Active Site Mutants of Pig Citrate Synthase: Effects of Mutations on the Enzyme Catalytic and Structural Properties†

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ABSTRACT: We examined the catalytic efficiency of 18 pig citrate synthase mutants. The residues mutated were selected according to two criteria: the conservation of that residue in all known citrate synthase sequences, and the importance of that residue in substrate−amino acid interactions suggested by the extensive crystal structure information on the enzyme and its complexes. Several changes were made at certain residues to probe the effects of size, hydrogen bonding, and charge on the kinetics of the enzyme. The mutations, as expected, affected the k_{cat}s and K_ms for OAA and acetyl-CoA to varying degrees. The catalytic efficiency of each of the mutants was determined by the k_{cat}/K_m for the individual substrates, OAA and acetyl-CoA. All mutations affected k_{cat}. There was only one mutant, Asp327Asn, in which the K_ms primarily were affected. Most mutations affected both k_{cat} and K_m and included the following: His274Gly, His274Arg, Asp375Gly, Asp375Asn, Asp375Glu, Asp375Gln, His320Gly, His320Gln, His320Asn, His320Arg, Arg401His, Gly275Val, and Gly275Ala. The mutations, Arg401Gly, Arg401Lys, His235Gln, and Asn242Glu, had smaller effects on k_{cat} and K_m. The CS mutant Arg401Lys exhibited a modestly improved k_{cat}/K_m for both substrates compared to the nonmutant enzyme. X-ray crystallographic studies at 2.7 Å resolution of one of the mutants, His274Gly, have been undertaken. The mutant enzyme crystallizes in an "open" conformation essentially isomorphous to wild type. The refined model has good geometry and a crystallographic *R* factor of 0.187 for 11 441 reflections observed between 6.0 and 2.7 Å resolution. The refined model revealed a localized relaxation of the structure to relieve strain imposed by a high-energy main and side chain conformation of His274 in the nonmutant, but otherwise the mutation does not result in major structural alterations. Preliminary electrostatic calculations provide support for the concept that the transition state in the rate-limiting step of the citrate synthase catalyzed reaction may be an "enolized" version of acetyl-CoA that is neither neutral nor fully negatively charged and that a possible role for the catalytically essential His274 is to stabilize this by charge delocalization mediated by a hydrogen bond. These results provide the basis for further studies of the effects of these changes on the several reactive intermediates, activated substrates, and transition states which may occur along the reaction coordinate for this type of Claisen enzyme.

The current working hypothesis for the mechanism of the CS-catalyzed reaction[1] is shown in Figure 1A. First, binding of OAA to the enzyme active site causes a large conformational change from an open to a closed form in which His320 causes polarization of the OAA carbonyl bond, thereby increasing the positive charge on the 2-carbon of OAA, making it more susceptible to condensation with acetyl-CoA. Second, binding of acetyl-CoA to the CS−OAA complex is followed by a concerted acid−base reaction where Asp375 is the general base, and His274 is the general acid. This causes an enolization of acetyl-CoA to yield an "enolized" intermediate, the proposed nucleophile, that may be neutral or partially negatively charged (Figure 1B). Additional conformational changes are believed to accompany this step. Third, the nucleophile condenses stereospecifically with the si-face of the activated OAA to give citryl-CoA. Citryl-CoA is hydrolyzed, possibly utilizing Asp375, to yield citrate and CoA. Lastly, a conformation change from a closed form to an open form occurs to release the products. This scheme was derived from a variety of experiments, including crystallography (Remington *et al.*, 1982; Karpusas *et al.*, 1990; Liao *et al.*, 1991; Remington, 1992; Usher *et al.*, 1994), site-directed mutagenesis (Evans *et al.*, 1988a,b, 1989; Alter *et al.*, 1990; Zhi *et al.*, 1991), NMR of ^{13}C-labeled substrate (Kurz *et al.*, 1992, 1995), and isoelectric focusing (Kurz *et al.*, 1992).

We have prepared additional mutants of CS in order to obtain a more detailed knowledge of the CS mechanism. Studies on mutant forms of this enzyme, thus far, have examined two of the three catalytic residues, His274, and Asp375 (Evans *et al.*, 1989; Alter *et al.*, 1990; Zhi *et al.*, 1991; Kurz *et al.*, 1992, 1995). In this paper, we report on the initial rate kinetic constants of mutants of the three

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[1] Abbreviations: CS, citrate synthase; OAA, oxalacetate; PCS, recombinant pig citrate synthase; PHCS, authentic pig heart citrate synthase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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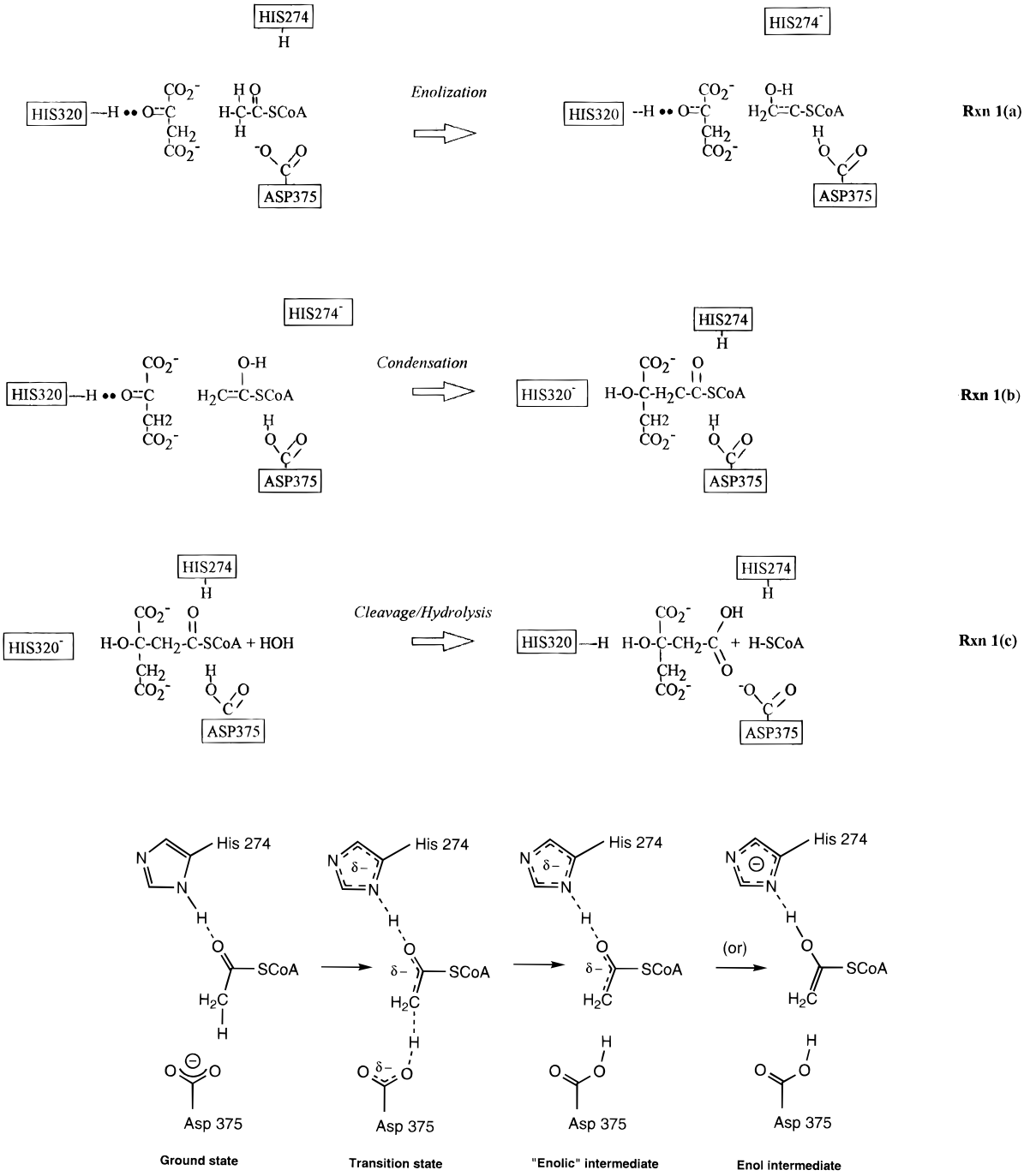

FIGURE 1: (A) Working hypothesis for the reaction mechanism of PCS. Three consecutive partial chemical reactions occur at each of the two active sites of the dimer: (a) enolization, (b) condensation, and (c) cleavage/hydrolysis. Neutral His274 and His320 are shown and represent current thinking on this subject. (B) Schematic showing the proposed charge distributions for the ground state, transition state and two possible intermediates in the rate-limiting step of the CS catalyzed reaction, in which a proton is abstracted from the nucleophilic methyl carbon of acetyl-CoA.

catalytic residue(s) and five other residues in the active site or putatively involved in a conformation change. The residues that were mutated were chosen because of their conservation in all sequenced CSs (Table 1), and their various roles were deduced from the crystal structure of the enzyme.

MATERIALS AND METHODS

Mutagenesis. The approach involves creating mutations by *in vitro* site-directed double base pair mismatched oligonucleotide-mediated mutagenesis and selection in *dut*⁻ strains of *Escherichia coli* as described (Kunkel, 1985; Evans *et al.*, 1989; Alter *et al.*, 1990). The cDNA fragments (Table 2) that encode the nonmutant PCS and mutant PCSs (His320 to Gly, Gln, Asn, and Arg; Asp327 to Asn; Gly275 to Ala and Val; Arg401 to His, Gly, and Lys; His235 to Gln; and Asn242 to Glu) were prepared, ligated in-frame into an expression vector, and characterized by restriction enzyme analysis. Mutagenesis rates were greater than 80%, and mutations were confirmed by DNA sequencing.

Enzyme Preparation. The cells, *Escherichia coli* DEK15, containing the mutant and nonmutant CS expression vectors (Evans *et al.*, 1988a,b, 1989) were grown in the presence of antibiotics at 30 °C in either L-broth (Maniatis *et al.*, 1982) or supplemented M9 (Evans *et al.*, 1989). The ability of

Table 1: Homologous Active Site Amino Acids in 20 CS Proteins[a]

	acetyl-CoA binding residues											citrate-OAA binding residues					
	273	314	315	316	317	318	319	321	322	368	375	238	274	320	329	401	421
Ph	L	V	V	P	G	Y	G	A	V	tmk	D	H	H	H	R	R	R
Ck	L	V	V	P	G	Y	G	A	V	K	D	H	H	H	R	R	R
Y1	L	V	V	P	G	Y	G	A	V	K	D	H	H	H	R	R	R
Y2	L	V	I	P	G	Y	G	A	V	K	D	H	H	H	R	R	R
Nc	L	V	V	P	G	Y	A	A	V	K	D	H	H	H	R	R	R
Tt	L	V	V	P	G	Y	G	A	V	A	D	H	H	H	R	R	R
At	L	V	I	P	G	Y	G	G	V	E	D	H	H	H	R	R	R
Ta	L	R	L	M	G	F	G	R	V	K	D	H	H	H	R	R	R
C2	L	·	M	S	G	Y	G	G	V	R	D	H	H	H	R	R	R
Cg	L	R	L	M	G	F	G	R	V	R	D	H	H	H	R	R	R
Rp	A	R	L	M	G	F	G	R	V	R	D	H	H	H	R	R	R
Cb	A	R	L	M	G	F	G	R	V	K	D	H	H	H	R	R	R
Bh	A	R	L	M	G	F	G	R	V	K	D	H	H	H	R	R	R
Pa	A	K	L	M	G	F	G	R	V	R	D	H	H	H	R	R	R
Aa	A	R	L	M	G	F	G	R	V	R	D	H	H	H	R	R	R
Ec	A	R	L	M	G	F	G	R	V	K	D	H	H	H	R	R	R
B1	L	K	I	M	G	F	G	R	V	K	D	H	H	H	R	R	R
B2	L	R	L	M	G	F	G	R	V	R	E	H	H	H	R	R	R
B3	L	K	I	M	G	F	G	R	V	K	D	H	H	H	R	R	R
Ms	L	K	V	M	G	F	G	R	V	T	D	H	H	H	R	R	R

[a] Alignments determined using the PC Gene Intelligenetics Sequence Software Program and PILEUP. Amino acids are numbered according to the pig citrate synthase protein sequence. Single-letter amino acid abbreviations are used. Trimethylysine is indicated by tmk. Organism names are abbreviated as follows: Ph, pig heart CS; Ck, chicken CS; Y1, mitochondrial yeast CS; Y2, peroxisomal yeast CS; Nc, *Neurospora crassa*; Tt, *Tetrahymena thermophilia* CS; At, *Arabidopsis thaliana* CS; Ta, *Thermoplasma acidophilum* CS; C2, *Cucurbita* peroxisomal CS; Cg, *Corynebacterium glutamicum* CS; Rp, *Rickettsia promazekii* CS; Cb, *Coxiella burnetii* CS; Bh, *Bartonella henselae* CS; Pa, *Pseudomonas aeruginosa* CS; Aa, *Acinetobacter anitratum* CS; Ec, *Escherichia coli* CS; B1, *Bacillus subtilis* CS(Z); B2, *Bacillus subtilis* CS(A); B3, *Bacillus coagulans* CS(A); Ms, *Mycobacterium smegmatis*.

Table 2: Nucleotide Sequences for Mutant PCSs

His274 →	Gly	GGG	CCC	CTA	**GGT**	GGG	CTG	GCA
His274 →	Arg	GGG	CCC	CTA	**CGA**	GGG	CTG	GCA
Asp375 →	Gly	CCC	AAT	GTG	**GGA**	GCT	CAC	AGT
Asp375 →	Asn	CCC	AAT	GTG	**AAC**	GCT	CAC	AGT
Asp375 →	Glu	CCC	AAT	GTG	**GAG**	GCT	CAC	AGT
Asp375 →	Gln	CCC	AAT	GTG	**CAG**	GCT	CAC	AGT
His320 →	Gly	GGC	TAT	GGC	**GGC**	GCA	GTA	CTA
His320 →	Gln	GGC	TAT	GGT	**CAG**	GCA	GTA	CTA
His320 →	Asn	GGC	TAT	GGC	**AAT**	GCA	GTA	CTA
His320 →	Arg	GGC	TAT	GGC	**CGT**	GCA	GTA	CTA
Arg401 →	His	GGG	GTA	TCA	**CAT**	GCA	CTG	GGT
Arg401 →	Gly	GGG	GTA	TCA	**GGT**	GCA	CTG	GGT
Arg401 →	Lys	GGG	GTA	TCA	**AAG**	GCA	CTG	GGT
Asp327 →	Asn	AGG	AAG	ACT	**AAC**	CCA	CGA	TAT
Gly275 →	Ala	CCC	CTA	CTA	**GCT**	CTG	GCA	AAT
Gly275 →	Val	CCC	CTA	CTA	**GTT**	CTG	GCA	AAT
His235 →	Gln	TAC	CTC	ACC	**ATT**	**CAA**	AGT	GAC
Asn242 →	Glu	GAA	GGT	GGC	**GAA**	GTA	AGT	GCT

the transformants to complement the *glt*A mutation was tested by growing cells on M9 with either 2.0 mM glutamate or 100 mM acetate as the carbon source.

Protein Purification. An affinity chromatography method was developed using a Reactive Yellow 3 agarose column (Sigma Chemical Co., St. Louis, MO) to purify CS and the CS mutant proteins. The main advantage of this purification procedure over the ATP-Sepharose method (Evans *et al.*, 1988b; Alter *et al.*, 1990) was the ability to purify the mutant and nonmutant CS enzymes in the absence of substrates, which when tightly bound may interfere with other mechanism studies in which stoichiometric enzyme amounts are required. Nonmutant and mutant CS proteins were purified individually from the respective *E. coli* transformants following a modification of the standard procedure (Evans *et al.*, 1988b). In order to increase the yield of the mutant CS proteins from the reactive yellow column, the mutant proteins were dialyzed against 2 × 2 L of buffer B at lower ionic strength (5 mM). The dialyzate was loaded onto 15 mL of the Reactive Yellow column (1.5 × 9.0 cm) equilibrated with buffer B. The column was washed with 10 column volumes of buffer B at 0.5 mL/min to elute the nonspecifically adsorbing proteins, and then PCS or the PCS mutants were eluted with a linear gradient of NaCl (100 mL, 10−500 mM in 50 mM Tris-HCl, pH 7.5). Each fraction was measured for protein concentration, CS activity, and ionic strength. The peak fractions containing the mutant PCS proteins (180 mM NaCl) were verified by their co-elution with a [U-^{14}C]-labeled PCS standard and by specific α-PCS immunoreaction. The nonmutant PCS purified identically as the enzyme isolated from pig heart muscle (PHCS). The other mutant PCS proteins, with the exceptions noted in the Results, also purified like the nonmutant. The eluted proteins were concentrated by Amicon ultrafiltration or precipitated with an 80% saturated solution of ammonium sulfate then stored at 4 °C.

Protein Quantitation. Proteins were determined by the Bradford method (Bradford, 1976) and by absorbance at 280 nm using the extinction coefficient ($\epsilon_{cm}^{1\%} = 17.8$) (Singh *et al.*, 1970).

Determination of Protein Purity. The purity of PCS and the PCS mutants was evaluated by 7% SDS−polyacrylamide gel electrophoresis (Laemmli, 1970) using calibrated molecular weight standards (Sigma Chemical Co., St. Louis, MO). The gels were stained for 1 h at room temperature with a solution of Coomassie Brilliant Blue R and destained overnight in 30% methanol, 10% glacial acetic acid.

Quantitative Immunoblot Analysis of CS. Two solid phase protein immunodetection methods (a radiochemical and a horseradish peroxidase coupled method) have been used successfully in this laboratory with our CS antibodies (Nemeth *et al.*, 1991). Gel electrophoresis was carried out on SDS−polyacrylamide slab gels (7%) (Laemmli, 1970) which were calibrated with the prestained molecular weight standards as above. The relative densities of the radiolabeled bands were determined for each experiment using an LKB 2222-020 Ultroscan XL laser densitometer at 633 nm. Peaks were integrated using the LKB internal integrator and line printer and expressed as mean ± SEM.

PCS Enzyme Assay. The effect of each active site amino acid substitution on the overall PCS reaction rate was studied by initial velocity kinetic analysis of the purified mutant enzymes. The forward steady state reaction for PCS and the mutants was monitored continuously for 30 min. PCS activity was determined by measuring the increase in absorbance at 412 nm when 5,5′-dithiobis(2-nitrobenzoate) (DTNB) reacts with the enzymically produced reduced CoA. Routine PCS assays were performed as previously reported (Srere *et al.*, 1963). Specific activity is defined in units/mg of protein, where 1 unit is defined as the liberation of 1 μmol of CoA/min at 25 °C using $\epsilon = 13.6$/mM/cm, and protein as measured by the method of Bradford (1976). Kinetic constants were determined by measuring the initial velocity of the PCS reaction when one substrate was saturating and the other substrate was limiting. Data were plotted, and the kinetic constants were calculated by three different methods using an enzyme kinetics software package (Trinity Software/Campton, NH). Results from Eadie−Hofstee, unweighted Lineweaver−Burk, and Hanes−Wolf plots were in agreement with each other.

Sequencing. The cDNA amplification products were purified (Maniatis *et al.*, 1982) and subcloned into M13mp18 and pUC19. Sequencing (Sanger *et al.*, 1977) was performed by using the universal and reverse M13/pUC primers, and oligonucleotide primers derived from the internal PCS coding sequence located every 200 bp apart (Evans *et al.*, 1988a). Sequencing was performed on both strands and on four independent recombinant clones encoding the same cDNA sequence. Sequencing reactions were loaded in the order ACGT and GATC to confirm the DNA sequences.

Protein Sequence Analysis of PCS. Amino acid sequence information was acquired by automated Edman degradation with a model 477A sequencer from the Applied Biosystems Division of Perkin Elmer (Foster City, CA) using the manufacturer's standard programming and chemicals. For N-terminal sequence analysis, 5 μg (110 pmol of monomer) of enzyme was repurified for sequencing by SDS−PAGE followed by electroblotting to Immobilon-SQ paper (Millipore Corp., Bedford, MA). The protein band was located by staining with Coomassie Blue and excised according to the method of Matsudaira (1987).

X-ray Crystal Analysis of PCS. The mutant His274Gly was the only one of the mutants that could be crystallized. Crystals were grown by the hanging drop method of vapor diffusion. Approximately 10 μL of protein [absorbance of 15 at 280 nm (1 cm)] in 25 mM Hepes (pH 7.5) was mixed with an equal volume of the well solution [1 mL 10%−15% (w/v) PEG 4000, 50 mM KPO_4, pH 7.5], sealed, and heated to 37 °C for 10 min, and then allowed to equilibrate at room temperature for several weeks. Well-formed tetragonal crystals appeared in 1−2 weeks, reaching maximum dimensions of about $0.4 \times 0.4 \times 1.0$ mm^3 and appeared to be approximately isomorphous to the "open-form" crystals described previously for PHCS (Remington *et al.*, 1982). Data were collected on a Xuong−Hamlin multiwire detector and reduced with the supplied software (Howard *et al.*, 1985). Cell dimensions were somewhat different to wild type, so rigid body refinement was initiated using the open-form structure (Protein Data Bank entry 1CTS), followed by standard crystallographic refinement using the TNT suite of programs (Tronrud *et al.*, 1987) and manual inspection with Frodo (Jones, 1982). No solvent or substrate molecules were incorporated into the atomic model. Temperature factors were restrained such that bonded atoms were to have similar values and to lie within the range of 5−75 Å^2.

RESULTS

Primary Structure of CS and Mutants. When the amino acid composition of PHCS was compared to the PCS, the two were the same except for the amino acids contributed by the mitochondrial leader sequence in the cDNA clone. The presence of a trimethyllysine in the PHCS was established and was not found in the PCS and is consistent with the lack of the methylase in *E. coli* (Evans *et al.*, 1988b). The primary N-terminal amino acid sequences were the same for PHCS and the *in vitro* expressed PCS, except for the five-amino acid N-terminal extension in the recombinant CS due to the subcloning strategy. N-terminal sequencing confirmed that *E. coli* efficiently cleaves the N-terminal Met in the expressed recombinant enzyme. PHCS had a variable N-terminus one to three amino acid residues longer than the reported N-terminus (Bloxham *et al.*, 1982). The amino acids present in the first 3 Edman cycles were consistent with the addition of one to three amino acids from the N-terminal leader sequence. The major peak was Ala, and this was assigned as the N-terminal residue. The variability observed in the first residue could be explained by differences in the cleavage of the mitochondrial leader sequence in PHCS. We reconstructed the PCS clone, deleted the five amino acids, and placed an initiator methionine residue before the first Ala in the mature enzyme. The reconstructed PCS clone was completely resequenced, and only one base pair substitution was found in the entire 1383 bp of sequence. When expressed in *E. coli* with the vector used in this work,[2] this slightly shorter mRNA was not as stable as the mRNA encoding the slightly longer CS protein. Consequently, to improve yield of the mutant proteins, mutagenesis was performed on the slightly longer CS clone. We previously established that the observed kinetic properties of nonmutant PCS (recombinant) and PHCS (native) are the same (Evans *et al.*, 1988b). The sequence of each of the PCS mutants (Table 2) was determined (Sanger *et al.*, 1977), and only

[2] In other recent unpublished work, Dr. Kurz, using a different vector and our PCS cDNA, has prepared PCS with the proper length with no reduction in enzyme yield, verifying our initial observations.

Table 3: Pig Citrate Synthase Mutant Proteins Expressed in *E. coli*[a]

mutation	growth acetate	PCS activity (units/mg)
CS$^-$	−	0.0 ± 0.0
nonmutant	+	4.9 ± 0.13
His274 → Gly	−	0.005 ± 0.001
His274 → Arg	−	0.004 ± 0.004
Asp375 → Gly	−	0.006 ± 0.003
Asp375 → Asn	−	0.001 ± 0.001
Asp375 → Glu	−	0.012 ± 0.003
Asp375 → Gln	−	0.004 ± 0.003
His320 → Gly	−	0.019 ± 0.003
His320 → Gln	−	0.001 ± 0.001
His320 → Asn	−	≤0.001
His320 → Arg	−	0.003 ± 0.002
Arg401 → His	−	0.004 ± 0.001
Arg401 → Gly	+	5.80 ± 0.04
Arg401 → Lys	+	4.8 ± 0.14
Asp327 → Asn	−	0.036 ± 0.003[b]
Gly275 → Ala	+	4.9 ± 0.13
Gly275 → Val	−	0.110 ± 0.003
His235 → Gln	+	4.54 ± 0.1
Asn242 → Glu	+	8.4 ± 0.1

[a] CS and protein assays on crude cell lysates were performed in triplicate on $n = 3-6$ independent cultures. [b] Reactions were run at 25 °C in 100 mM Tris-HCl, pH 8.1, containing 100 μM each OAA and acetyl-CoA. For the Ala275 mutant these concentrations of substrates were not saturating.

Table 4: Kinetic Constants for Pig Citrate Synthase Mutants[a]

mutation	k_{cat} (s^{-1})	K_m OAA (μM)	k_{cat}/K_m [b] (OAA)	K_m AcCoA (μM)	k_{cat}/K_m [b] (AcCoA)
nonmutant	90.1[c]	4.6[c]	19.6	7.8[c]	11.6
recombinant	96.0[c]	4.9[c]	19.6	7.7[c]	12.5
His274 → Gly	0.10[d]	3.5[d]	0.03	5.4[d]	0.02
His274 → Arg	0.15[d]	3.8[d]	0.04	6.5[d]	0.02
Asp375 → Gly	0.06[d]	3.27	0.02	*e*	0.008 [f]
Asp375 → Asn	0.01[d]	1.18[g]	0.008	*e*	0.001 [f]
Asp375 → Glu	0.23[d]	0.6[d]	0.38	4.1[d]	0.06
Asp375 → Gln	0.02[d]	2.19[g]	0.009	*e*	0.002 [f]
His320 → Gly	0.22	54.0	0.004	109.0	0.002
His320 → Gln	0.53	85.0	0.006	126.0	0.004
His320 → Asn	0.02	19.9	0.001	6.5	0.003
His320 → Arg	0.01	1.0	0.010	6.8	0.001
Arg401 → His	0.01	22.0	0.001	18.5	0.001
Arg401 → Gly	49.7	2.7	18.4	4.4	11.3
Arg401 → Lys	66.1	2.6	25.4	4.1	16.1
Asp327 → Asn	56.1	63.1	0.89	59.2	0.95
Gly275 → Ala	14.0	6.9	2.03	15.9	0.88
Gly275 → Val	0.01	34.7	0.001	15.2	0.001
His235 → Gln	59.2	3.0	19.7	4.7	12.6
Asn242 → Glu	83.2	10.0	8.3	5.3	15.7

[a] Reactions were run at 25 °C in 100 mM Tris-HCl (pH 8.1). Steady state reactions contained between 100 and 1000 μM each substrate where one was limiting (3−100 μM) and the second was saturating. Kinetic constants were calculated by three different methods as described under Materials and Methods. [b] k_{cat}/K_m units are s^{-1} M^{-1}. [c] From Evans *et al.* (1988b). [d] From Alter *et al.* (1991). [e] All bind the transition state analog inhibitor CMCoA 10-fold tighter in the ternary complex than the nonmutant, but nonlinear Lineweaver−Burk plots made K_m determination difficult. [f] Assuming that the K_m of the mutant is similar to the nonmutant. [g] K_d.

the site-directed mutated residue was changed in a 50-amino acid stretch on either side of the mutated residue. Therefore, changes in the activity, physical properties, and structure of PCS due to single amino acid changes could be assessed.

Growth Studies. Preliminary assessment of the mutant enzymes activities was made by the growth of each of the *E. coli* DEK15 *glt*A transformants. Table 3 summarizes the mutations, *in vivo* activities, and the aerobic growth characteristics of each *E. coli* DEK15 *glt*A transformants containing mutant or nonmutant CS on minimal acetate media. All mutants grew on glutamate.

The activities of the PCS mutant enzymes correlated with the acetate growth phenotypes. In general, acetate$^-$ cells contained PCS activities 2−3 orders of magnitude lower than the wild type when enzyme activity was assayed in crude cell lysates (Table 3). The Arg401Gly, Arg401Lys, His235Gln, and Asn242Glu mutants were acetate$^+$ and had PCS activities equal to or greater than the nonmutant, while Arg401His and all the mutants of His274, Asp375, and His320 were acetate$^-$ and had 10^2-10^3-fold decreases in PCS activity. The mutation of Asp327 to Asn327 resulted in an acetate$^-$ phenotype and a 100-fold lower PCS activity in cell lysates. Mutations of Gly275Ala and Gly275Val were also isolated and differed in their phenotypes and CS activities. An increase in the length of the side chain from one to two carbons in the Gly275Ala and Gly275Val mutants dramatically decreased the activity of the enzyme. Gly275Ala grew on acetate and had PCS levels like the nonmutant, while Gly275Val was acetate$^-$ and had a 50-fold lower level of PCS activity in cell lysates.

Purification of the CS Mutants. Nonmutant and mutant PCS proteins were expressed and purified from *E. coli*. The purification method is rapid and convenient, and final enzyme recoveries were routinely between 30% and 40% of the initial enzyme activity. An initial assessment of the effects of the amino acid substitutions on the overall conformation of the protein can be obtained from the behavior of the mutant proteins during affinity column chromatography. His320Arg had a higher affinity for Reactive Yellow agarose which was similar to its characteristics on ATP-Sepharose (Alter *et al.*, 1990). Both of the active site Arg replacements caused the proteins to elute over a broad ionic strength range (150−300 mM NaCl) with significant portions of the mutant protein eluting in fractions trailing a smaller protein peak at 180 mM NaCl. The eluted fractions containing the mutant proteins were pooled and concentrated. Final recoveries of enzyme were approximately 3-fold lower in the purified PCS Arg mutant samples than the other PCS mutant proteins.

Upon inspection by SDS−PAGE and by protein blot analysis, the PCS mutants were homogeneous preparations (MW 50 000) that reacted with the α-PCS antibodies like authentic PCS (data not shown). There was no indication of the presence of degradation products in the purified Arg mutant PCS samples as evidenced by the absence of Coomassie staining or immunoreacting protein bands of lower molecular weight in protein gels and blots.

Kinetic Characterization of the PCS Mutants. All the mutations tested in this work had an effect on the kinetics of PCS (Table 4). Mutation of the residues His274, Asp375, and His320 had major effects on k_{cat}. All the mutants of His274, Arg375, and His320Arg had lower K_ms for OAA and acetyl-CoA (1.4−8-fold) than the nonmutant enzyme. Mutation of His320 to Asn, Gln, and Gly had higher K_ms for OAA and acetyl-CoA (4.3−18.5-fold) than the nonmutant enzyme. Asp327 is a residue that occurs in the layer of residues surrounding the active site, and mutation to Asn had a major effect on increasing the K_ms of both substrates. The Asp327Asn mutation resulted a small 1.6-fold reduction in k_{cat}, a 14-fold increase in the K_m for OAA, and an 8-fold increase in K_m for acetyl-CoA, resulting in a 12−22-fold decrease in the k_{cat}/K_ms for each substrate. A 10^4-fold

Table 5: Data Collection and Atomic Model Statistics for His274Gly

space group	$P4_12_12$
unit cell *a* (Å)	78.0
unit cell *c* (Å)	198.0
resolution (Å)	2.7
no. of obsd reflections	33 702
no. of unique reflections	12 743
R_{merge}[a]	0.027
completeness (%)[b]	72
crystallographic *R* factor[c]	0.187
shell resolution (Å)	2.84−2.7
completeness (outermost shell)	37
R factor (shell)	0.30
no. of protein atoms	3323
no. of solvent atoms	0
average *B* factor (Å^2)	26.1
deviations from ideality	
bond lengths (Å)	0.015
ideal bond angles (deg)	2.5
no. of inverted chiral centers	0
temperature factors (Å^2)[d]	1.9

[a] $R_{merge} = \sum|I - \langle I\rangle|/\sum I$ where $\langle I\rangle$ is the average of the individual measurements of intensities *I*. [b] Completeness is the number of observed reflections divided by the number of theoretically possible expressed as a percentage. [c] Crystallographic *R* factor $= \sum|F_{obs} - F_{calcd}|/\sum F_{calcd}$. [d] This parameter is the root mean square difference in temperature factors between bonded atoms and is constrained to be similar by the refinement procedure.

Table 6: Free Energies ΔG_s of Transfer from Water to the Low-Dielectric Active Site of Citrate Synthase[a]

	ε				
charge location	80	10	4	2	1
Asp375 (ground state)	0	8	20	38	71
all atoms (transition state)	−89	−84	−76	−64	−43
"enolized" intermediate	−51	−45	−36	−22	3
His274 (neutral enol)	76	83	94	111	141
$\Delta\Delta G_s$ (ground to transition state)	−89	−92	−96	−102	−114

[a] In kcal mol^{-1} for a net charge of (−1) distributed equally over the ground state or the proposed transition state or intermediates in the citrate synthase catalyzed reaction as suggested by Figure 1B. The self-energies were calculated using DELPHI on a grid of 0.33 Å as described in the text, with the ground state in water taken as the reference state. $\Delta\Delta G_s$ is the change in energy from the ground state to the transition state and represents that component of the transition state stabilization due to the polarization of the surrounding solvent.

reduction in k_{cat} and an increase in the K_ms for OAA and acetyl-CoA were also observed when Arg401, a residue which forms a hydrogen bond with citrate, was replaced with His. The Arg401His mutant had 5- and 2.5-fold increases in the K_ms for OAA and acetyl-CoA, respectively, resulting in a 10^5-fold decrease in k_{cat}/K_m. It should be noted, however, that other substitutions for Arg401, Arg401Gly and Arg401Lys, had moderate decreases in the k_{cat} and the K_ms for OAA and acetyl-CoA. Hence, only a modest or no change in the k_{cat}/K_m for the individual substrates was observed in these mutants. The mutants, His235Gln and Asn242Glu, behaved in a similar fashion. His235 and Asn242 are conserved residues in CS that occur in the second shell of residues surrounding the active site pocket, but mutation of these residues did not greatly affect k_{cat} or K_ms.

Mutation of Gly275 to Val, in which a putative hinge region residue is mutated, caused a 10^4-fold reduction in k_{cat} and increases in the K_ms for OAA and acetyl-CoA by 7- and 2-fold, respectively, resulting in a 10^5-fold decrease in k_{cat}/K_m. Gly275Ala caused a 7-fold reduction in k_{cat} and a 1.5−2-fold increase in the K_ms for OAA and acetyl-CoA. These results may be a consequence of the mutation interfering with the substrate-induced conformational change from the open to the closed form (or *vice versa*) when an amino acid of larger size occupies this critical hinge position in the enzyme structure.

Structural Characterization of a PCS Mutant. The mutant His274Gly crystallized essentially isomorphous to the wild type pig heart enzyme in space group $P4_12_12$, with one subunit in the asymmetric unit, although the unit cell parameters differed by about 1%. Crystallographic data collection and atomic model statistics for the mutant His274Gly are presented in Table 6. The final *R* factor for all 11 441 reflections observed between 6.0 and 2.7 Å resolution was 0.187, and the model has good geometry. No electron density was observed for residues preceding residue 4, or beyond residue 434, so these are not included in the atomic model. No solvent molecules were included.

Overall, the atomic model is very similar to the "open form" of the enzyme described previously (Remington *et al.*, 1982). After optimal superposition of the α-carbons of the wild type structure on the mutant, an rms deviation of 0.48 Å is obtained, with a shift of 1.0 Å and rotation of 1.7°, caused by a slight repacking of the molecules in the crystal. An overlay of the α-carbon backbones is shown in Figure 2A. There is a large cleft between the small domain (residues 275−381) and the large domain which consists of the remaining residues, with the exception that residues 426−434 form a helix that crosses over to become part of the large domain of the second subunit. The only difference made by the mutation is a slight collapse of the main chain at residue His274 into the cavity created by removal of the methylimidazole side chain. A stereoview of the active site is shown in Figure 2B. No substrates are bound in this crystal form of the enzyme, thus the model does not directly address the effects this change may have on substrate binding.

Electrostatic Calculations. Electrostatic self-energies for models of the ground state, proposed transition state, and two different models of the intermediate in the CS-catalyzed reaction were calculated using the program DELPHI (Sharp & Honig, 1990; Gilson *et al.*, 1988; Gilson & Honig, 1988) using an dielectric constant of 2 for the interior of the atoms and several external fixed dielectric constants. The grid spacing was 0.33 Å, and standard van der Waals radii were assumed. A model of the active site was constructed using atomic coordinates of the carboxylate of Asp375 (four atoms), the carboxylate of carboxymethyldethiacoenzyme A (four atoms), and the side chain of His274 (six atoms) as determined by Usher *et al.* (1994). An overall net negative charge of −1 was assumed, and this was distributed equally over (1) Oδ1 and Oδ2 of Asp375 in the ground state; (2) Oδ1 and Oδ2 of Asp375, the methyl and carbonyl oxygen of acetyl CoA, and Nδ1 and Nϵ2 of His274 in the proposed transition state as suggested in Figure 1B; (3) Nδ1 and Nϵ2 of His 274 and the methyl and carbonyl oxygen of the "enolized" intermediate; and (4) Nδ1 and Nϵ2 of His 274 as a model of the neutral enol intermediate. The change in the total system energy term output by the QDIFFXS module of the DELPHI package, upon subtracting the value obtained for the ground state in water ($\epsilon = 80$), is the change in self-

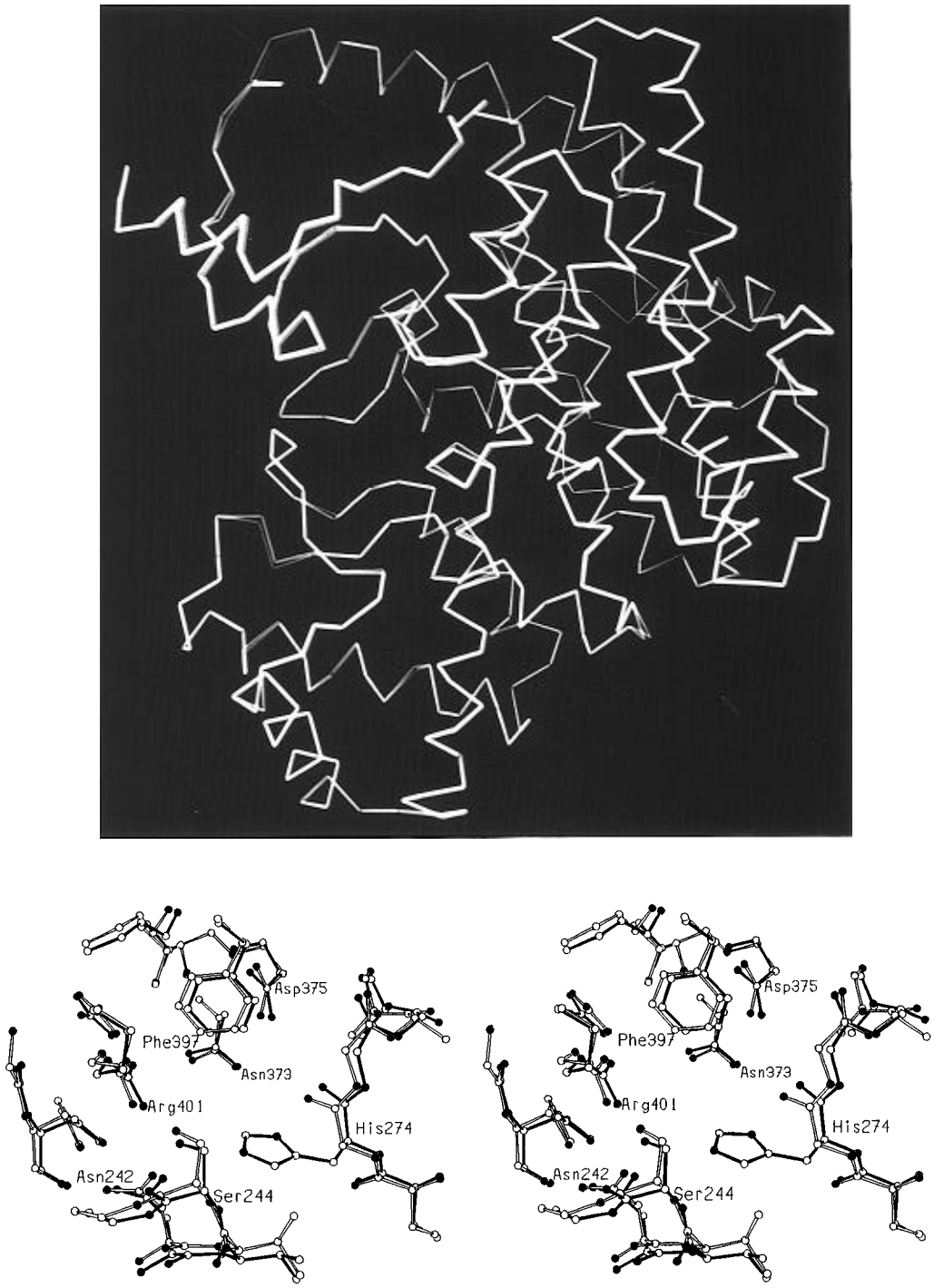

FIGURE 2: (A, top) Overlay of the α-carbon backbones of the wild-type and mutant His274Gly. The site of the mutation is essentially in the center of the image where the largest deviation of the two backbones occurs. (B, bottom) Stereoview of an overlay of the models of the wild-type and mutant His274Gly of CS. The active site is in the "open" configuration in the absence of substrates. Carbon atoms are filled, and nitrogen and oxygen atoms are shaded, and the bonds of wild type are filled. The removal of the histidine side chain results in a slight collapse of the main chain toward the active site.

energy of the system of charges due to the polarization of the surrounding solvent as discussed by Gilson and Honig (1988). These quantities correspond to the free energies of transfer of the ground state, transition state, and intermediate models from water to the enzyme active site for the proposed values of the protein dielectric. Of course, this treatment ignores the energetics of bond formation and breakage and entropic effects not related to the electrostatic component of the reaction, but these should not depend strongly on the local dielectric constant.

DISCUSSION

Previous kinetic and structural studies of the mechanism of CS have established that activation of both substrates, OAA and acetyl-CoA, and large protein conformation changes are integral parts of the catalytic strategy of the enzyme (Remington *et al.*, 1982; Evans *et al.*, 1989; Alter *et al.*, 1990; Zhi *et al.*, 1991; Kurz *et al.*, 1992). These earlier investigations have led to a working hypothesis of the mechanism of the enzyme (Figure 1). Recent advances in gene technology, protein engineering, and structural biology

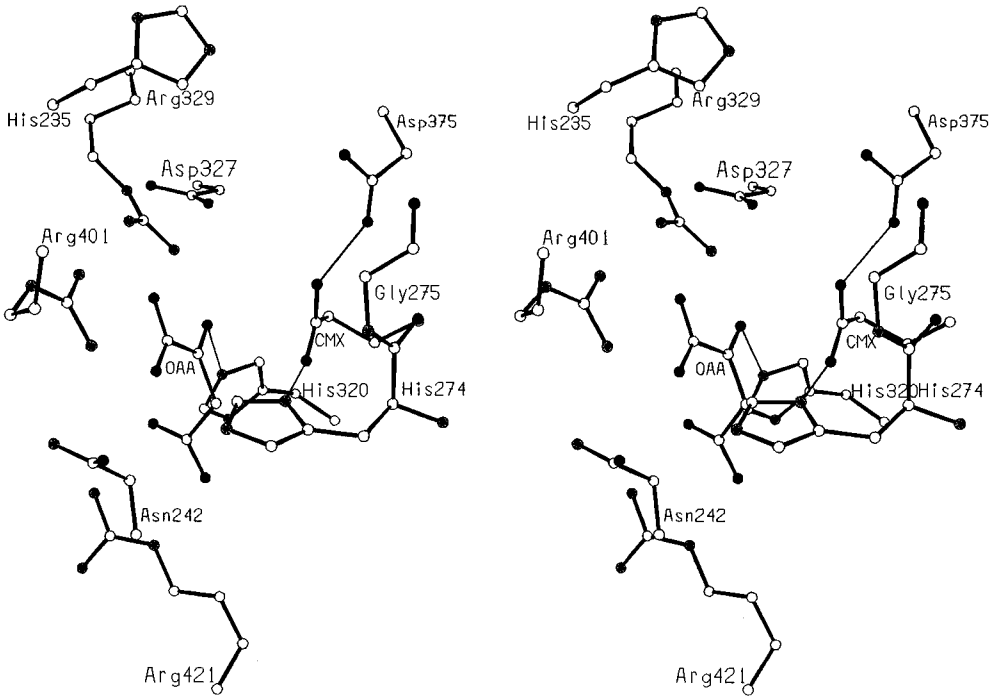

FIGURE 3: Stereodiagram showing the configuration of the active site of CS, with bound oxaloacetate and an inhibitor, carboxymethyldethiacoenzyme A. All of the amino acids discussed in this work are shown in their configurations as determined by Usher *et al.* (1994). Carbon atoms are filled, and nitrogen and oxygen atoms are shaded. Hydrogen bonds from the critical catalytic residues His320, His274, and Asp375 to the substrate or inhibitor are indicated with thin lines.

allow the selective substitution of residues implicated as catalytically important with other residues which cannot interact with substrates or intermediates or must interact differently as a consequence of their changed size or charge. The resulting pattern of changes in the catalytic constants of the enzyme then allow a rough classification (Fersht *et al.*, 1987a,b) of the changes in the binding energies in the bound substrates and in the transition state. If a mutation lowers k_{cat} with little or no effect on K_ms, then the stability of one or more transition states within the central complex is decreased. While further work will be required to characterize this destabilization in terms of chemical and conformational intermediates, product dissociation steps are unlikely to be responsible on the basis of the weak binding of products to all CSs studied so far. In spite of the present lack of ability to assign the effect of a mutation to a particular elementary step, the distinctions between the classes of effect on bound states as suggested by Fersht (1987) remain useful.

On the basis of the extensive crystallographic data (Remington *et al.*, 1982; Karpusas *et al.*, 1990; Liao *et al.*, 1991; Remington, 1992; Usher *et al.*, 1994) we report preliminary data on residues in two general classes: (1) Residues that interact directly with substrates, products, or intermediates through one or more hydrogen bonds or salt bridges. These include His274, Asp375, His320, and Arg401. (2) Residues whose importance has been deduced either from their invariance or conservative replacement throughout evolution or from their apparent role in the conformation change from open to closed forms (hinge residues). These include Asp327, Gly275, His235, and Asn242. Figure 3 is a stereodiagram of the active site of PCS with bound oxalacetate and an inhibitor, carboxymethyldethiacoenzyme A. All the amino acids discussed in this paper are shown. Their relative positions with respect to citrate are shown and indicate the tight fit of the reactant with these residues in the active site of PCS.

The results of the mutagenesis experiments reported in this paper demonstrate that each of the three residues, His274, Asp375, and His320 in the active site, may be considered catalytically essential. Replacement of any one of these residues by mutation to Gly resulted in reductions in k_{cat} by 10^3 with small decreases in the K_ms of the substrates for His274 and Asp375 and a 10-fold increase for His320. Similar results have been reported for the equivalent of these residues in the allosteric *E. coli* CS (Pereira *et al.*, 1994). It can reasonably be inferred that these residues are primarily involved in transition state stabilization. The actions of His274 and Asp375 are presumed to be coordinated (Karpusas *et al.*, 1990; Alter *et al.*, 1990). A general acid−base mechanism was proposed (Karpusas *et al.*, 1990; Alter *et al.*, 1990; Liao *et al.*, 1991; Remington, 1992; Usher *et al.*, 1994) in which Asp375 and His274 act in concert to form and stabilize a reactive form of acetyl-CoA. On the basis of the structural studies of transition state analog inhibitor complexes as well as by the mutagenesis experiments reported here, this picture appears essentially correct although a number of significant questions remain (Figure 1, vide infra): (1) the question of whether His274 participates as a charged or neutral residue; (2) the question of how CS effects the rate enhancement; and (3) the question of how a number of different conformations that CS adopts during the course of catalysis relate to specific enzyme−substrate, enzyme−intermediate, and enzyme−product interactions along the reaction coordinate. The question of whether His274 participates as a charged or neutral residue is central to understanding the reaction path in CS and the unusual behavior of catalytic histidines acting as general acids. An example of a similar catalytic histidine, His95, in triose phosphate isomerase was shown to be neutral in NMR studies (Lodi & Knowles, 1991), and quantum calculations support these conclusions (Karplus *et al.*, 1992).

That charge delocalization can account for the stabilization of a reactive intermediate in the CS-catalyzed reaction follows from a largely overlooked feature of the Born theory of solvation (Born, 1921). Born showed that the energy E (the "self-energy") required to create a spherical charge Q of radius R in a medium of dielectric constant ε is inversely proportional to its size, that is

$$E = Q^2/(2\varepsilon R) \quad (1)$$

(Born, 1920). Considering the transfer of the ion from water (εw) to the lower dielectric of the enzyme active site (εs), the required energy is

$$\Delta E = (Q^2/2R)(1/\varepsilon s - 1/\varepsilon w) \text{ or } (166Q^2/R)(1/\varepsilon s - 1/\varepsilon w)$$

where the latter expression yields unit charge in kcal/mol and radius in Å. For example, doubling the radius of a charge reduces its energy by

$$\Delta E = 166Q^2(^1/_4 - ^1/_2)/(\varepsilon R)$$

which is 41.5 kcal/mol in the vacuum (initial $R = 1$ Å), so the effect can be substantial. Thus, if an enzyme can delocalize the charge on the transition state or an intermediate over neighboring residues, as suggested in Figure 1B, it can stabilize it by effectively increasing its "radius", and the effect will depend on the local dielectric constant.

Preliminary calculations using the program DELPHI have been used to estimate the magnitude of this effect, assuming different constant dielectric values. DELPHI has previously been shown to reproduce the experimental free energies of ion hydration in solution with reasonable accuracy (Jayaram *et al.*, 1989; Gilson & Honig, 1988). The resulting estimates for the self-energies of the various proposed charge distributions in the active site of CS are shown in Table 6, where all values correspond to a hypothetical reference state of 1M in vapor or aqueous phases at 298 K, relative to the ground state in water. The electrostatic self-energy of the transition state is lower than that of the ground state, and the difference does depend on the local dielectric constant.

The dielectric constant in the active site of CS is unknown, but on the basis of both experimental evidence and theoretical calculations, several authors have suggested that it is appropriate to assume that it is in the range 2−10 (Harvey, 1989; Gilson & Honig, 1986; Gilson *et al.*, 1985; Mathew, 1985). The trends in self-energies for different dielectrics are apparent from Table 6. As the ground state is moved to increasingly lower dielectric, its energy increases as expected, and this is presumably offset by binding energy. The energy of the hypothetical transition state also increases, but the rate of change is different, suggesting that the transition state is stabilized relative to the ground state, also as expected. Furthermore, it is clear that the proposed "enolized" intermediate, in which the charge is smeared over both the histidine and the intermediate, is stabilized substantially over the proposed neutral enol intermediate in which the final charge resides solely on the histidine (Figure 1B).

For the conservative choice of $\varepsilon s = 4$, the ground state is destabilized on the enzyme, but the difference between the ground state and transition state energies, $\Delta\Delta G_s$, is about 7 kcal/mol less than for the solution reaction ($\varepsilon w = 80$, Table 6). From the Arrhenius equation,

$$k = A \exp(-\Delta G^{\ddagger}/RT)$$

assuming a standard preexponential factor of $A = 10^{13}$ s^{-1} for the pseudo-unimolecular enzyme catalyzed reaction, this corresponds to a rate increase of 8×10^7 over the solution reaction *solely from charge delocalization*. This result strongly suggests that a neutral His 274 may be more effective in stabilizing an "enolized" intermediate than if it were positively charged, as the net overall charge on the intermediate would then be 0. On a final note, the energetic cost calculated for the transfer of Asp375 from water to the vacuum (last column of Table 6) agrees well with the experimental values for desolvation of acetate [79−87 kcal/mol, as previously reported by Gilson and Honig (1988)].

The replacement of His274 with Gly had the predicted deleterious effect on k_{cat} with little effect on either K_m. The substitution of Arg at this position had closely similar consequences even though Arg bears a permanent positive charge and is considerably larger than His. Simulation analysis of triose phosphate isomerase bearing a permanent positive charge at His95 resulted in a thermodynamic trap for the intermediate (Karplus *et al.*, 1992). It should also be noted that thermodynamic studies on the PCS mutant His274Arg resulted in a stabilization against thermal denaturation in complexes with OAA (Zhi *et al.*, 1991). His274 is both a catalytic and a hinge residue involved in the CS conformational change. In the His274Arg mutant, it is likely that what is responsible for the large reduction in k_{cat} is not just the change in charge, but rather a structural change induced by the mutation. The detailed mechanism by which His274 stabilizes the reactive intermediate/transition state will require further study.

Substitutions of Asn, Gln, and Glu for the proposed general base, Asp375, while significantly reducing k_{cat} concomitantly lowered the K_m for OAA 2−7-fold while having little effect on the K_m for acetyl-CoA. This result is consistent with a change from an ordered steady state bireactant enzyme (in which $K_a = k_{cat}/k_1$) toward an ordered prior-equilibrium system in which K_a approaches zero. The K_ds for OAA to wild type and mutants have been measured, and these mutants were found to have affinity for OAA comparable to the wild type. Since it is well-known that the tight binding of OAA in comparison with that of acetyl-CoA is responsible for the preferred ordered mechanism of the wild type, it is likely that the pattern of changes in kinetic constants represents a change in mechanism from steady state to prior-equilibrium ordered.

It is not yet certain whether the activated intermediate of acetyl-CoA is a neutral enol as shown in Figure 1A or a strong hydrogen-bonded enolate (Figure 1B). Catalytic enhancement has been attributed in part to the alignment of His274 and Asp375 with the enol(ate) intermediate in the active site which may allow for a concerted reaction to occur. Besides the theoretical energetic problem of a charged intermediate species, the enolate, the crystallographic (Remington, 1992) and physical chemical (Zhi *et al.*, 1991; Kurz *et al.*, 1992) evidence argues for a neutral enol intermediate. However, the existence of a short strong hydrogen bond has been postulated for enzymes that must abstract a proton with a pK > 20, using amino acid residues whose pK is ∼6−8 [see Gerlt and Gassman (1993) for review]. While the hydrogen bond distances involving the transition state analog inhibitor are indeed very short, the rate enhancement

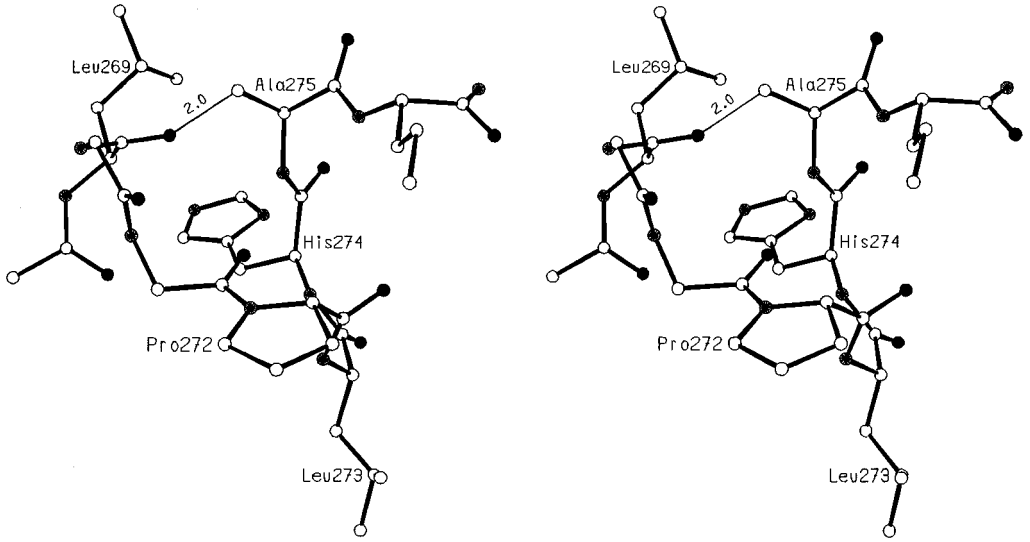

FIGURE 4: Hypothetical model of the mutation Gly275Ala, showing an extremely bad contact of the methyl group of Ala275 with the carbonyl oxygen of Leu269 in the "open" form of the enzyme. This contact would not occur in the closed conformation and would destabilize the open form and presumably interfere with the closed to open conformational change.

provided by a single, very short, strong hydrogen bonded enolate has been questioned, and enzyme stabilization of the intermediate by charge delocalization seems to be a viable alternative (Remington *et al.*, 1982; Remington, 1992; Usher *et al.*, 1994; Schwartz *et al.*, 1995).

The substitutions at the other catalytic residue, His320, provide a particularly interesting set of mutations. His320 interacts directly with the carbonyl oxygen of OAA, an interaction likely to be important in OAA carbonyl polarization (Kurz *et al.*, 1995), but His320 is also the putative proton donor in the formation of the alcohol of citrate. The most conservative replacements of His320 are with the neutral residues, Asn and Gln, which are closely isosteric to His and capable of hydrogen bonding interactions with the carbonyl of OAA. However, neither of these residues is at all likely to serve as a proton donor. The Arg substitution introduces a permanent positive charge as well as a larger side chain. Hydrogen bonding interactions are still possible, but proton donation from Arg to the carbonyl is highly unlikely. The Gly substitution eliminates any interaction with the carbonyl and introduces a much smaller residue. k_{cat} is greatly impaired in all these cases, and K_ms for OAA and acetyl-CoA are modestly increased (in free energy terms) except for the Arg mutant where the K_ms are modestly decreased. Again, the picture is of a residue primarily important in transition state stabilization.

Another residue that interacts directly with substrates and products is Arg401 which makes a salt bridge with the carboxylate of citrate. Mutations at Arg401 had minimal effects except when Arg401 was mutated to His, which resulted in a 10^3-fold decrease in k_{cat} and a 3−4-fold increase in the K_ms of both substrates. Mutation of Arg401 to His in the CS active site caused the largest reductions in k_{cat} and in each k_{cat}/K_m of any of the 18 mutants. When the equivalent of Arg401 is mutated to Leu in the *E. coli* CS, the k_{cat} of the enzyme was reduced 10^4-10^5-fold, and no OAA binding could be detected (Pereira *et al.*, 1994). This may reflect differences in ionization states between the functional groups, conformational changes induced by the mutation, or both. pH dependence studies of kinetic constants of this mutant may provide insight into this issue. Evidence for the contribution of this important salt bridge interaction in the enzymatic reaction of CS was obtained when Arg401 was mutated to Lys401. Arg401Lys had a normal k_{cat} but an increase in k_{cat}/K_m for each substrate when compared to the nonmutant. Arg401Lys would therefore be considered to be catalytically modestly more efficient than the nonmutant enzyme (Plaut & Knowles, 1972; Knowles & Albery, 1977).

Further experimentation will be necessary to establish unequivocally the protonation state of the catalytic histidines, the role of the conformational change in the catalytic cycle, and the exact structures of the proposed activated intermediates, particularly whether activated acetyl-CoA is a neutral enol or a short "strong-hydrogen-bonded" enolate (Cleland, 1992; Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994; Usher *et al.*, 1994).

The second class of residues investigated includes several residues that appear important from their conservation in all known CS sequences but are not believed to interact directly with substrate, products, or intermediates.

Asp327, a residue conserved in all known CSs, occurs in the second shell of residues around the active site of CS. Mutation of Asp327 increased the K_m of both substrates and reduced k_{cat} by 62%. Transformants containing the Asp327Asn mutation had an acetate$^-$ phenotype, but the purified enzyme was catalytically active. This is the first example of a phenotype in which cells contain catalytic amounts of CS but are unable to grow on acetate. This paradox can be explained by examining the differences between the affinities for OAA and acetyl-CoA of the nonmutant and mutant enzymes. Although k_{cat} was reduced by less than half of the nonmutant by mutation of Asp327 to Asn, an apparent 10-fold decrease in the affinity of this mutant for each of the substrates rendered the cells containing this PCS mutant protein incapable of growing on acetate as a sole carbon source. Thus, Asp327 is a residue essential for the recruitment and binding of substrates to the active site of PCS.

Gly275 occurs at the "hinge" region in the CS active site. "Hinge" residues are those whose backbone conformational angles change during the course of the conformational transition from the "open" to the "closed" form of the

enzyme. Development of steric hindrance in such a residue would presumably interfere with this conformation change and allow an assessment of its importance to the catalytic cycle of the enzyme. Mutation of Gly275 to Ala and Val progressively decreased the activity of the enzyme. This is interesting since neither Ala275 nor Val275 makes contact with the substrates. This indicates that conformational changes are at least as important as the catalytic residues. Val275 had large effects on the K_m of the substrates and a 10^4-fold decrease in k_{cat}. These observations argue that the catalytic strategy of CS involves not only the correct juxtaposition of the essential proton-donating and -accepting catalytic residues, but also that the activation energy of the transition state is lowered in the nonmutant enzyme by enzyme−substrate induced conformational strain at the active site. It is not likely that Ala275 will interfere directly with His274 or other catalytic residues. They are both in a helix so that their side chains point in directions that are 90° apart, so there is no possibility of interference with the His274 side chain by Ala275. In the mutations Gly275Ala and Gly275Val, model studies with both the open and closed forms of the enzyme suggest that even Ala would be very destabilizing to the *open* form of the enzyme and would produce extremely bad contacts with the carbonyl oxygen of Leu269 (2.0 Å, minimum is about 3.2 Å) and the side chain of Thr394 (Figure 4). Val would be even worse in this position. Since the backbone conformational angles of Gly275 change from (−86°, −31°) in the open state to (−63°, −24°) in the closed state, Ala275 would be somewhat less strained in the closed state. However, the side chain methyl group would still make bad contacts with the side chains of Leu269 and Thr394. Thus, it appears that any side chain larger than Gly at position 275 would hinder the conformational transition, but the *open* state would be destabilized more than the closed state. Val275 probably disrupts the active site substantially in either conformation of the enzyme. Structural studies in solid and solution states may resolve these alternative possibilities.

The crystallographic model of the mutant His274Gly reveals that strain in the active site of CS is reduced by the mutation in at least two ways, which explains the result that this mutation drastically stabilizes the enzyme to thermal denaturation (Zhi *et al.*, 1991). First, the main chain conformational angles of His274 in the closed conformation had previously been shown to be in a "disallowed" region of the Ramachandran diagram, with (Φ,Ψ) of (−120°, −120°), which is allowed for glycine (Ramachandran & Sasisekharan, 1968). Second, the side chain conformational angle of wild type, χ^2, is essentially 0° in either conformation of the enzyme. This eclipsed conformation results in unfavorable van der Waals interactions between Nδ1 and the α-carbon of residue 274. The conformation is enforced upon the residue by hydrogen bonds between the backbone amide and hydroxyl group of Ser244 to Nϵ2 and is probably necessary for proper binding of substrate. Removal of the side chain relieves these contacts, but the loss of the two hydrogen bonds presumably offsets this stabilizing effect to some degree. Therefore, the 10^3-fold decrease in k_{cat} for this mutant form of CS may be attributed to the absence of an ionizable proton donor at the active site for the chemical reaction of the enzyme to proceed and also to a conformational change.

The mutagenesis experiments reported here demonstrate that the active site residues His274, Asp375, and His320 are critically important to the catalytic efficiency of the enzyme. These experiments also point to the importance of conformational movements *via* an essential hinge region.

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