The effect of replacing the conserved active-site residues His-264, Asp-312 and Arg-314 on the binding and catalytic properties of *Escherichia coli* citrate synthase

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The first step in the overall catalytic mechanism of citrate synthase is the binding and polarization of oxaloacetate. Active-site residues Arg-314, Asp-312 and His-264 in *Escherichia coli* citrate synthase, which are involved in oxaloacetate binding, were converted by site-directed mutagenesis to Gln-314, Asn-312 and Asn-264 respectively. The R314Q and D312N mutants expressed negligible overall catalytic activity at pH 8.0, the normal assay pH, but substantial activities for the partial reactions that reflect the cleavage and hydrolysis of the substrate intermediate citryl-CoA. However, when the pH was lowered to 7.0, the overall reaction of the mutants became significant, in contrast to the wild-type enzyme, whereas the two mutants exhibited reduced activities for the partial reactions. This result is consistent with the existence of a rate-limiting step between the two partial reactions for these mutants that is pH-dependent.

The K_m for oxaloacetate for the two mutants was increased 10-fold and was paralleled by an increase in the K_m for citryl-CoA, whereas the K_m for acetyl-CoA was increased only 2-fold. Overall, there was a striking parallel between the results obtained for these two mutants, which suggests that they are functionally linked in the *E. coli* enzyme. The equivalent of these two residues form a salt bridge in the pig heart citrate synthase crystal structure. The H264N mutant, in which the amide nitrogen of asparagine should mimic the δ -nitrogen of histidine, showed negligible activity in terms of both overall and partial catalysis, which may result from a hindrance of conformational change upon oxaloacetate binding. The affinity of this mutant for oxaloacetate appeared to be greatly reduced when investigated using indirect fluorescence and chemical modification techniques.

INTRODUCTION

Citrate synthase (EC 4.1.3.7) belongs to an important group of enzymes that catalyse the formation of a new C—C bond. The availability of the complete amino acid sequence (Bloxham et al., 1981, 1982) and the X-ray crystal structure (Remington et al., 1982) of the pig heart enzyme has led to the identification of active-site residues responsible for substrate binding, namely three histidine residues, His-274, His-238 and His-320, and three arginines, Arg-329, Arg-401 and Arg-421 (in the other subunit). With the advent of the *E. coli* citrate synthase sequence (Ner et al., 1983; Bhayana and Duckworth, 1984), comparison with the pig heart citrate synthase was possible (Bloxham et al., 1982; Bell et al., 1983). Although the overall amino acid sequence identity between pig heart and *E. coli* citrate synthase is only 27%, there is a high conservation of sequence within the active-site regions, enabling the equivalent residues in the *E. coli* enzyme, His-264, His-229, His-306, Arg-314, Arg-387 and Arg-407, to be identified. There is as yet no crystal structure for the *E. coli* enzyme; however, the *E. coli* amino acid sequence has been modelled using the pig heart structure (D. P. Bloxham, unpublished work; Duckworth et al., 1987).

Oxaloacetate binds first to the enzyme, inducing a conformational change which results in the complete formation of the site for the binding of the second substrate, acetyl-CoA. His-264, suspected to be located near the hinge point between the two domains of the enzyme (Remington et al., 1982), was mutated to an asparagine in order to disrupt this conformational change upon oxaloacetate binding. In addition, this histidine plays a key role in the enolization of acetyl-CoA (Karpusas et al., 1990; Alter et al., 1990; Man et al., 1991).

The most crucial feature in the binding of oxaloacetate is the resulting polarization of the carbonyl group. This polarization has been demonstrated by n.m.r. (Kurz et al., 1985) and Fourier transform i.r. spectroscopy (Kurz and Drysdale, 1987). The primary group involved in this polarization must be His-305 in *E. coli*, as this protonated histidine initially forms a hydrogen bond with the carbonyl oxygen and subsequently transfers this proton during catalysis. However, associated with this histidine is Arg-314, while comparison with the pig heart crystal structure would suggest that this arginine will form a salt bridge with Asp-312. The role of Arg-314 is unclear but an interesting comparison is possible with lactate dehydrogenase, where reduction of the carbonyl group of pyruvate also involves a hydrogen-bonded histidine as the source of the transferred proton. This histidine also has an attendant arginine, mutation of which to glutamine results in greatly reduced polarization of the carbonyl function in a substrate analogue of pyruvate (Clarke et al., 1986). Therefore it was of considerable interest to mutate Arg-314 to Gln-314 in citrate synthase and to examine the effect of this mutation on oxaloacetate binding and catalysis.

The role of Asp-312 was also of interest, particularly because a similar conserved aspartate is seen in lactate dehydrogenase, although in this case it is directly associated with the catalytic histidine (Clarke et al., 1988). Given the clearly defined role of the catalytic histidine residue (His-305) in binding and catalysis, it was decided not to attempt a mutation of this histidine, especially as such a mutation has already been described (Anderson et al., 1989) as producing an essentially inactive enzyme.

A major advantage of citrate synthase for mutagenesis studies is that the enzyme activity can also be analysed in terms of two partial reactions, namely the hydrolysis and cleavage of the

citryl-CoA intermediate. The cleavage of citryl-CoA back to the substrates oxaloacetate and acetyl-CoA represents a formal reversal of the condensation stage. This more detailed analysis allows the effect of mutations to be investigated in terms of the participation of catalytic residues in the individual partial reactions (Man et al., 1991).

The results presented in this paper demonstrate the importance of three active-site residues, His-264, Arg-314 and Asp-312, in binding and catalysis of the enzyme. In particular, a strong parallel is observed between the Arg-314 and Asp-312 residues in terms of the effect of charge removal on the properties of the enzyme. This linkage of the effects of mutation supports the proposal that, as in the pig heart citrate synthase, these two residues are closely associated in the *E. coli* enzyme, probably as a salt bridge. A major change in the kinetic properties that was associated with the R314Q and D312N mutations resulted in the exposure of a rate-limiting step between the two partial reactions of citryl-CoA formation and hydrolysis, and this complements a similar observation that has been reported for the citrate synthase from *Sulfolobus solfataricus* (Lill et al., 1992).

EXPERIMENTAL

Materials

Citryl-CoA was prepared and purified as described by Lohlein-Werhahn and Eggerer (1980). All other chemicals were obtained from Sigma Chemical Co.

Site-directed mutagenesis

The 2.1 kb *SacI*–*SalI* fragment from plasmid pDB₂ (Ner et al., 1983) was subcloned into a bacteriophage M13mp19; this was followed by oligonucleotide-mediated mutagenesis (Zoller and Smith, 1983) and enrichment for mutants by the method of Kunkel (1985). The following mutagenic primers, purified as described previously (Taylor et al., 1990) were used: mp1 (Arg-314 to Gln-314), 5'-ACGACCCGCAAGGCCACCGTA-3'; mp2 (Asp-312 to Asn-312), 5'-AAAATTACAACCCGCGCGC-3'; mp3 (His-264 to Asn-264), 5'-GACCTGCGAACGGCGGTGC-3'. The mutated codons are underlined and the altered nucleotides are emboldened. In each case, the mutated genes were re-sequenced using specific primers before recloning the gene, as an *SacI*–*BglII* fragment, into pDB₂. Plasmids specifying the mutant and non-mutant forms of citrate synthase were then introduced into the recombinant-deficient *E. coli* strain DEK15, which carries a deletion in the *gluA* gene, the structural gene for citrate synthase (Walsh and Koshland, 1985).

Purification of wild-type and mutant citrate synthases

A volume (2 l) of Luria broth containing ampicillin (25 µg/ml, final concentration) was inoculated with 1 ml of an overnight culture of *E. coli* DEK15 containing PDB₂ and with forced aeration at 37 °C for 24 h. Bacteria were isolated and the enzyme was purified as described previously (Handford et al., 1988). Enzyme fractions eluted after DEAE-cellulose ion-exchange chromatography were further purified by f.p.l.c. on a Mono-Q (Pharmacia) column.

Enzyme assays

The 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) assay of citrate synthase (Srere, 1963) and its partial reactions (citryl-CoA

cleavage and citryl-CoA hydrolysis) have been described previously (Bayer et al., 1981; Handford et al., 1988; Man et al., 1991). All spectrophotometric assays were performed at room temperature in a total volume of 0.8 ml with a Hitachi U2000 spectrophotometer. The rates of the reactions were determined for various concentrations of substrate and these were analysed using the ENZFITTER computer programme (Leatherbarrow, 1987) to calculate the K_m and k_{cat} values.

ANS binding to citrate synthase

Aliquots (1–20 µl) of 10 mM solution of the fluorescent probe 8-anilino-1-naphthalenesulphonic acid, Mg salt (ANS) were added to a cuvette containing 50 µg of protein in 1 ml of Tris/HCl, pH 8.0, 1 mM EDTA. Emission was monitored at 518 nm after excitation at 365 nm (5 nm slit width). This was repeated in the presence of a range of concentrations (20 µM–5 mM) of oxaloacetate. The fluorescence of ANS in free solution was also determined and subtracted from values obtained in the presence of protein to give a measure of bound ANS.

Reaction of citrate synthase with DTNB

Normal assay conditions

Purified citrate synthase (0.1–0.5 mg) was incubated with DTNB to determine the number and rate of exposed cysteines reacting with DTNB as previously described (Man et al., 1991).

Denaturing conditions

Purified citrate synthase (0.1 mg) was incubated with 4 M urea in the presence of 100 µM DTNB. Release of the thiol groups was followed spectrophotometrically at 412 nm. The experiment was repeated in the presence of increasing concentrations of oxaloacetate (0.1 mM–10 mM) as previously described (Handford et al., 1988).

NADH binding of citrate synthase

Small aliquots (1 or 2 µl) of 0.2 mM NADH were added sequentially to 1 ml of 20 mM Tris/HCl, pH 8.0, 1 mM EDTA, containing 50 µg of purified citrate synthase. Fluorescence emission was monitored at 460 nm after excitation at 350 nm. The fluorescence of free NADH in solution was also measured and subtracted from the above values to obtain a measure of NADH binding to citrate synthase as previously described (Handford et al., 1988).

RESULTS

Purification of wild-type and mutant forms of citrate synthase from *E. coli*

The wild-type and mutant proteins were isolated and purified from *E. coli* as described in the Experimental section. In each case, the citrate synthase protein was the major protein in the soluble fraction of the cell, and was obtained with a yield of 50 mg/l. The wild-type and mutant proteins ran as a single band with a M_r of 50000 on SDS/PAGE.

Studies on binding and catalysis of wild-type and mutant citrate synthases under normal assay conditions

The residues Arg-314 and Asp-312 were converted to a glutamine and an asparagine respectively to investigate the effects of

Table 1 Kinetic constants for overall reactions of citrate synthase

All assays were performed in 0.1 M Tris/HCl buffer, pH 8.0 or 7.0. The enzyme was incubated with varying concentrations of substrate (25 μ M–1 mM) and 100 μ M DTNB for 2–30 min. The rates of the reactions were obtained by measuring A_{412} . Results shown are the means $\pm$ S.D. of at least three determinations; 1 unit is 1 μ mol of substrate transformed/min. n.d., not determined; WT, wild-type.

Sample	Overall activity (units/mg)		Oxaloacetate K_m (μ M)		Acetyl-CoA K_m (μ M)	
	pH 7	pH 8	pH 7	pH 8	pH 7	pH 8
WT	12 $\pm$ 4.7	81 $\pm$ 0.1	77 $\pm$ 18	50 $\pm$ 14	120 $\pm$ 20	350 $\pm$ 8
H264N	n.d.	< 0.001*	n.d.	n.d.	n.d.	n.d.
D312N	0.041 $\pm$ 0.018	0.002 $\pm$ 0.0005*	800 $\pm$ 150	n.d.	390 $\pm$ 9	n.d.
R314Q	0.036 $\pm$ 0.023	0.003 $\pm$ 0.0002*	1000 $\pm$ 200	n.d.	290 $\pm$ 14	n.d.

* Assays performed using 1 mM oxaloacetate and 250 μ M acetyl-CoA.

Table 2 Kinetic data for the partial reactions of citrate synthase

All assays (0.8 ml) were performed in 0.1 M Tris/HCl buffer, pH 8.0. The enzyme was incubated with citryl-CoA and either 100 μ M DTNB or 165 μ M NADH plus 1 unit of malate dehydrogenase for 2–30 min. Results shown are the means $\pm$ S.D. of at least three determinations. Assays for citryl-CoA hydrolysis and citryl-CoA lyase activities were performed by measuring at A_{412} and A_{340} respectively; 1 unit is 1 μ mol of substrate transformed/min.

Sample	Citryl-CoA hydrolysis (unit/mg)	Citryl-CoA lyase (unit/mg)	Utilization of citryl-CoA (nmol) in	
			Hydrolysis	Lyase
WT	9 $\pm$ 0.7	3.1 $\pm$ 0.6	30*	14*
H264N	0.007 $\pm$ 0.0004	0.003 $\pm$ 0.0004	—	—
D312N	0.53 $\pm$ 0.04	0.35 $\pm$ 0.0321†	40†	21†
R314Q	0.23 $\pm$ 0.01	0.11 $\pm$ 0.01	39†	25†

* Assays were performed using 55 μ M citryl-CoA.

† Assays were performed using 80 μ M citryl-CoA.

Table 3 Kinetic constants for the partial reactions of citrate synthase

All assays were performed in 0.1 M Tris/HCl buffer, pH 8.0 or 7.0. The enzyme was incubated with various concentrations (25–500 μ M) of citryl-CoA and 100 μ M DTNB for 2 min. The rates of the reactions were obtained by measuring A_{412} . Results shown are the means $\pm$ S.D. of at least three determinations; 1 unit is 1 μ mol of substrate transformed/min.

Sample	Citryl-CoA hydrolase activity			Citryl-CoA lyase activity			Hydrolase (k_{cat}/K_m)/lyase (k_{cat}/K_m)
	V_{max} (unit/mg)	K_m (μ M)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	V_{max} (unit/mg)	K_m (μ M)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	
WT	53 $\pm$ 13	57 $\pm$ 9	8×10^5	7.6 $\pm$ 1.5	26 $\pm$ 5	2.5×10^5	3.3
D312N, pH 8.0	3 $\pm$ 0.1	460 $\pm$ 9	6×10^3	2.4 $\pm$ 0.4	400 $\pm$ 4	5×10^3	1.2
D312N, pH 7.0	0.2 $\pm$ 0.08	440 $\pm$ 2	3×10^2	0.2 $\pm$ 0.02	440 $\pm$ 2	3×10^2	1.0
R314Q, pH 8.0	4 $\pm$ 0.1	830 $\pm$ 25	4×10^3	2.2 $\pm$ 0.5	410 $\pm$ 6	4.5×10^2	1.0
R314Q, pH 7.0	0.2 $\pm$ 0.1	830 $\pm$ 6	2×10^2	0.1 $\pm$ 0.04	430 $\pm$ 9	2×10^2	0.8

mutation on binding and catalysis. The overall reaction at pH 8.0 was very low, being $\sim 3 \times 10^{-3}$ % of the wild-type rate (Table 1) and too low to allow detailed kinetic analysis. However, the partial reactions showed a significant rate (Tables 2 and 3). Citryl-CoA hydrolysis by the D312N mutant showed an activity of 6 % of the wild-type value and the activity of the R314Q mutant was 3 % of the wild-type value (Table 2). Citryl-CoA lyase activity of the D312N mutant was 11 % of that of the wild-type whereas the activity of the R314Q mutant was 3.5 % of the wild-type value (Table 2). Moreover, when the actual amounts of product from each reaction were determined after the reaction had reached completion, the ratio of products from the two reactions was broadly maintained for the wild-type and the two

mutant enzymes (Table 2). The K_m values of citryl-CoA calculated for the R314Q and D312N mutants at pH 8.0 were 823 and 455 μ M respectively (Table 3). These values were considerably higher than that determined for the wild-type (K_m 57 μ M) (Table 3). When values of the K_{cat}/K_m ratio for hydrolase and lyase were compared for wild-type and mutant enzymes (Table 3), it was found that both mutations had a slightly greater effect on the efficiency of the hydrolase activity. Moreover, it was clear that the effects of the two mutations, D312N and R314Q, on catalysis were very similar.

His-264 was converted to an asparagine because in the pig heart crystal structure it is the δ -nitrogen of His-264 that hydrogen bonds to the acetyl-CoA-derived carboxyl in the enzyme-product

complex. It is proposed that the δ -nitrogen of histidine can be mimicked by the amide nitrogen of asparagine (Leatherbarrow and Fersht, 1987). The H264N mutation rendered the enzyme essentially inactive in terms of both the overall and partial reactions (Table 1). The very low apparent rates that were observed were not significant and using the normal indirect assay conditions involving DTNB that are employed as a result, no kinetic parameters for substrate binding could be determined (see below).

Studies on binding and catalysis of the mutants at pH 7.0

Because the initial polarization of the carbonyl group of oxaloacetate and the subsequent condensation reaction will require a protonated His-305, it is possible that the very low overall activity observed at pH 8 with the R314Q and D312N mutants was attributable in part to an unfavourable change in the pK_a of this histidine. Therefore, it was of interest to investigate the activities of the mutants at pH 7, which would facilitate protonation of this histidine, although it should be noted that in the wild-type enzyme overall activity is reduced at pH 7.

When the overall activity was measured at pH 7, a value of 0.3% of wild-type activity was achieved for both mutants (Table 1). Thus a lowering of the pH increased the rate of the overall reaction at least 10-fold. On the other hand, the activities of the partial reactions were reduced at least 10-fold at pH 7.0 compared with pH 8.0 for the two mutants (Table 3). Again, this effect can be explained by a change in the pK_a of His-305 in the mutants, as citryl-CoA should bind to the unprotonated form of His-305 before catalysis. However, it should be noted that K_m for citryl-CoA did not change significantly on lowering the pH to 7.0.

These results are most readily explained in terms of an overall inhibition of activity of the partial reactions at pH 7 in line with that observed for the inhibition of the overall reaction of the wild-type enzyme at this pH. On the other hand, the stimulation of the mutant enzyme at pH 7.0 from a very low rate at pH 8.0 could be caused by the facilitation of the rate-limiting step between the citryl-CoA formation and hydrolysis reactions.

The higher overall activity at pH 7 for the mutant enzymes allowed kinetic measurements to be made to determine the binding of the normal substrates, oxaloacetate and acetyl-CoA. The binding of oxaloacetate was decreased dramatically, as indicated by the increase in K_m values to 1 mM for the R314Q mutant and 800 μ M for the D312N mutant (Table 4). The K_m value for acetyl-CoA binding of mutant R314Q (K_m 294 μ M) and D312N (K_m 385 μ M) was slightly higher than for the wild-type (K_m 190 μ M).

DTNB reactivity of the binding mutants

E. coli citrate synthase contains seven cysteines (Ner et al., 1983; Bhayana and Duckworth, 1984), one of which is exposed and reacts with DTNB (Talgoy et al., 1979). The DTNB reactivity of wild-type and the binding mutants were similar, as shown by the rate at which the cysteine reacted with DTNB (Table 4). They all showed exposure of one of their seven cysteines, as implied by the 1:1 ratio of mol of DTNB reduced/mol of protein (Table 4). This indicates that the overall structure of the mutants was not drastically altered. This result should be compared with the results of mutagenic studies involving the catalytic residue Asp-362, where DTNB reactivity was enhanced in mutants in which the negative charge was lost (Man et al., 1991).

In an alternative use of DTNB under denaturing conditions,

Table 4 DTNB reactivity of wild-type (WT) and mutant *E. coli* citrate synthase

All incubations were performed using 0.15–0.5 mg of protein in 0.1 M Tris/HCl buffer, pH 8.0. The protein was incubated with 100 μ M DTNB and the rate of reactivity was obtained by measuring A_{412} . Results shown are the means $\pm$ S.D. of at least three determinations.

Citrate synthase	Rate of DTNB reaction (nmol/min per nmol of protein)	Extent of DTNB reaction (mol of DTNB reduced/mol of protein)
WT	0.15 $\pm$ 0.01	1.0 $\pm$ 0.05
D312N	0.20 $\pm$ 0.03	1.2 $\pm$ 0.10
R314Q	0.15 $\pm$ 0.02	0.9 $\pm$ 0.09
H264N	0.12 $\pm$ 0.02	0.9 $\pm$ 0.03

Figure 1 Thiol reactivity of wild-type (WT) and H264N mutant citrate synthase

Assays were performed using 100 μ M DTNB in the presence of 4 M urea. The enzyme was incubated with different concentrations of oxaloacetate: ●, 0 mM WT; ★, 0.1 mM WT; ▲, 1 mM WT; ■, 4 mM WT; ○, 0 mM H264N; □, 4 mM H264N; ◇, 10 mM H264N.

the ability of oxaloacetate to protect the enzyme from cysteine exposure, and hence DTNB reactivity, was used to test for oxaloacetate binding with the H264N mutant. The results (Figure 1) show that whereas almost complete protection against DTNB reactivity was achieved in the wild-type with 4 mM oxaloacetate, only partial protection was achieved with the Asn-264 mutant, even with oxaloacetate concentrations as high as 10 mM. Thus it would appear that the binding of oxaloacetate to the H264N mutant was greatly reduced.

ANS binding of the mutants

An alternative procedure for detecting large changes in protein conformation is to study the binding of the fluorescent probe ANS (Kollmann-Koch and Eggerer, 1989). The binding of this hydrophobic fluorescent reagent to the wild-type and mutant enzymes in the presence of oxaloacetate was investigated. The ability of oxaloacetate to induce a large conformational change on binding to citrate synthase, and hence a change in ANS fluorescence, was used to evaluate the binding of oxaloacetate to wild-type and the H264D mutant. It was observed that whereas oxaloacetate reduced the increase in fluorescence on ANS binding in the wild-type, no such reduction was observed with the mutant (results not shown), thus confirming the greatly reduced ability

of the mutant to bind oxaloacetate and induce a conformational change in the protein.

NADH binding to the mutant enzymes

Unlike pig heart citrate synthase, the enzyme from *E. coli* binds NADH as an allosteric effector at a site distinct from the active site (Duckworth et al., 1987). The binding of NADH may be readily monitored by fluorescence enhancement, and when this was performed with wild-type and mutant enzymes no significant change in binding affinity was detected, thus indicating a lack of gross structural change in the proteins on mutation. The K_d values for NADH were 1.87, 2.63, 2.95 and 2.55 μ M for the wild-type, H264D mutant, R314Q mutant and D312N mutant enzymes respectively.

DISCUSSION

Following the identification of the residues involved in the binding of oxaloacetate in pig heart citrate synthase using X-ray crystallographic data (Remington et al., 1982), homology studies with *E. coli* citrate synthase have become possible (Bloxham et al., 1982; Bell et al., 1983). The corresponding amino acids in the *E. coli* enzyme were determined, which enabled subsequent site-directed mutagenesis of these active-site residues in this enzyme.

Effect of mutations on substrate binding

The results presented in this paper indicate that the active-site residues Arg-314, Asp-312 and His-264 are required for the binding of oxaloacetate. The K_m values for oxaloacetate indicated that oxaloacetate-binding affinity in the R314Q and D312N mutants was reduced. The lack of significant enzyme activity with the H264N mutant prevented the calculation of an actual K_m for oxaloacetate affinity. However, ANS binding experiments demonstrated that no conformational change could be detected in the presence of oxaloacetate and hence the binding of this substrate may be minimal. This result was confirmed by DTNB protection experiments, which also indicated a lack of oxaloacetate binding.

Zhi et al. (1991) performed conformational stability studies on pig heart synthase and demonstrated that His-274 mutations cause a reduced conformational flexibility of the protein, which may cause a decrease in the overall catalytic efficiency of the enzyme. Mutations of this histidine have already been performed on the pig heart enzyme (Alter et al., 1990) to produce catalytically deficient enzymes in terms of overall enzyme activity. Oxaloacetate binding of these mutants was not affected, as demonstrated by the K_m values for this substrate; however, no studies on the partial reactions were performed.

It is interesting that whereas we found no catalytic activity and reduced oxaloacetate binding with the H264N mutant in *E. coli*, the corresponding H274R and H274G mutations in the pig heart enzyme showed no effect on oxaloacetate binding and retained significant overall catalytic activity. This would confirm that the His-274 residue is not directly involved in the binding of oxaloacetate but achieves an effect indirectly via conformational disturbances resulting from the mutation. It would appear that mutation of the pig heart His-274 to arginine or glycine still allows the required conformational changes. It is possible that the active-site region for the *E. coli* enzyme is more sterically constrained than the pig heart enzyme and is thus more sensitive

to amino acid replacements (W. J. Man, Y. Li, C. D. O'Connor and D. C. Wilton, unpublished work).

The results with the *E. coli* enzyme are consistent with the predicted location of these residues within the enzyme based on comparison with the pig heart crystal structure. Arg-314 is predicted to be within hydrogen-bonding distance of the C-1 carboxyl group and the carbonyl oxygen of oxaloacetate and to form a salt bridge with Asp-312. His-264 has been assumed to be located at the hinge point of the enzyme, again based on comparison with the pig heart enzyme (Remington et al., 1982), and any alteration of this residue may have adverse effects on the conformational change induced on oxaloacetate binding, so preventing catalysis.

Effect of mutations on catalysis

The first step in the overall reaction is the condensation of acetyl-CoA with oxaloacetate, which requires the removal of a proton from the methyl group of acetyl-CoA. A major source of substrate activation in this process must be the polarization of the carbonyl group of oxaloacetate, and such polarization has been observed upon binding of oxaloacetate to the enzyme using n.m.r. (Kurz et al., 1985) and Fourier transform i.r. spectroscopy techniques (Kurz and Drysdale, 1987). The mechanism of this polarization is unknown. However, Arg-314 may be implicated because of its interaction with the carbonyl oxygen of oxaloacetate. Moreover, in the case of lactate dehydrogenase, which involves an analogous carbonyl polarization of pyruvate, the involvement of the equivalent arginine has been demonstrated using mutagenesis. Therefore, this arginine in *E. coli* citrate synthase along with its presumptive counterion, Asp-312, were mutated to a glutamine and asparagine respectively in order to remove the formal charge on these residues and to assess this change in active-site charge on catalysis.

The remarkable feature of these mutations was that in both cases overall activity was negligible at pH 8.0 whereas both partial reactions (citryl-CoA cleavage and hydrolysis), which together should reflect the overall activity, were inhibited to a much smaller extent. This strongly suggests that a step between citryl-CoA formation and hydrolysis had become rate-limiting in these mutants. The nature of this step is unknown, but must involve a conformational change involving both the Arg-314 and Asp-312 residues acting in unison, as the mutation of either of these residues produced a very similar effect.

Strong support for the conclusion that there is a rate-limiting conformational change during catalysis has come from recent work with the citrate synthase from *S. solfataricus* (Lill et al., 1992). In this study, the enzyme was subjected to limited proteolysis with trypsin which decreased the overall enzyme activity to 4% of the native enzyme, but the rates of both partial reactions remained unaffected. It was suggested that the communication between the lyase and hydrolase forms of the enzyme was inhibited by this proteolysis. Taken together, these two results highlight the importance of the conformational changes that occur between the two catalytic forms of the enzyme and which have been discussed in terms of the pig heart crystal structures (Remington et al., 1982; Wiegand and Remington, 1986). Moreover, it is possible that conformational changes may be related to the change in charge distribution at the active site as catalysis proceeds. This explanation is consistent with our observations that the change in the overall charge at the active site with the R314Q and D312N mutant enzymes has a dramatic effect on the complete overall reaction, although the rates of the individual partial reactions were much less affected (Table 3). Thus, the change in active-site charge has resulted in a primary

effect on a conformational step between the formation and hydrolysis of citryl-CoA. Arg-314 has also been mutated to a leucine residue, which rendered the enzyme incompetent with respect to its overall activity; however, no data were available on the rates of the partial reactions of this mutant (Anderson and Duckworth, 1989).

The overall mechanism of citrate synthase is controversial in terms of the active-site residues involved in the deprotonation of acetyl-CoA and the subsequent condensation reaction. Based on the pig heart crystal structures with various substrate analogues, and supported by mutagenesis studies on this enzyme, an attractive mechanism has been proposed for the enolization step (Karpusas et al., 1990; Alter et al., 1990). This mechanism involves the catalytic aspartate (Asp-362 in *E. coli*) acting as a base to remove the methyl proton while the protonated histidine (His-264 in *E. coli*) acts as an electrophile and stabilizes the enolate. Our mutagenesis studies with the *E. coli* enzyme involved, in particular, a D362E mutation which resulted in the overall reaction and the hydrolysis reaction being 98% inhibited. However, the condensation reaction, which was measured in terms of citryl-CoA cleavage back to substrates, was not affected by this mutation (Man et al., 1991). Therefore, we were led to propose a mechanism in which the deprotonation step is catalysed by His-264 acting as a base while there is minimal involvement of the aspartate at this stage of the overall mechanism. The results presented in the present paper highlight the crucial importance of a conformational change between the condensation and hydrolysis steps in the overall reaction and, therefore, the possible added complexity of interpreting data based on externally added citryl-CoA as a substrate. Also, because of the conformational change that occurs during catalysis, mechanistic information based on the crystal structure enzyme-inhibitor complexes must be interpreted with caution.

In summary, His-264, Arg-314 and Asp-312 are important in the binding and catalysis of citrate synthase. The ability to measure a number of parameters of citrate synthase activity, including the partial reactions, has allowed us to evaluate the effect of some of these mutations at various stages in the overall catalytic pathway. The results have demonstrated that Arg-314 and Asp-312 appear to act in unison, consistent with a structural linkage between these two groups. In addition, a rate-limiting step between citryl-CoA formation and hydrolysis expressed in the R314Q and D312N mutants has been identified and is

consistent with a significant conformational change during catalysis.

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