the active site of carboxypeptidase A (Hilvert et al., 1986). In the present situation, the H297N substitution has been demonstrated to be without effect on the conformation of the active site, although the racemase activity has been totally inactivated by the substitution. However, with the use of two mechanistic probes for abstraction of the α-proton from (*S*)-mandelates, the H297N mutant enzyme is catalytically indistinguishable from the wild-type enzyme. This retention of full activity in a partial reaction allows the impairment observed in the overall racemization reaction to be assigned to the absence of His 297 and its role as a general acid/base catalyst in generating or protonating a kinetically viable intermediate.

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Conformational Stability of Pig Citrate Synthase and Some Active-Site Mutants†

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ABSTRACT: The conformational stabilities of native pig citrate synthase (PCS), a recombinant wild-type PCS, and six active-site mutant pig citrate synthases were studied in thermal denaturation experiments by circular dichroism and in urea denaturation experiments by using DTNB to measure the appearance of latent SH groups. His^{274} and Asp^{375} are conserved active-site residues in pig citrate synthase that bind to substrates and are implicated in the catalytic mechanism of the enzyme. By site-directed mutagenesis, His^{274} was replaced with Gly and Arg, while Asp^{375} was replaced with Gly, Asn, Glu, or Gln. These modifications were previously shown to result in 10^3–10^4-fold reductions in enzyme specific activities. The thermal unfolding of pig citrate synthase and the six mutants in the presence and absence of substrates showed large differences in the thermal stabilities of mutant proteins compared to the wild-type pig citrate synthase. The functions of His^{274} and Asp^{375} in ligand binding were measured by oxalacetate protection against urea denaturation. These data indicate that active-site mutations that decrease the specific activity of pig citrate synthase also cause an increase in the conformational stability of the protein. These results suggest that specific electrostatic interactions in the active site of citrate synthase are important in the catalytic mechanism in the chemical transformations as well as the conformational flexibility of the protein, both of which are important for the overall catalytic efficiency of the enyzme.

Citrate synthase catalyzes the stereospecific condensation of acetyl coenzyme A and oxalacetate to form citrate. It is an excellent enzyme for studying the relationships between the chemical structure of the protein and its physical, chemical, and functional properties. The sequence of the enzyme from pig heart was derived from amino acid sequence analysis (Bloxham et al., 1981, 1982); the mammalian enzyme has a high degree of homology with the citrate synthases isolated from yeast, plant, and bacterial sources (Alter et al., 1990); the three-dimensional structures of the pig heart citrate syn-

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thase with and without substrate ligands have been determined (Remington et al., 1982; Wiegand et al., 1984; Karpusus et al., 1990), and the cDNA encoding pig citrate synthase was prepared and cloned (Evans et al., 1988).

In eukaryotes, citrate synthase is a dimeric enzyme. From X-ray diffraction data, it was demonstrated that the enzyme monomers are composed of a large and a small domain connected by a "hinge" region, that the active site occurs in a cleft between the large and small domains, and that the enzyme undergoes large conformational changes during the course of catalysis (Wiegand et al., 1984). This latter assumption was examined in detail recently by Karpusas et al. (1990), who presented evidence that after the large conformational change that occurs upon oxalacetate (OAA)[1] binding, all steps of the reaction occur with crystallographically identical forms of the enzyme. Each dimer contains two functionally independent active sites with binding sites for both oxalacetate and acetyl coenzyme A (Weidman et al., 1973; Srere, 1965a; Johansson & Pettersson, 1974). The protein is largely α-helical with amino acid residues from both monomers of the dimeric enzyme forming an integral part of the active site (Remington et al., 1982). The helices from the two subunits pack together tightly to give a globular molecule with four pairs of α-helices sandwiched at the interdomain interface. His^{274} and Asp^{375} are two of many residues which are conserved in citrate synthases, interact directly with substrate molecules, and have been implicated in the catalytic mechanism of the enzyme (Karpusus et al., 1990). From site-directed mutagenesis experiments, these two residues were shown to be catalytically essential amino acids involved in substrate binding (Evans et al., 1989; Alter et al., 1990; Remington et al., 1982). Since conformational changes are presently a questionable property of the catalytic mechanism of citrate synthase, it was hypothesized that mutations that affect catalytic activity may also affect the conformational changes of the protein which then may be examined experimentally. Therefore, we were interested in determining the unfolding characteristics of the enzyme and probing the relationships between chemical structure and conformational stability by studying the unfolding properties of pig citrate synthase in active-site mutants of the enzyme by thermal denaturation and denaturation in 4.0 M urea.

Materials and Methods

Purification of the Nonmutant and Mutant PCS Proteins. Oligonucleotide-directed mutagenesis was performed by the method of Kunkel (Kunkel, 1985; Kunkel et al., 1987) with a cDNA template encoding pig citrate synthase that contained uracil residues in place of thymine (Evans et al., 1989). The DNA fragments that encode the mature forms of the nonmutant and six mutant forms of the pig citrate synthase that lack the mitochondrial leader peptide sequence were subcloned in-frame into the pT7-7 vector that contains the T7 RNA polymerase promoter and induced by a temperature shift and expressed in a *glt*A mutant of *Escherichia coli* that lacks endogenous *E. coli* citrate synthase protein (Evans et al., 1988). The induced mutant and nonmutant proteins represented 10–15% of the total cellular protein and were purified individually following a modification of the standard agarose–ATP chromatography procedure (Evans et al., 1989; Mukherjee & Srere, 1976). Each PCS mutant protein and the nonmutant enzyme eluted in a similar manner from the column by the addition of the substrates oxalacetate and coenzyme A to the elution buffer. The mutant proteins were judged homogeneous by SDS–PAGE (Laemmli, 1970).

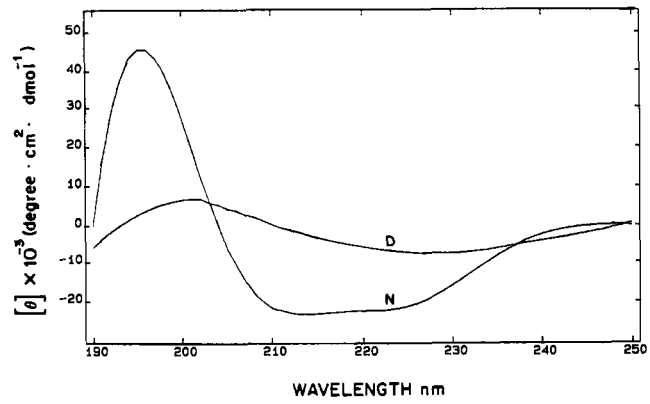

FIGURE 1: Circular dichroism spectra of native and unfolded pig heart citrate synthase. The far-UV spectra of native pig heart citrate synthase, the pig citrate synthase clone, and six active-site mutants were similar and characterized by a strong maximum in the 190–195-nm region and minima at 208 and 222 nm. Native (N) proteins were in 5.0 mM Tris-HCl, pH 7.5, buffer (0.07–0.45 mg/mL) at 25 °C and unfolded in the same solution at 60 °C (D, denatured).

Thermal Denaturation Monitored by Circular Dichroism. The secondary structure of pig citrate synthase is readily probed by circular dichroism. This technique is particularly diagnostic for α-helical proteins, like pig citrate synthase, which have far-UV CD spectra that include a maximum at 190 nm, a crossover at 200 nm, and double minima at 208 and 222 nm (Brahms & Brahms, 1980; Manavalan & Johnson, 1983; Chen et al., 1984; Wu & Yang, 1970). The CD spectra of pig citrate synthase (Figure 1) and each of the mutant enzymes (data not shown) display these α-helical features. For spectra, multiple scans (three or more) were obtained for each sample and averaged. Each spectrum was recorded in 1-nm wavelength increments and the signal acquired for 1 s at each wavelength. The observed ellipticity, θ, was background-corrected and measured for three different protein concentrations (0.07–0.45 mg/mL). The mean residue ellipticity, $[\theta]_{222}$, of pig heart citrate synthase was calculated by using a mean residue weight of 114 and was 20069 ± 1575 deg·cm^2/dmol. The abundance of α-helical structural elements within native pig heart citrate synthase was estimated between 60 and 80% from the CD spectra by an iterative procedure that compares the secondary structural elements of a polylysine standard in 5% increments. This is consistent with values of 78% ± 7% (West et al., 1990), 50–55% (Wu & Yang, 1970), and 72% from X-ray crystallography (Remington et al., 1982). Each of the mutant enzymes had the same overall helical content as the wild-type enzyme.

The stability of pig citrate synthase and the mutants toward thermal denaturation was measured by monitoring changes in α-helical content of the proteins by the increase in mean residue ellipticity at 222 nm as a function of temperature. The ellipticity of a 1.0 μM sample of the PCS mutant proteins was measured by using an AVIV 60 DS spectrometer. Thermal denaturation was monitored by changes in the circular dichroism spectrum in 1.5 °C steps. The samples were equilibrated 1.0 min at each temperaure, and the signal was recorded for 1.0 min. This rate of heating was found adequate to achieve equilibrium at each temperature. The midpoint of the unfolding transition was determined for each enzyme in the presence and absence of substrates. When protein solutions were heated to the T_m and then cooled to 20 °C, the ellipticity

[1] Abbreviations: PCS, pig citrate synthase made by recombinant DNA methodology; CD, circular dichroism; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; acetyl-CoA, acetyl coenzyme A; OAA, oxalacetate; DTNB, 5,5′-dithiobis(2-nitrobenzoate).

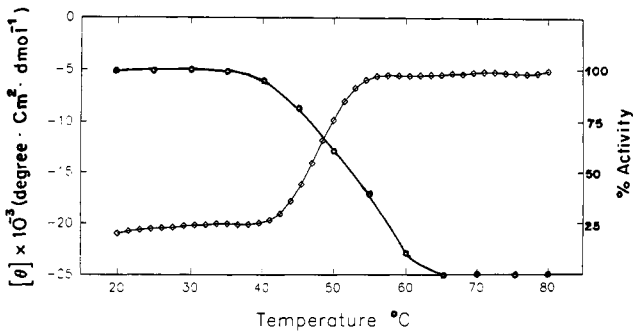

FIGURE 2: Thermal unfolding curve for pig heart citrate synthase. The change in ellipticity, $[\theta] \times 10^{-3}$ (degrees centimeter squared per decimole), at 222 nm (□) and the percent activity remaining (○) were measured as a function of temperature in 5.0 mM Tris-HCl, pH 7.5, buffer.

returned to 80% of its starting value. However, when protein solutions were heated to 15 °C above the T_m and then cooled to 10 °C, an increase in the turbidity of the sample was detected, and the ellipticity returned to only 5–6% of the starting value. The partial irreversibility of the unfolding transitions, therefore, appears to result from protein aggregation that occurs at high temperature. The aggregation of citrate synthase also was demonstrated in light-scattering experiments and shown to predominate at high local concentrations of unfolded protein (Buchner et al., 1991).

Urea Denaturation followed by -SH Group Release and Protection by Oxalacetate. Urea denaturation was followed by the exposure of enzyme -SH groups spectrophotometrically with the use of Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoate) (DTNB) (Srere, 1965b, 1966). Reactions were routinely followed in 10^{-4} M DTNB at 412 nm in a Beckman DU50 spectrophotometer equipped with thermospacers. Denaturation before reaction with DTNB was reversible and followed first-order reaction kinetics. The differences in the apparent first-order rate constants for urea denaturation were measured when the enzyme or the mutants were added to a mixture of 4.0 M urea and increasing concentrations of oxalacetate.

Protein and Enzyme Determination. Samples were assayed for protein (Bradford, 1976) and citrate synthase activity (Srere et al., 1963).

RESULTS

Thermal Denaturation of Pig Citrate Synthase. The thermal unfolding reactions of the pig heart and nonmutant pig citrate synthases followed single-step coincident transitions (Figure 2). In the absence of substrates, the mean residual ellipticity of wild-type pig citrate synthase increased with temperature and had a T_m of 48 °C. Changes in the secondary structure of pig citrate synthase through the thermal transition region were paralleled by decreases in the activity of the enzyme. The enzyme activity was maximal between 20 and 37 °C, decreased at temperatures above 40 °C, and was undetectable at temperatures above 65 °C. The transition midpoint for the decrease in pig citrate synthase activity as a function of temperature was 52 °C. Consistent with the law of mass action, the thermal stability of pig citrate synthase increased with 4-fold higher concentrations of protein, indicating that the dimer state is significantly populated in the transition zone of the denaturation curve.

Differences in the Thermal Stability between Mutant and Nonmutant Forms of Pig Citrate Synthase. Conformational changes are observed when pig citrate synthase binds oxalacetate (Srere, 1965b, 1966), and it has been shown that the

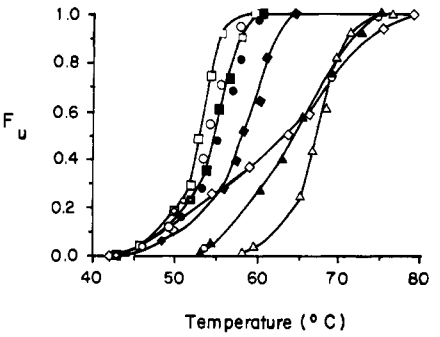

FIGURE 3: Fraction of unfolded mutant and nonmutant pig citrate synthase as a function of temperature in the presence of 50 μM oxalacetate. The fractions of unfolded (F_u) mutant and nonmutant pig citrate synthase were calculated by using eq 1 and thermal unfolding data from circular dichroism. (□) Gly^{274}; (○) PCS clone; (■) PCS from heart; (●) Glu^{375}; (♦) Arg^{274}; (◇) Gly^{375}; (▲) Asn^{375}; (△) Gln^{375}.

enzyme cystallizes in an open conformation in the absence of substrates and in a closed conformation in the presence of oxalacetate, transition-state analogues, and products (Remington et al., 1982; Karpusas et al., 1990). The early suggestion was that a certain degree of conformational flexibility is required for the overall citrate synthase reaction to occur, and recent studies indicate that the same crystal conformation (closed) participates in all steps of the reaction following binding of OAA (Karpusas et al., 1990). Therefore, the unfolding properties of pig citrate synthase and six active-site mutants of the enzyme were measured in the absence of and in the presence of 50 μM oxalacetae or 50 μM coenzyme A. The fraction of unfolded protein, F_u, was calculated by using

$$F_u = (y_F - y_{obs})/(y_F - y_u) \quad (1)$$

where y_{obs} is the observed mean residual ellipticity at 222 nm and y_F and y_u are the ellipticity values characteristic of the folded and unfolded conformations. The thermal unfolding curves for citrate synthase and six mutants in the presence of oxalacetate are shown in Figure 3. The fractional unfolded protein was calculated and plotted as a function of temperature. Large differences in the thermal stabilities of citrate synthase and the mutants were observed. The unfolding of pig heart citrate synthase and the pig citrate synthase clone followed identical denaturation curves with single coincident transitions and melting temperatures of 54 °C in the presence of oxalacetate. Mutation of His^{274} to Gly^{274} or Arg^{274}, or mutation of Asp^{375} to Glu^{375} had little effect on the thermal transition of pig citrate synthase in the presence of oxalacetate. Replacement of Asp^{375}, however, with the residues Gly, Asn, or Gln caused a large stabilization of the protein to thermal denaturation, a shift of the unfolding curves to the right, and an increase in the T_m of the protein by 5–10 °C. In addition, the mutants, Gly^{375} and Asn^{375} had broad thermal transition zones which may indicate the presence of multiconfomer populations in the unfolding transitions of these mutants.

A comparison of the activities and the melting temperatures of pig citrate synthase and the mutants in the absence and presence of oxalacetate and coenzyme A is given in Table I. The purified pig heart citrate synthase and the cloned pig citrate synthase protein had similar specific activities, 125 and 115 units/mg, respectively, and the unfolding characteristics of the two proteins were identical in the presence or absence of substrates. The melting curves for the nonmutant enzymes followed single coincident curves with a T_m of 48 °C in the absence of substrates. A large increase in the stability of the wild-type protein was observed when thermal denaturations were conducted in the presence of both substrates. The T_m

Table I: Activity and Melting Temperatures of Pig Citrate Synthase Mutants

protein	activity[a] (units/mg)	T_m[b] (°C)	T_m(CoA) (°C)	T_m(OAA) (°C)	T_m(OAA + CoA) (°C)
wild type (pig heart)	125 ± 15	47.8 ± 0.3	50.0 ± 1.4	57.5 ± 2.1	66.5 ± 0.7
wild type (pig clone)	115 ± 10	47.7 ± 1.8	51.3 ± 3.3	56.3 ± 0.4	66.0 ± 1.4
$His^{274} \rightarrow Gly$	0.16 ± 0.12	51.5 ± 1.4	52.0 ± 1.4	56.0 ± 4.2	63.0 ± 0.1
$His^{274} \rightarrow Arg$	0.18 ± 0.02	57.6 ± 0.6	57.5 ± 0.7	58.0 ± 0.1	58.0 ± 0.3
$Asp^{375} \rightarrow Gly$	0.07 ± 0.01	53.8 ± 1.3	56.0 ± 1.4	62.5 ± 1.6	65.4 ± 2.3
$Asp^{375} \rightarrow Asn$	0.01	53.6 ± 3.1	55.5 ± 2.8	64.0 ± 1.4	69.7 ± 1.3
$Asp^{375} \rightarrow Glu$	0.28 ± 0.02	45.7 ± 2.4	51.0 ± 2.8	56.3 ± 1.8	61.0 ± 2.8
$Asp^{375} \rightarrow Gln$	0.02	57.6 ± 0.8	59.0 ± 1.0	69.1 ± 1.5	71.3 ± 0.4

[a] The activities of the pig citrate synthase enzyme and mutants were determined from initial steady-state kinetics of a reaction run at 25 °C in 100 mM Tris-HCl (pH 8.1) containing 100 μM acetyl coenzyme A and 50 μM oxalacetate and assuming substrate concentrations are saturating (Alter et al., 1990). [b] The specific rotations of the pig citrate synthase enzyme and mutants were measured at 222 nm as a function of temperature in a 0.2-mL cell containing 5.0 mM Tris-HCl (pH 7.5) and 1.0 μM enzyme. The midpoint of the thermal denaturation curve (T_m) was determined in the presence or absence of substrate (50 μM) oxalacetate and/or 50 μM acetyl coenzyme A). Experiments were performed in triplicate, and the mean and standard deviation of T_m were determined.

Table II: Parameters Characterizing the Thermal Unfolding of Pig Citrate Synthase Mutants in the Presence of Oxalacetate[a]

protein	T_m[b] (°C)	ΔT_m[c] (°C)
wild type (pig heart)	57.9 ± 2.1	
wild type (pig clone)	55.7 ± 0.8	
$His^{274} \rightarrow Gly$	56.0 ± 4.5	−1.9
$His^{274} \rightarrow Arg$	56.8 ± 2.7	−1.1
$Asp^{375} \rightarrow Gly$	62.5 ± 1.6	4.6
$Asp^{375} \rightarrow Asn$	63.7 ± 1.6	5.8
$Asp^{375} \rightarrow Glu$	56.8 ± 2.3	−1.1
$Asp^{375} \rightarrow Gln$	69.0 ± 1.0	11.1

[a] The specific rotations of the pig citrate synthase enzyme and mutants were measured at 222 nm as a function of temperature in a 0.2-mL cell containing 5.0 mM Tris-HCl (pH 7.5), 50 μM oxalacetate, and 1.0 μM protein. [b] T_m is the midpoint of the thermal unfolding curve in degrees centigrade when $\Delta G = 0$. [c] ΔT_m is the difference between T_m values of the mutant and wild-type enzyme.

of wild-type pig citrate synthase increased 3, 10, and 19 °C in the presence of coenzyme A, oxalacetate, or both substrates, respectively. When His^{274} was replaced with Gly or Arg, or when Asp^{375} was replaced with Gly, Asn, Glu, or Gln, a 10^3–10^4 decrease in enzyme specific activity was detected. The Arg^{274} mutant was not protected from thermal denaturation by the addition of substrates, while the Gly^{274} mutant was stabilized by substrates and had thermal transitions like the wild-type enzyme. Although replacement of Asp^{375} with Glu^{375} inactivated the enzyme, the Glu^{375} mutant unfolded like the wild-type enzyme and had melting temperatures in the presence and absence of substrates similar to those for the wild-type enzyme. When the negatively charged carboxyl group at position 375 was replaced with the residues Gly, Asn, or Gln, a large increase (5–10 °C) in the stability of the protein was detected in the absence of substrates. When thermal denaturations were conducted in the presence of coenzyme A, oxalacetate, or both substrates, the T_m of these mutant pig citrate synthases increased proportionally compared to the increases in the T_m of the wild-type enzyme in the presence of substrates.

The differences in the stabilities among the mutant and nonmutant pig citrate synthase proteins were estimated under the conditions near the midpoint of the thermal unfolding curves (Table II). The pig citrate synthase prepared from the cloned cDNA was identical in every functional and physical property tested compared to the citrate synthase purified from pig heart. Therefore, differences in the conformational stability between the pig citrate synthase molecules differing by a single amino acid substitution at the active site could be readily assessed. Large increases in the ΔT_m (from 4.6 to 11.1 °C) were measured for the Gly^{375}, Asn^{375}, and Gln^{375} mutant proteins.

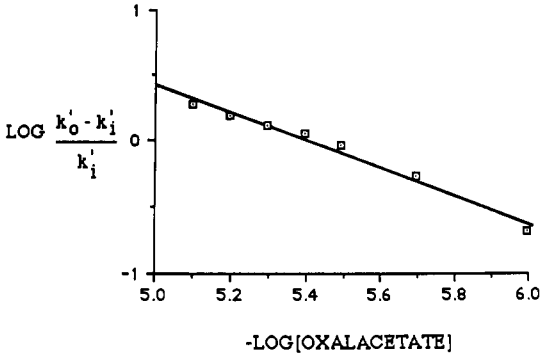

FIGURE 4: Effect of oxalacetate concentration on urea-induced exposure of –SH groups in pig heart citrate synthase. Urea concentration, 4 M; temperature, 24 °C; DTNB concentration 10^{-4} M; Tris-HCl, pH 8.2, 0.1 M in 1.0 mL.

Differences in the Sulfhydryl Group Accessibility between Mutant and Nonmutant Pig Citrate Synthases. When pig citrate synthase is unfolded in the presence of a denaturant, such as urea or guanidine hydrochloride, there is a progressive loss of secondary structure, and 4 mol of –SH/mol of enzyme become available for reaction with DTNB (Srere, 1966; West et al., 1990). Oxalacetate protects the enzyme against the unfolding and the loss of enzyme activity, and the rate constants for the protection against –SH group exposure and activity loss are quite similar (Srere, 1966). Since the interaction between oxalacetate and pig citrate synthase takes place at the active site, an apparent dissociation constant for oxalacetate and pig citrate synthase in the presence of urea can be determined by plotting the log of the difference in the first-order rate constants in the presence and absence of oxalacetate versus the negative log of the oxalacetate concentration as shown for the native enzyme (Figure 4). The rates of –SH group appearance in 4.0 M urea as a function of oxalacetate concentration for the nonmutant enzyme and the Gly^{274}, Gly^{375}, Asn^{375}, Glu^{375}, and Gln^{375} mutants were similar. Like the thermal unfolding data in which the Arg^{375} mutant was not stabilized further by the addition of substrates, the rate of –SH reaction for the Arg^{274} mutant also was unaffected by oxalacetate concentration.

From the first-order rate plots, the K_d for oxalacetate of the nonmutant and pig mutant citrate synthases were calculated and compared to the K_ms for oxalacetate with these proteins (Table III). The wild-type pig enzyme and the pig citrate synthase clone had similar K_ds (1.06 and 1.20 μM) and K_ms (5.0 μM) for oxalacetate and agree with previously reported values (Srere, 1966). The mutants Gly^{274}, Gly^{375}, Glu^{375}, and Gln^{375} had somewhat higher K_ds for oxalacetate between 2.19 and 9.6 μM. A K_d for the Arg^{274} mutant could not be de-

Table III: K_d of Pig Citrate Synthase Mutants for Oxalacetate

protein	K_d[a] (μM)	K_m[b] (μM)
wild type (pig heart)	1.20 ± 0.02	5.0
wild type (pig clone)	1.06 ± 0.07	5.0
His^{274} → Gly	5.91 ± 0.35	3.5
His^{274} → Arg	c	3.8
Asp^{375} → Gly	3.27 ± 0.83	
Asp^{375} → Asn	1.18 ● 0.08	
Asp^{375} → Glu	9.60 ± 0.21	0.6
Asp^{375} → Gln	2.19 ± 0.54	

[a] Dissociation constants were calculated from first-order rate constants as described previously (Srere, 1966). Experiments were performed in triplicate, and data are reported as mean and SEM. [b] From Alter et al. (1990). Mutants Asp^{375} → Gly, Asp^{375} → Asn, and Asp^{375} → Gln were not determined since these PCS mutants were catalytically inactive. [c] The sulfhydryl group reactivity of the His^{274} → Arg mutant PCS protein was not protected by the presence of oxalacetate.

termined since this mutant was not protected by oxalacetate, and the K_d of the Asn^{375} mutant for oxalacetate (1.18 μM) did not differ from that of the wild-type pig enzyme.

Discussion

Pig citrate synthase is well suited to structure/function studies by site-directed mutagenesis. Its three-dimensional structure is known to 1.7-Å resolution (Karpusas et al., 1990). Its reaction mechanism has been well characterized (Eggerer & Remberger, 1964; Buckel & Eggerer, 1969). The cDNA encoding pig citrate synthase has been isolated and expressed in *E. coli*, and active-site mutants of the enzyme have been prepared and expressed in *E. coli* (Evans et al., 1988; Alter et al., 1990).

The citrate synthase of all eukaryotes studied thus far is a dimer composed of identical M_r 50000 subunits. The enzyme crystallizes in two main conformations depending upon the substrate ligands bound: (1) an open tetragonal form; (2) a closed monoclinic structure (Remington et al., 1982). The enzyme is composed predominantly of α-helices, accounting for 80% of its secondary structure, with 20 α-helices in each subunit and only 1 short stretch of β-sheet. Each subunit consists of a large and a small domain containing 15 and 5 helices, respectively, with the active site occurring in a cleft between the large and small domains. Amino acid residues from both subunits contribute amino acid side chains to the active site, and the helices from the two subunits pack together tightly to give a globular molecule. The existence of two main conformations by crystallographic studies of the enzyme confirmed previous solution studies (Srere, 1965b, 1966) that indicated that on binding of oxalacetate citrate synthase undergoes a large conformational change. The crystallography studies have shown that in the transition of the open form to the closed form the small domain rotates 18° relative to the large domain when OAA is bound.

Three consecutive partial chemical reactions describe the course of catalysis by pig citrate synthase: enolization, condensation, and hydrolysis (Eggerer & Remberger, 1964; Buckel & Eggerer, 1969). The X-ray crystal structure of the carboxymethyl coenzyme A (a transition-state analogue)–oxalacetate–enzyme complex indicated that the carboxylate of carboxymethyl coenzyme A is hydrogen-bonded simultaneously to His^{274} and Asp^{375} (Karpusas et al., 1990), and site-directed mutagenesis experiments are consistent with a concerted general acid–base catalytic mechanism for the enzyme (Alter et al., 1990). By site-directed mutagenesis of pig citrate synthase, we replaced His^{274} with Gly or Arg, and replaced Asp^{375} with Gly, Asn, Glu, or Gln. Changes in pig citrate synthase catalytic activity and structure were compared with the properties of the native enzyme by the unfolding of the mutant proteins by thermal denaturation and by the protection against urea-induced exposure of SH groups in the presence by oxalacetate. Essentially the largest decreases in enzyme specific activity were measured in those active-site mutants which showed the largest increases in thermal stability.

Mutations created by site-directed mutagenesis of RNase T_1 (Shirley et al., 1989), λ repressor (Pakula & Sauer, 1989), iso-1-cytochrome *c* (Das et al., 1989), and T4 lysozyme (Alber et al., 1987; Matsumura et al., 1988) demonstrate that proteins can be significantly stabilized or destabilized by discrete changes in critical amino acid residues. We show here that mutation of the catalytic residues His^{274} and Asp^{375} has dramatic effects not only on the enzyme activity but also on the conformational stability of pig citrate synthase. Since the amino acid composition, α-helical content, and overall folded conformations of the mutant pig citrate synthase proteins (Alter et al., 1990) do not differ significantly from those of the wild-type enzyme, the larger T_m values of Gly^{375}, Asn^{375}, and Gln^{375} mutants reflect an increase in the thermal stability of the folded conformation of these mutant proteins due to specific changes in the "pocket" structure of the active site of citrate synthase. Alternatively, mutations also could alter the association–dissociation constant for dimer formation (Gittelman & Matthews, 1990; Bowie & Sauer, 1989). This latter explanation is less likely for citrate synthase since the strength of the interaction between the monomers of citrate synthase has been examined crystallographically (Remington et al., 1982) and is not easily disrupted, confirming previous physical studies (Wu & Yang, 1970). Discrete mutations in the active site of citrate synthase that are far from the dimer interface and do not change the overall conformation of the native protein are more likely to affect the thermostability of the enzyme due to specific structural changes within the enzyme cleft.

In the X-ray crystal structure of pig citrate synthase, His^{274} is a catalytic active-site residue that occurs in a strained, conformationally unfavorable orientation (Remington et al., 1982). Asp^{375} also is a catalytic active-site residue and is close to His^{274} in the three-dimensional structure of pig citrate synthase. Asp^{375} interacts with the same carboxyl of citrate as His^{274} (Karpusas et al., 1990) and participates in the enolization and hydrolysis reactions. It is possible that mutations which increase the thermal stability of the enzyme may also relax the conformational strain in the active site of citrate synthase. The increased stability of the Gly^{375}, Asn^{375}, and Gln^{375} mutants and the similar unfolding characteristics of the wild-type, Gly^{274}, and Glu^{375} mutants suggest that hydrophobic interactions, hydrogen bonding, and favorable electrostatic interactions at the active site may all contribute to the increased stability of these mutant forms. Replacement of Asp^{375} with Gly, Asn, and Gln removed a charged residue from a hydrophobic environment, increased the hydrophibicity of the active-site pocket, and significantly increased the conformational stability of the protein. Asn^{375} and Gln^{375} are two residues that are able to form favorable hydrogen bonds in the active site which may also contribute to the increase in the thermal stability of these two mutants (Table III). The data reported here suggest that in the wild-type enzyme, Asp^{375} may exist in an electrostatically unfavorable hydrophobic environment which intrinsically destabilizes the folded conformation and increases the tendency of Asp^{375} to be protonated during catalysis. This conclusion is supported by the observations that (1) active-site mutations that reduced the catalytic efficiency of pig citrate synthase also caused an increase in

the conformational stability of the protein, (2) amino acid substitutions that replaced charged catalytic residues with more hydrophobic amino acids inactivated the enzyme, increased the thermal stability of the protein, and increased the net positive charge at the active site of pig citrate synthase, and (3) the mutant proteins were stabilized by substrates and bound substrates with similar kinetic and dissociation constants.

Mutation of His^{274} was interesting since replacement of this residue with either Gly^{274} or Arg^{274} reduced the specific activity of the enzyme to the same extent. His^{274} is a critical active-site residue since it not only contributes to the binding site of oxalacetate but it also occurs at the "hinge" region between the large and small domains, forms a hydrogen bond with the terminal carboxylate of citrate (Remington et al., 1982), and participates in the enolization partial reaction (Alter et al., 1990). Mutation of His^{274} to Gly caused a 10^3 decrease in enzyme activity and had little effect on the thermal stability of the enzyme, suggesting that His^{274} is an essential catalytic residue. Replacement of His^{274} with Arg^{274}, however, also caused a 10^3 decrease in enzyme activity, but this mutant was not protected by oxalacetate from either thermal or urea denaturation. It is possible that replacement of His^{274} with a residue of a different size and shape, Arg^{274}, altered the arrangement of the groups in the active-site conformation such that this PCS mutant was not protected by OAA in thermal and urea denaturation experiments. These results suggest that His^{274} not only is an essential catalytic residue but also is important for the conformational integrity of the active site of citrate synthase when OAA binds.

Therefore, amino acid substitutions which change the electrostatic interactions of two important catalytic residues, His^{274} and Asp^{375}, in the active-site "pocket" of pig citrate synthase had the primary effect to reduce the enzyme activity by removing essential catalytic amino acids. In addition, the data presented here indicate that some conformational flexibility may be necessary for the overall reaction to occur. The X-ray crystal structures ultimately will determine the conformations of these mutant forms of citrate synthase and relate the relative contributions of each substituent to the overall stability and activity of the enzyme. These studies are in progress.

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Registry No. CoA, 85-61-0; His, 71-00-1; Asp, 56-84-8; citrate synthase, 9027-96-7; oxalacetic acid, 328-42-7.

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