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# Mutation of Essential Catalytic Residues in Pig Citrate Synthase†

Gerald M. Alter,‡ Joseph P. Casazza,§ Wang Zhi,‖ Peter Nemeth,‖ Paul A. Srere,‖ and Claudia T. Evans*,‖

*Pre-Clinical Science Unit, Department of Veterans Affairs Medical Center, and Biochemistry Department, University of Texas Southwestern Medical Center, 4500 South Lancaster Road, Dallas, Texas 75216, Wright State University, Dayton, Ohio 45435, and NIAAA Laboratory of Metabolism and Molecular Biology, Rockville, Maryland*

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ABSTRACT: Two amino acid residues, $His^{274}$ and $Asp^{375}$, were replaced singly in the active site of pig citrate synthase (PCS) with $Gly^{274}$, $Arg^{274}$, $Gly^{375}$, $Asn^{375}$, $Glu^{375}$, and $Gln^{375}$. The nonmutant protein and the mutant proteins were expressed in and purified from *Escherichia coli*, and the effects of these amino acid substitutions on the overall reaction rate and conformation of the PCS protein were studied by initial velocity and full time course kinetic analysis, behavior during affinity column chromatography, and monoclonal antibody reactivity. Native and mutant proteins purified similarly had a subunit molecular weight of 50 000 and were homologous when examined with 10 independent a-PCS monoclonal IgGs or with a polyclonal anti-PHCS serum. No activity was detected for $Asn^{375}$ or $Gln^{375}$. The $k_{cat}$s of the other purified mutant proteins, however, were decreased by about $10^3$ compared to the nonmutant enzyme activity. The $K_m$ for oxalacetate was decreased 10-fold in the $Glu^{375}$ protein and was reduced by half in $Gly^{274}$ and $Arg^{274}$ PCSs, while the $K_m$ for acetyl-CoA was decreased 2-3-fold in $Gly^{274}$, $Arg^{274}$, and $Gln^{375}$ PCSs. A mechanism is proposed that electrostatically links $His^{274}$ and $Asp^{375}$.

The overall reaction catalyzed by citrate synthase (EC 4.1.3.7) is an aldol condensation reaction and is the first step of the Krebs tricarboxylic acid cycle (Wiegand & Remington, 1986; Srere, 1972). The reaction is stereospecific with respect to oxalacetate (OAA)[1] such that the acetyl group of acetyl coenzyme A adds to the si face of the keto moiety of oxalacetate with a concomitant inversion of the configuration of the methyl hydrogens (Eggerer et al., 1970; Retey & Arigoni, 1970). Three consecutive partial chemical reactions describe the course of catalysis by citrate synthase: enolization, condensation, and hydrolysis (Eggerer & Remberger, 1964; Buckel & Eggerer, 1969). The enzyme from pig heart is a dimeric protein of identical subunits whose X-ray crystal structure has been determined and whose mechanism has been intensively studied. The sequence of the enzyme from pig heart was derived from amino acid sequence analysis (Bloxham et al., 1981, 1982) and from the cDNA sequence (Evans et al.,

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* Address correspondence to this author of the Veterans Affairs Medical Center, 4500 S. Lancaster Rd., Dallas, TX 75216.
‡ Wright State University.
§ NIAAA Laboratory of Metabolism and Molecular Biology.
‖ University of Texas Southwestern Medical Center.

[1] Abbreviations: PCS, pig citrate synthase made by recombinant DNA methodology; PHCS, pig heart citrate synthase; $His^{274}$, $Asp^{375}$, PCS is the nonmutant pig citrate synthase from its unmodified cDNA expressed in and purified from *E. coli*; the mutant PCSs are designated by the residues substituted for $His^{274}$, or $Asp^{375}$; these are $Gly^{274}$, $Arg^{274}$, $Gly^{375}$, $Asn^{375}$, $Glu^{375}$, and $Gln^{375}$; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; acetyl-CoA, acetyl coenzyme A; OAA, oxalacetate.

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1988a). Each dimer contains two functionally independent active sites with binding sites for both oxalacetate and acetyl coenzyme A (Weidman et al., 1973; Srere, 1965; Johansson & Pettersson, 1974).

The enzyme was shown to crystallize in two main forms, an "open" tetragonal and a "closed" monoclinic configuration (Remington et al., 1982). These studies indicated that (1) the enzyme monomers are composed of a large and a small domain connected by a "hinge" region, (2) amino acid residues from both polypeptide chains of the dimeric enzyme contribute to each active site, and (3) the active site occurs in a cleft between the large and small domains (Remington et al., 1982). The regions of the enzyme involved in substrate binding and the active-site amino acids involved in hydrogen bonding also were identified (Remington et al., 1982). From X-ray diffraction of the monoclinic form of pig heart citrate synthase, $His^{274}$ was shown to form a hydrogen bond with a terminal carboxylate of citrate (which originated from acetyl-CoA), and, therefore, it was postulated that $His^{274}$ may participate in the enolization partial reaction. In addition, $His^{274}$ by its interaction with other residues contributes to the formation of the binding site of oxalacetate. $Asp^{375}$, which is close to $His^{274}$ but does not form a salt bridge with it, interacts with the same carboxyl of citrate as $His^{274}$. It was proposed an aspartyl carboxy may be involved in both the enolization and hydrolysis partial reactions. This report describes specific mutations of the $His^{274}$ and $Asp^{375}$ residues in pig citrate synthase. Changes in citrate synthase catalytic activity and structure were compared with the properties of the native enzyme by kinetic analysis, its purification behavior, and molecular surface epitope mapping using an a-PCS monoclonal antibody family.

## Experimental Procedures

*Materials.* Agarose–ATP (type 4) was obtained from Pharmacia Inc., Piscataway, NJ; DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], oxalacetate, egg lysozyme, and pig heart citrate synthase were from Sigma, St. Louis, MO; $^{125}I$ protein A (47 mCi/mg) was from Amersham, Arlington Heights, IL; and protein molecular weight standards were from Bio-Rad, Richmond, CA. Acetyl coenzyme A was prepared by the method of Simon and Shemin (1953). All other chemicals used were of the highest purity available.

*Purification of the Nonmutant and Mutant Pig Citrate Synthase Proteins.* Oligonucleotide-directed mutagenesis was performed as described previously (Evans et al., 1989) with a cDNA template encoding pig citrate synthase (Evans et al., 1988a) that contained uracil residues in place of thymine (Kunkel, 1985; Kunkel et al., 1987). The DNA fragments that encode the mature forms of the nonmutant and six mutant forms of pig citrate synthase that lack the mitochondrial leader peptide sequence were subcloned as previously described (Evans et al., 1988a), and the proteins were expressed in a *gltA*$^-$ *Escherichia coli* (Evans et al., 1989). Nonmutant and the mutant pig citrate synthase proteins were purified individually following a modification of the standard procedure (Evans et al., 1989; Mukherjee & Srere, 1976) and assayed for protein (Bradford, 1976) and citrate synthase activity (Srere et al., 1963).

*Immunological Studies of Mutant and Nonmutant PCS.* The purity of the wild-type and mutant PCS proteins was determined by SDS–PAGE (Laemmli, 1970) and protein staining. The proteins were transferred to nitrocellulose and incubated with a polyclonal antiserum directed against pig heart citrate synthase (Evans et al., 1988a). The second antibody was alkaline phosphatase conjugated anti-rabbit Ig (Sigma), and the immunoreactions were developed as described previously (Kispal et al., 1989). The antibody detected 0.01 μg of purified nonmutant or mutant PCS protein.

Table I: Partial Nucleotide Sequences for Producing Mutant PCSs

| | | |
|---|---|---|
| PHCS | Amino Acid Sequence | Gly-Pro-Leu-His-Gly-Leu-Ala |
| $His^{274}$ | Unmodified cDNA Sequence | GGG CCC CTA **CAT** GGG CTG GCA |
| $Gly^{274}$ | | GGG CCC CTA **GGT** GGG CTG GCA |
| $Arg^{274}$ | | GGG CCC CTA **CGA** GGG CTG GCA |
| PHCS | Amino Acid Sequence | Pro-Asn-Val-**Asp**-Ala-His-Ser |
| $Asp^{375}$ | Unmodified cDNA Sequence | CCC-AAT-GTG-**GAT**-GCT-CAC-AGT |
| $Gly^{375}$ | | CCC-AAT-GTG-**GGA**-GCT-CAC-AGT |
| $Asn^{375}$ | | CCC-AAT-GTG-**AAC**-GCT-CAC-AGT |
| $Glu^{375}$ | | CCC-AAT-GTA-**GAG**-GCT-CAC-AGT |
| $Gln^{375}$ | | CCC-AAT-GTG-**CAG**-GCT-CAC-AGT |

The surface of the mutant PCS proteins was examined with an a-PHCS monoclonal antibody family. Monoclonal antibody producing hybridoma cell lines were raised against the pig heart citrate synthase enzyme by the conventional method (Kohler & Milstein, 1975) in the Biotechnological Facility of the University Medical School of Pecs (Hungary). IgG1 monoclonal antibody fractions were purified from ascitic fluid of hybridoma-bearing mice by protein G affinity column chromatography (Pharmacia, Sweden). The purified nonmutant and mutant PCS samples were examined immunoserologically by the ELISA method (Engall & Perlmann, 1972) with 10 a-PHCS monoclonal antibody subclones that recognize independent surface epitopes of pig heart citrate synthase. PHCS, nonmutant PCS, and mutant PCS proteins were immobilized onto plastic microtiter plates (Corning) and incubated with the monoclonal antibodies. The free binding sites were saturated with 1% bovine serum albumin. The immunoreactions were detected with anti-mouse Ig horseradish peroxidase labeled goat antisera (Fisher Scientific Co.) and quantiated at $OD_{490nm}$ with a Bio-Rad multiwell plate ELISA reader.

The mutant and nonmutant PCS samples were characterized further by protein dot and electroblot analysis using monoclonal a-PHCS G12. The immunoreaction was detected by using anti-mouse Ig horseradish peroxidase with 3-amino-9-ethylcarbazole in 0.1 M, pH 5.2, citric acid buffer. The positive reaction was visualized as a red-brown precipitate.

*Kinetic Studies of the Mutant and Nonmutant Pig Citrate Synthase Proteins.* The time courses for the forward citrate synthase reaction catalyzed by nonmutant and mutant PCS proteins were monitored spectroscopically (Srere et al., 1963). Previously, it was demonstrated that the kinetics of the forward citrate synthase reaction did not differ between the PCS purified from *E. coli* and the pig heart enzyme (Evans et al., 1988b). Kinetic analyses were routinely run at 25 °C in 100 mM Tris-HCl buffer at pH 8.1 and specific activities determined in the presence of 100 μM acetyl-CoA and 50 μM oxalacetate. Michaelis constants for oxalacetate and acetyl coenzyme A were determined from full time course measurements using a progress curve fitting program (Boeker, 1984).

## Results

*Purification of Mutant Forms of Pig Citrate Synthase from E. coli.* The nonmutant and mutant PCS proteins prepared from the modified cDNA as indicated in Table I were purified from *E. coli* total cell lysates. Figure 1 is the agarose–ATP elution profile of the nonmutant enzyme and the $Gly^{375}$ mutant PCS protein. Each PCS mutant protein and the nonmutant enzyme eluted in a similar manner from the column. Mutant

Table II: Reactions of Recombinant PCS with Monoclonal Antibodies to PHCS

| | a-PHCS monoclonal antibodies | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| OD | G12 | C11 | C3 | B11 | G4 | F11 | E11 | H6 | E2 | G3 | mixed |
| +++ >1.0 | | | | | | PHCS NM[a] $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ | | | PHCS | PHCS | PHCS NM $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ |
| ++ 0.5–1.0 | PHCS NM $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ | PHCS NM $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ | PHCS NM $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ | | | | PHCS NM $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ | PHCS NM $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ | NM $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ | NM $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ | |
| + <0.5 | | | | PHCS NM $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ | PHCS NM $Gly^{274}$ $Arg^{274}$ $Gly^{375}$ $Asn^{375}$ $Glu^{375}$ $Gln^{375}$ | | | | | | |

[a] NM, nonmutant ($His^{274}$, $Asp^{375}$). Duplicate experiments were performed with different antigen concentrations and monoclonal antibody dilutions. The relationships between the monoclonal antibodies and antigens remained constant.

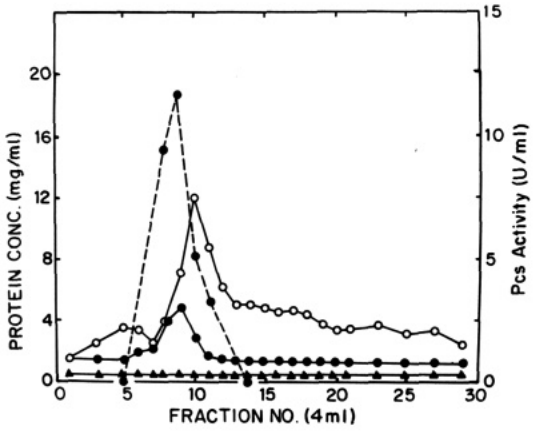


FIGURE 1: Agarose–ATP chromatography of nonmutant ($His^{274}$, $Asp^{375}$) and mutant PCS. Two agarose–ATP columns (15-mL bed volumes) were simultaneously equilibrated with 5 mM $KPO_4$ buffer, pH 7.5, and loaded with either the nonmutant ($His^{274}$, $Asp^{375}$) or the mutant PCS protein that was expressed in *E. coli* and equilibrated in the same buffer. The samples were eluted with 5 mM $KPO_4$ buffer, pH 7.5, containing 50 mM KCl, 100 μM oxalacetate, and 100 μM coenzyme A. (▲—▲) No PCS enzyme control (milligrams per milliliter); (●—●) nonmutant PCS (milligrams per milliliter); (●---●) nonmutant PCS (units per milliliter); (○—○) $Gly^{375}$ (milligrams per milliliter).

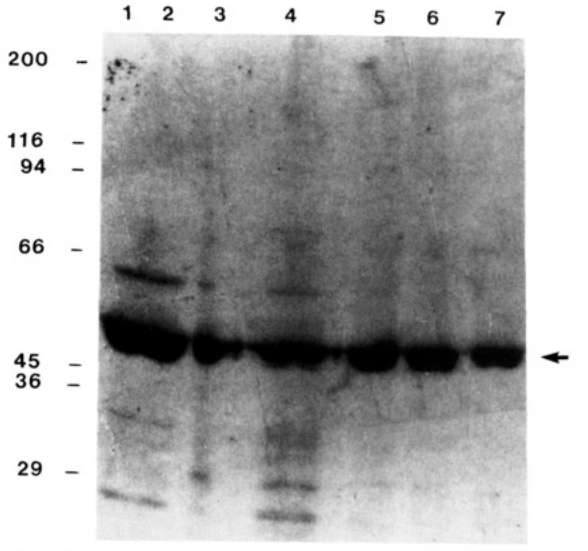


FIGURE 2: SDS–PAGE of the purified nonmutant and mutant PCS proteins. 10% SDS–PAGE gels were loaded with each purified PCS protein (3 μg). Lane 1, Nonmutant PCS enzyme, 2, $Gly^{274}$; 3, $Arg^{274}$; 4, $Gly^{375}$; 5, $Asn^{375}$; 6, $Glu^{375}$; 7, $Gln^{375}$. The sizes of the molecular weight standards are marked, and the position of purified PHCS is marked by the arrow.

enzymes were insufficiently active to follow their elution from affinity columns kinetically as was done for the native enzyme. Since mutant and native enzymes are very similar as judged immunologically (see below), polyvalent antibodies were used in slot-blot analyses to verify that the protein peak eluted was the mutant citrate synthase. As shown in Figure 1, protein peaks corresponded to citrate synthase. Similar results were found for other mutant citrate synthases. SDS–PAGE of the purified mutant and the nonmutant PCS proteins demonstrated that each protein was greater than 99% homogeneous and had a molecular weight of 50000 (Figure 2). SDS–PAGE and protein blot analysis using either the polyclonal antiserum or the monoclonal a-PHCS G12 indicated that both purified mutant and nonmutant PCS proteins reacted like the authentic pig heart citrate synthase.

*Monoclonal Antibody Binding.* The PCSs were compared immunoserologically by using the monoclonal a-PHCS IgG1s. ELISA and dot-blot analyses indicated epitopes probed by monoclonal antibodies were similarly or identically presented in all the citrate synthase proteins tested. Table II summarizes the immunological data. Also as judged by dot-blot analysis, mutant, nonmutant, and citrate synthases from pig heart were immunologically indistinguishable (very similar). The electrotransferred denatured mutant and nonmutant enzymes showed similar reactivity with a-PHCS G12 monoclonal antibody in Western blot analyses (data not shown).

*Kinetic Characterization of the Wild-Type and Mutant Forms of Pig Citrate Synthase.* The overall condensations

Table III: Kinetic Parameters of Pig Citrate Synthases Expressed in *E. coli*[a]

| protein | $k_{cat}$ ($s^{-1}$) | $K_m$(OAA) ($\mu$M) | $K_m$(AcCoA) ($\mu$M) | $\Delta\Delta G^*$ (OAA)[d] (kcal) | $\Delta\Delta G^*$ (AcCoA) (kcal) |
|---|---|---|---|---|---|
| $His^{274}$, $Asp^{375}$ (nonmutant) | 96 ± 6.5 | 6.0 ± 3.7 | 16.3 ± 1.5 | | |
| $Gly^{274}$ | 0.10 ± 0.05 | 3.5 ± 2.5 | 5.4 ± 2.3 | 3.7 | 3.4 |
| $Arg^{274}$ | 0.15 ± 0.02 | 3.8 ± 0.3 | 6.5 ± 3.1 | 3.6 | 3.3 |
| $Glu^{375}$ | 0.23 ± 0.04 | 0.6 ± 0.3 | 4.1 ± 1.6 | 2.2 | 2.7 |
| $Gly^{375}$ | 0.06 ± 0.01[b] | | | 4.4[e] | 4.4[e] |
| $Asn^{375}$ | 0.01 ± 0.01[b,c] | | | | |
| $Gln^{375}$ | 0.02 ± 0.02[b,c] | | | | |

[a] Reactions run at 25 °C in 100 mM Tris-HCl, pH 8.1. [b] Determined from initial steady-state kinetics of a reaction run at 25 °C in 100 mM Tris-HCl, pH 8.1, containing 100 $\mu$M acetyl coenzyme A and 50 $\mu$M oxalacetate and assuming substrate concentrations are saturating. [c] Values not significantly greater than background. [d] Calculated by using $\Delta\Delta G^* - RTkb[(k_{cat}/K_m)/(k_{cat_n}/K_{m_n})]$ where n indicates nonmutant. [e] Calculated by assuming $K_m$s of the mutant and nonmutant are the same.

Table IV: Homologous Citrate Synthase Active-Site Amino Acids

| | acetyl coenzyme A binding region residues | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| enzyme[b] | 46[a] | 164[a] | 273[a] | 314 | 315[a] | 316 | 317[a] | 318[a] | 319 | 321 | 322 | 324 | 368 | 375[a] |
| PHCS | R | R | L | V | V | P | G | Y | G | A | V | R | TMK | D |
| YCS1 | R | K | L | V | v | P | G | Y | G | A | V | R | K | D |
| YCS2 | R | K | L | V | I | P | G | Y | G | A | V | R | K | D |
| ATCS | R | K | L | V | I | P | G | Y | G | G | V | R | E | D |
| ECCS | F | R | A | R | L | M | G | F | G | R | V | K | K | D |
| PACS | F | R | A | K | L | M | G | F | G | R | V | K | R | D |
| AACS | F | R | A | K | L | M | G | F | G | R | V | K | R | D |
| RPCS | F | R | A | R | L | M | G | F | G | R | V | K | R | D |

| | citrate/oxalacetate binding region residues | | | | | |
|---|---|---|---|---|---|---|
| enzyme[b] | 238[a] | 274[a] | 320[a] | 329[a] | 401[a] | 421[a] |
| PHCS | H | H | H | R | R | R |
| YCS1 | H | H | H | R | R | R |
| YCS2 | H | H | H | R | R | R |
| ATCS | H | H | H | R | R | R |
| ECCS | H | H | H | R | R | R |
| PACS | H | H | H | R | R | R |
| AACS | H | H | H | R | R | R |
| RPCS | H | H | H | R | R | R |

[a] Hydrogen bonding amino acid (Evans et al., 1989). Alignments determined by using the Microgenie sequence software program (Beckman). [b] PHCS, pig heart citrate synthase; YCS1, mitochondrial yeast citrate synthase; YCS2, cytosolic yeast citrate synthase; ATCS, *Arabidopsis thaliana* citrate synthase; ECCS, *Escherichia coli* citrate synthase; PACS, *Pseudomonas aeruginosa* citrate synthase; AACS, *Acinetobacter anitratum* citrate synthase; RPCS, *Rickettsia promazekii* citrate synthase.

of oxalacetate and acetyl-coenzyme A to citrate by purified nonmutant and mutant PCSs were assayed (Table III) and indicated that the $His^{274}$ and $Asp^{375}$ PCS mutants were much less active than the nonmutant enzyme. Replacement of $His^{274}$ with either Gly or Arg caused a 600–700-fold reduction in $k_{cat}$ while mutation of $Asp^{375}$ to Gly caused an approximate 1600-fold decrease in $k_{cat}$. Changing $Asp^{375}$ to either $Asn^{375}$ or $Gln^{375}$ caused a decrease in enzyme activity to a value below the limits of our present methods of detection.

Full time course reactions were measured for mutant enzymes with sufficient activity and for the nonmutant PCS. Data from time courses were fitted by progress curve analysis assuming a bimolecular reaction, A + B → P. Observed and predicted time courses were in excellent agreement. $K_m$s and $k_{cat}$s derived from this analysis are summarized in Table III. The $k_{cat}$s derived from detailed analyses are in good agreement with those derived from initial steady-state kinetic assays performed at high substrate concentrations (footnote *b*, Table III). The $K_m$ for oxalacetate decreases 10-fold for the $Glu^{375}$ mutant enzyme and is decreased to about half for both $Gly^{274}$ and $Arg^{274}$ relative to nonmutant PCS. The $K_m$ for acetyl coenzyme A is decreased 2–3-fold for each mutant PCS enzyme measured. Values for corresponding $\Delta\Delta G^*$s for the reactions are also listed in Table III.

## Discussion

$His^{274}$ and $Asp^{375}$ are two of several residues which are conserved in citrate synthases, seem to interact directly with substrate molecules (Table IV), and have been implicated in the catalytic mechanism of the enzyme (Wiegand & Remington, 1986). To evaluate their significance, these residues were changed by using site-directed mutagenesis. The mutated enzyme was characterized both structurally using immunological means and functionally using enzyme kinetic analyses. Properties were compared with the nonmutant enzymes. In two mutant citrate synthases, the $His^{274}$ was replaced with a Gly and an Arg, respectively, while in the other mutant enzymes $Asp^{375}$ was replaced with a Gly, Asn, Glu, or Gln.

*Purification of Citrate Synthases.* Mutant citrate synthase protein was isolated by using the same protocol as the native enzyme (Figure 1). Since ammonium sulfate fractionation cuts containing citrate synthase were the same in mutant and native proteins, the solubility and therefore surface properties of citrate synthases are similar. This suggests the structure of citrate synthase is not drastically changed by the modifications introduced by mutagenesis. This contention is further supported by the similar binding and elution of mutant and native enzymes during ATP–agarose affinity chromatography. Citrate synthase probably interacts with column-bound ATP at the subsite of the acetyl-CoA binding region that normally interacts with CoA's adenosyl phosphate moiety. The adenosyl phosphate site is adjacent to the location where the acetyl portion of the acetyl-CoA binds, where the acyl-CoA condensation with OAA occurs, and where the mutations that are reported here were introduced (Wiegand & Remington, 1986). Thus, affinity chromatography results indicate modifications

of the catalytic region studied here do not disorganize the adjacent CoA binding region.

Monoclonals were raised and selected by using citrate synthase isolated from pig hearts. Results in Table II indicate all monoclonal antibodies recognized pig heart citrate synthase expressed by *E. coli* as well as mutated citrate synthase. Further, at least semiquantitatively, they bind equally well to mutated and native citrate synthasae expressed in *E. coli*. Additional studies with the monoclonal antibodies will indicate whether conformational changes occur in the mutant and native pig citrate synthases.

*Catalytic Role of Active-Site Residues.* The large reductions in catalytic efficiencies of mutant citrate synthases (Table III) attest to the importance of $His^{274}$ and $Asp^{375}$ in this enzyme's catalytic mechanism. Mutations at positions 274 and 375 uniformly have a greater impact on $k_{cat}$ than on $K_M$ (Table IV). Though this suggests changes in turnover are greater than perturbation of binding, the $K_M$s are themselves complex functions of rate constants and reflect other processes as well as binding. The effect of mutations on the catalytic function of an enzyme then can conveniently be judged by comparing the transition-state free energy for a reaction catalyzed by mutant and nonmutant enzymes. Transition-state free energies, which are a function of $k_{cat}/K_M$s, are summarized in Table III (Fersht, 1985). Though some activity was observed with the $Gly^{375}$ mutant, it was insufficient to accurately determine $k_{cat}$ and $K_M$. Therefore, approximate $k_{cat}$ and activation parameter values were estimated (footnotes, Table III). Mutants $Asn^{375}$ and $Gln^{375}$ were virtually inactive.

At pH 8.1, the pH at which citrate synthase activity is assayed, aspartic acid and histidine side chains in aqueous solutions are normally deprotonated. However, apparent $pK_a$s of protein side chains can vary significantly. This is likely in the case of citrate synthase. X-ray crystallographic evidence suggests $Asp^{375}$ and $His^{274}$ are located close to each other in the enzyme's three-dimensional structure (Remington & Wiegand, 1982), making the protonation state of one side chain effect the other. In such an environment, the dissociation of a proton from the Asp is encouraged by favorable electrostatic interactions between negatively charged Asp and a positively charged His side chains. At the same time, the apparent $pK_a$ of the histidine side chain is raised owing to the same electrostatic influence. As a result, $His^{274}$ and $Asp^{375}$ likely act initially as acid and base catalyst, respectively, at the assay pH. Examples of similar $pK_a$ changes have been reported (Nishikura, 1978; Kilmartin et al., 1973).

Another consequence of a close arrangement of His and Asp residues is that their protonation state should be coordinated. When one residue accepts or donates a hydrogen ion, a proton on the other residue would be destabilized or stabilized, respectively. The advantage of such an arrangement is that coordinated acid-base catalysis can occur (Jencks, 1969).

The effects of mutations on the activity of citrate synthase are discussed in terms of a model in which $Asp^{375}$ is ionized while $His^{274}$ is protonated in the oxalacetate–acetyl-CoA–enzyme complex enzyme, and the protonation state of these two amino acids is linked. The rate-limiting step is taken to be the enzyme-catalyzed enolization of acetyl-CoA as previously demonstrated by Eggerer (1965). Recently, Karpusas et al. (1990) have published X-ray crystallographic data on the chicken citrate synthase carboxymethyl-CoA complex and presented a partial mechanism for the action of citrate synthase. Using structural information, these researchers suggested the critical importance of $Asp^{375}$ and $His^{274}$ in the function of citrate synthase and postulated their role in the enolization and condensation steps of the citrate synthase reaction. Here, using mutants and kinetic analyses, we clearly show the functional importance of these residues and have limited the possible ways in which they contribute to catalysis. Using these complementary approaches, we suggest the same role for $Asp^{375}$ and $His^{274}$ in the enolization and condensation steps of the citrate synthase reaction.

*Replacements at $His^{274}$.* Gly substitution for the $His^{274}$ of citrate synthase causes about the same reduction in enzyme activity as Arg substitution at that position (Table III). This implies that a positively charged moiety at position 274, perhaps stabilizing a substrate through electrostatic interactions, is not the critical determinant for activity. However, a protonated histidine could also act as a general acid catalyst. This is quite consistent with the low activities of the mutants that have the nonprotic Gly or very basic Arg side chain at position 274. Since not all activity is lost with either substitution, water in concert with Arg in the $Arg^{274}$ mutant or with another active-site residue (i.e., $Asn^{242}$) in the $Gly^{274}$ mutant may act as a general acid catalyst. Nonetheless, the mutant active centers are catalytically much less efficient than the constellation of residues in the nonmutant enzyme's active site.

*Replacements at $Asp^{375}$.* When $Asp^{375}$, which can act as an acid–base catalyst and/or a hydrogen bond donor/acceptor, is replaced with residues having side chains that do not contain dissociable protons (i.e., Asn, Gln, and Gly), citrate synthase activity is substantially decreased (Table III). Since both Asn and Gln side chains can still form hydrogen bonds similar to Asp and since Asn is even isosteric with Asp, hydrogen bonding does not seem to be a critical role for $Asp^{375}$ in the nonmutant enzyme. However, acid–base properties of $Asp^{375}$ seem critical for the enzyme's function.

The reduced activity of $Glu^{375}$ relative to nonmutant citrate synthase is interesting. Though acid–base reactions are frequently characterized as having transition states that are "loose" or not as geometrically constrained as nucleophilic reactions (Fersht, 1985), Gandour (1981) has pointed out there are appreciable geometric constraints on the direction of proton release and approach. Model building indicates the orientations of the side chain carboxylic acids of Asp and Glu are sufficiently different, assuming the enzyme backbone does not change position, to explain the reduced activity of the $Glu^{375}$ mutant.

*Reaction Sequence for Citrate Synthase.* A reaction sequence for citrate synthase which is consistent with results reported here is presented in Figure 3. The enolization step is thought to be rate limiting (Eggerer, 1965). In this step, $His^{274}$ and $Asp^{375}$ act in concert as general acid and base catalysts, respectively. This maintains the net electrostatic neutrality of the His–Asp pair and allows enolization to occur without formation of an enolate ion. The $pK_a$ of $\alpha$-hydrogens of thioesters is estimated to be about 20 (Wlassics & Anderson, 1989; Lienhard & Wang, 1968), indicating a considerable catalytic advantage could result if concerted acid–base catalysis avoids its formation. The importance of coordinated catalysis in the nonenzymatic enolization of acetone has been reported [see Jencks (1969) and references cited therein; Remington, personal communication].

We postulate that in the condensation step of the reaction $His^{274}$ accepts a proton from the hydroxyl of the enol intermediate as it condenses with oxaloacetate. On the basis of X-ray structure studies (Remington et al., 1982), $His^{320}$ is well positioned to donate such a proton to oxaloacetate's carbonyl oxygen in this step. Protonation of $His^{274}$ is expected to in-

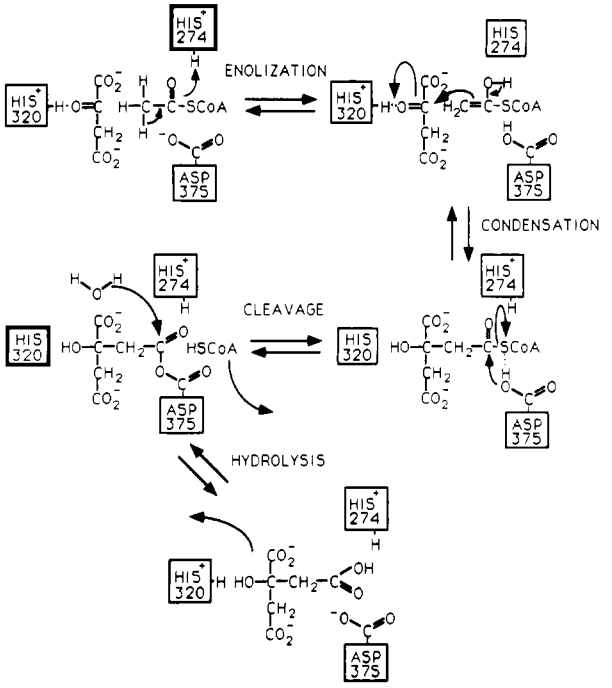


FIGURE 3: Proposed reaction sequence for citrate synthase.

crease the acidity of $Asp^{375}$ which may donate its proton to or share it with the thioester's sulfur. Since sulfonium ions seem to be very good leaving groups (Lillya & Miller, 1966), protonating the thioester sulfur is expected to activate CoA, making it the preferred leaving group [relative to an oxygen-containing leaving group (Jencks, 1969b)] from a tetrahedral intermediate formed in the cleavage step of the reaction (Figure 3).

Eggerer and co-workers (Löhlein-Werhahn et al., 1983) have provided strong evidence that citryl-CoA is an intermediate in the enzyme's reaction mechanism. The formation of an anhydride, internal to citryl-CoA (Buckel & Eggerer, 1969) or involving $Asp^{375}$ (Remington et al., 1982), has been suggested. Though its existence has not been proved and results reported here do not settle the issue, a mixed anhydride is included in the mechanism. Such a role for $Asp^{375}$ is quite consistent with the apparent inactivity of $Asn^{375}$, $Gln^{375}$, and $Gly^{375}$, whose side chains at position 375 cannot participate in anhydride formation. That $Gly^{375}$ retains some activity at all may indicate a water, sterically excluded by the larger side chains of $Asn^{375}$ and $Gln^{375}$, participates in the hydrolysis of citryl-CoA by that mutant enzyme. Interestingly, estimates of $Gly^{375}$ activation energy increases relative to nonmutant citrate synthase (Table III) are greater than those for $Gly^{274}$ and $Arg^{274}$. This is consistent with $Asp^{375}$ having a role in addition to coordinated acid–base catalysis. The smaller activation energy changes associated with $Glu^{375}$ may simply reflect the importance of correct position and orientation of the side chain carboxylic step of the reaction.

In the last step of the reaction, a water protonates $His^{320}$, providing a hydroxide ion that cleaves the mixed anhydride regenerating the enzyme's active site.

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# An Essential Proline in λ Repressor Is Required for Resistance to Intracellular Proteolysis†

John F. Reidhaar-Olson, Dawn A. Parsell, and Robert T. Sauer*
*Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*
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ABSTRACT: $Pro^{78}$ is a solvent-exposed residue at the N-terminal end of α-helix 5 in the DNA binding domain of λ repressor. Random mutagenesis experiments have suggested that $Pro^{78}$ is essential [Reidhaar-Olson, J. F., & Sauer, R. T. (1990) *Proteins: Struct., Funct., Genet.* (in press)]. To investigate the requirement for proline at this position, we constructed and studied the properties of a set of ten position 78 mutant proteins. All of these mutants have decreased intracellular activities and are expressed at significantly lower levels than wild type. Pulse–chase experiments show that the mutant proteins are rapidly degraded in the cell; the mutants examined had half-lives of 11–35 min, whereas the wild-type protein has a half-life of greater than 10 h. The rapid degradation of position 78 mutants is not suppressed by mutations that affect known *Escherichia coli* proteases. The $Pro^{78} \rightarrow$ Ala mutant could be overexpressed in a *dnaJ*⁻ strain and was purified. This mutant has full DNA binding activity in vitro, suggesting that its folded structure and ability to form active dimers are similar to those of wild type. The $PA^{78}$ mutant ($T_m$ = 48 °C) is less thermally stable than wild type ($T_m$ = 55 °C). Double-mutant studies show that this instability contributes to but is not the main cause of its rapid intracellular degradation and also suggest that proteolysis proceeds from the denatured forms of proteins containing the $PA^{78}$ substitution. The $PA^{78}$ mutation does not appear to introduce a new cleavage site for cellular proteases, nor does the mutation enhance susceptibility to proteases such as thermolysin and trypsin in vitro. The mutation does decrease the *m* value in GuHCl denaturation experiments and may alter the properties of the denatured polypeptide, allowing it to be specifically recognized by an *E. coli* protease or auxiliary degradation factor.

In studies of protein structure and function, it is important to know which side chains play the most significant roles. It is often possible to address this question by examining a family of genetically or phylogenetically related protein sequences (Hampsey et al., 1986; Bashford et al., 1987; Bowie et al., 1990). Residues that are highly conserved are likely to be crucial to some aspect of the structure or function of the protein. Residues that accept a variety of substitutions are clearly less important.

We have been studying the mutability of residues in the N-terminal domain of λ repressor (Reidhaar-Olson & Sauer, 1988, 1990). This domain consists of residues 1–92 and mediates the DNA binding activity of λ repressor (Sauer et al., 1979; Pabo et al., 1979). The structure of the N-terminal domain, both alone (Pabo & Lewis, 1982) and complexed with operator DNA (Jordan & Pabo, 1988), is known. In random mutagenesis experiments, we have found that most buried positions in the N-terminal domain are invariant or tolerate only conservative substitutions. In contrast, most surface positions tolerate a wide range of substitutions, although there are exceptions to this rule. One of the most notable exceptions occurs at the first residue of α-helix 5, $Pro^{78}$, which is more than 80% exposed to solvent in the crystal structure (Figure 1). Following random mutagenesis of this position, proline was the only residue recovered in a set of 28 functional sequences, suggesting that other substitutions result in nonfunctional proteins. However, examination of the crystal structure does not reveal any obvious role for the $Pro^{78}$ side chain; it is neither near the operator DNA nor involved in any obvious interactions that might stabilize the protein.

Here we report experiments aimed at understanding why proline is required at position 78. When other residues are substituted at this position, the resulting mutant proteins are subject to rapid degradation in vivo. Biochemical analysis of the $Pro^{78} \rightarrow$ Ala mutant shows that the purified protein has slightly decreased thermal stability, but the folded protein is as active as wild type in binding operator DNA. The thermal instability of the mutant contributes to but is not the sole cause of its rapid degradation. In fact, a multiply mutant protein that contains the $Pro^{78} \rightarrow$ Ala substitution but has wild-type thermal stability is still degraded rapidly and exhibits a defective phenotype in the cell. Overall, these results indicate that $Pro^{78}$ is an essential residue because it protects the wild-type protein from intracellular degradation.

## Materials and Methods

*Strains and Plasmids.* The following *Escherichia coli* K-12 strains were used in this work: strain X90 (Amann et al., 1983) is *ara* Δ(*lac pro*) *nalA argE*am *rif thi-1*/F′ *lac*⁺ *pro*⁺

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