values 2.7 and 4.2 times higher than that of the wild-type enzyme, respectively (6). Thus, the H-197 substitutions have more of an effect on enzyme activity than those of K-193 but do not significantly alter K_m values. Therefore, it seems that, of these three cationic residues in the first conserved region, H-184 is involved in the assembly of the tetrameric complex, H-197 is involved in catalysis, and K-193 has a major role in catalysis but is also significant in substrate binding.

None of the five H-266 mutations nor the four H-306 mutations resulted in enzymes which markedly differed kinetically from the wild type (Table 1). Even changes resulting in substantial charge and/or steric pertubations, such as substitution with valine or glutamate, did not affect enzyme activity. These results indicate that neither H-266 nor H-306 requires phosphorylation for enzyme activation, nor does either appreciably contribute to catalysis by the enzyme. This latter result is surprising in light of the study of Ko et al. (10), in which carboxyethylation by diethyl pyrocarbonate of these residues was shown to inactivate isocitrate lyase. Because in the diethyl pyrocarbonate study both H-266 and H-306 were modified, a double mutant of isocitrate lyase in which glutamate was substituted for both H-266 and H-306 was constructed. The H266E/H306E mutation decreased enzyme activity by 60% (Table 1), simply the amount expected when the slight effects on the activity of the two separate mutations are added together.

The apparent conflict between the present study and the carboxyethylation of H-266 and H-306 in the study of Ko et al. (10) is perplexing. Similar modification studies have identified His-298 in spinach ribulosebisphosphate carboxylase/oxygenase as an important active-site residue (8), a result which later crystallographic evidence verified (2). Rua et al. (20) also reported diethyl pyrocarbonate modification of H-306 in *E. coli* isocitrate lyase, but the modification was neither saturable nor significantly competitive with any of the substrates, indicating that this residue may not be in the active-site domain. However, that study was conducted in the absence of a divalent cation, such as Mg^{2+}, which is required for isocitrate lyase activity, making the results difficult to interpret. In light of these contrasting results with respect to the importance of H-266 and H-306, it should be noted that substrate-prevented chemical modification of an enzyme may be misleading. Thus, even though chemical modification studies can indicate the direction for further research, results obtained when residues are placed in an active-site domain as a result of chemical

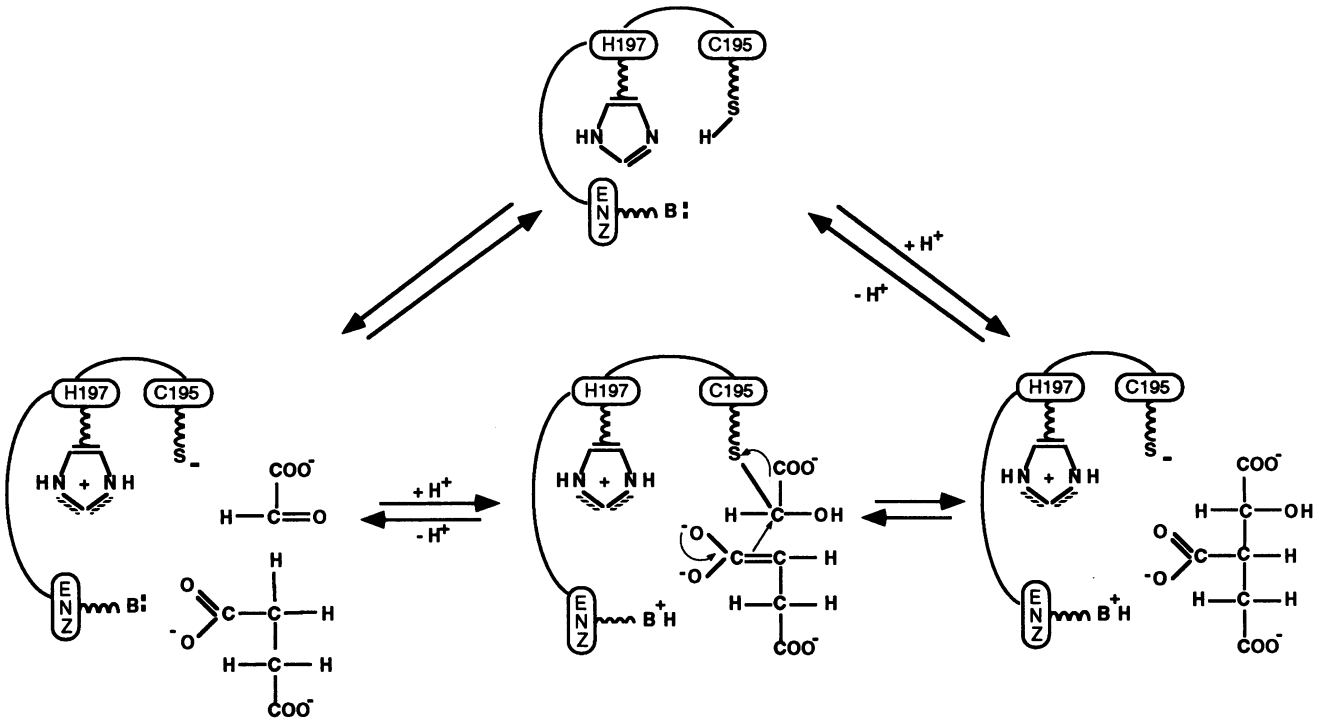

FIG. 3. Proposed catalysis by isocitrate lyase. The sequence shows a plausible role for His-197 (H-197) and Cys-195 (C-195) in the direction of the condensation reaction (i.e., the conversion of glyoxylate and succinate to isocitrate). The C-195 thiolate shown may form a tetrahedral intermediate of glyoxylate. The succinate anion, as postulated by Vanni et al. (22), could then attack the hemimercaptal carbon of glyoxylate, and the thiolate anion would act as an effective leaving group, especially when facilitated by reformation of the ion pair shown. Although not shown, the actual substrate is probably Mg^{2+}-isocitrate (22). As implied, the binding of Mg^{2+}-glyoxylate to free enzyme may position the side chains of C-195 and H-197 to enable the formation of the ion pair. ENZ, enzyme.

modification should be interpreted cautiously until verified by other methods.

Growth of isocitrate lyase-deficient *E. coli* JE10 cells expressing isocitrate lyase mutants. Tests of the growth of *E. coli* JE10 in minimal acetate medium while expressing various mutations of isocitrate lyase from plasmid pICL1 were conducted as described by Diehl and McFadden (6). In brief, 0.4 ml of stationary-phase cultures of JE10 were diluted to an optical density at 600 nm of 0.05 and then were added to 5 ml of M9 minimal (21) medium supplemented with 0.2% glucose or acetate, 100 μg of ampicillin per ml, and 20 μg of tetracycline per ml. After growth at 37°C, doubling times were determined by inspection of the linear portion of a plot of log optical density at 600 nm versus time.

In minimal glucose medium, the rates of growth of *E. coli* JE10 expressing any of the isocitrate lyase variants were similiar (doubling times, ca. 1.5 h at 37°C). In minimal acetate medium, both *E. coli* JE10 expressing wild-type isocitrate lyase from pICL1 and *E. coli* JM105 (the *aceA*$^+$ parental strain for JE10) (15) had doubling times of 3 h at 37°C, as did JE10 expressing any of the active H-266 and H-306 mutant enzymes and the H266E/H306E double mutant. These results are consistent with the in vitro assays of proteins derived from induced cells, which indicated no significant role for H-266 and H-306 with regard to catalysis or phosphorylation. However, *E. coli* JE10 containing plasmid pICL1 expressing inactive, poorly assembled H184L, H184K, and H184R mutant enzymes did not grow on acetate as the sole carbon source. Even the expression of the slightly active H184Q variant did not support the growth of JE10 at 37°C in M9 minimal acetate medium, probably because this mutant enzyme assembles poorly (Fig. 2).

In contrast, the assembled H-197 mutant enzymes, with <0.3% catalytic efficiency (Table 1), did support *E. coli* growth on acetate. The doubling times for JE10 cultures expressing the H197L, H197K, H197Q, and H197R mutant enzymes from plasmid pICL1 were 5.4, 5.9, 4.3, and 6.2 h, respectively, at 37°C. It is clear that the marked reduction in the catalytic efficiency of the mutant enzymes does not correlate well with the moderately reduced rates of growth on acetate of the corresponding mutants. The most plausible explanation is that for JE10 cells expressing wild-type isocitrate lyase from multicopy plasmid pICL1, the cleavage of isocitrate to glyoxylate and succinate is not the rate-limiting step in acetate assimilation. The present and earlier results (6) establish, however, that as the catalytic efficiency of isocitrate lyase in mutants is lowered, at some point the carbon flux across this step becomes limiting to growth on acetate and increases the doubling time.

Possible interaction of Cys-195 and His-197. Finally, the present study unambigiuosly demonstrates that H-197 is critical for *E. coli* isocitrate lyase activity and is most likely directly involved in catalytic mechanism. Since the K_m values for the H-197 mutant enzymes do not differ much from that of the wild-type enzyme, substrate binding seems relatively unaffected. Thus, H-197 probably does not significantly contribute to substrate binding but may work with another residue in the region to enhance catalysis. A very reasonable candidate is Cys-195, since this nearby conserved residue has been placed in the active site by affinity labeling with the substrate analog bromopyruvate (9) and substitution of this residue with Ser

results in an inactive enzyme in vitro (6). With regard to an interaction between the side chains of His-197 and Cys-195, the mechanism established for the thiol proteases is enlightening (4, 7). In these proteases, the imidazole group of the histidine is protonated by the thiol hydrogen from the proximal active-site cysteine, forming an ion pair. The thiolate anion then acts as a nucleophile to form a tetrahedral intermediate prior to the peptide cleavage reaction. In the case of isocitrate lyase, a similiar interaction may occur (Fig. 3). The thiolate anion may form a tetrahedral intermediate, a hemimercaptal, of glyoxylate in the condensation reaction. Our data suggest that the thiolate form of the enzyme is not obligatory in forming the hemimercaptal but that the parent thiol can also form the tetrahedral intermediate, as would be expected, but at a rate 300 to 400 times slower (Table 1). It is clear that the "crippled" enzyme functions reasonably well in supporting *E. coli* JE10 growth on acetate. Elucidation of the crystal structure of isocitrate lyase, which is in progress (1), should help test this proposed mechanism.

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