Conversion of citrate synthase into citryl-CoA lyase as a result of mutation of the active-site aspartic acid residue to glutamic acid

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The active-site aspartic acid residue, Asp-362, of *Escherichia coli* citrate synthase was changed by site-directed mutagenesis to Glu-362, Asn-362 or Gly-362. Only very low catalytic activity could be detected with the Asp→Asn and Asp→Gly mutations. The Asp→Glu mutation produced an enzyme that expressed about 0.8 % of the overall catalytic rate, and the hydrolysis step in the reaction, monitored as citryl-CoA hydrolysis, was inhibited to a similar extent. However, the condensation reaction, measured in the reverse direction as citryl-CoA cleavage to oxaloacetate and acetyl-CoA, was not affected by the mutation, and this citryl-CoA lyase activity was the major catalytic activity of the mutant enzyme. This high condensation activity in an enzyme in which the subsequent hydrolysis step was about 98 % inhibited permitted considerable exchange of the methyl protons of acetyl-CoA during catalysis by the mutant enzyme. The K_m for oxaloacetate was not significantly altered in the D362E mutant enzyme, whereas the K_m for acetyl-CoA was about 5 times lower. A mechanism is proposed in which Asp-362 is involved in the hydrolysis reaction of this enzyme, and not as a base in the deprotonation of acetyl-CoA as recently suggested by others. [Karpusas, Branchaud & Remington (1990) *Biochemistry* 29, 2213–2219; Alter, Casazza, Zhi, Nemeth, Srere & Evans, (1990) *Biochemistry* 29, 7557–7563].

INTRODUCTION

Citrate synthase (EC 4.1.3.7) belongs to an important group of enzymes that catalyse the formation of a new carbon–carbon 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 three potentially important conserved residues for catalysis, namely His-274, His-320 and Asp-375. Despite the absence of a crystal structure for *Escherichia coli* citrate synthase, both the gene sequence (Ner *et al.*, 1983) and the chemically determined amino acid sequence (Bhayana & Duckworth, 1984) are available. Although the overall amino acid identity between the pig and *E. coli* enzymes is only 27 %, there is a high conservation of sequence in the active-site regions, enabling three equivalent active-site residues, His-264, His-305 and Asp-362, to be identified, and suggesting that the two enzymes operate through similar mechanisms.

The reaction catalysed by citrate synthase is particularly suited to a detailed analysis using site-directed mutagenesis because the overall process has been suggested to involve three partial reactions that are experimentally measurable. These reactions are: (a) generation of a carbanion or equivalent from acetyl-CoA by base abstraction of a proton (Eggerer, 1965); (b) nucleophilic attack of the carbanion of acetyl-CoA on oxaloacetate to produce citryl-CoA; (c) hydrolysis of citryl-CoA to produce citrate and CoA. Incubation of citrate synthase with the 'presumptive' intermediate citryl-CoA under appropriate conditions allows the monitoring of the hydrolytic reaction (c), as well as the reverse reaction to produce acetyl-CoA and oxaloacetate (Srere, 1963; Bayer *et al.*, 1981; Lohlein-Werhahn *et al.*, 1983).

In a preliminary study we investigated the role of the active-site aspartic acid residue in *E. coli* by the replacement of this residue by glycine. This mutation produced a protein that was catalytically incompetent owing to the inability of the protein to bind carboxymethyl-CoA, a presumptive transition-state ana-

logue of acetyl-CoA (Handford *et al.*, 1988). In the present paper we investigate the role of this aspartic acid residue in more detail, and in particular we study properties of the conservative mutation in which this residue is replaced by glutamic acid. This particular mutation highlighted the role of the active-site aspartic acid residue in the hydrolysis reaction and showed that the residue was not catalytically involved in the condensation reaction to produce citryl-CoA.

During the course of this work two independent publications have appeared on the pig heart enzyme that implicate this aspartic acid residue directly in the condensation step (Karpusas *et al.*, 1990; Alter *et al.*, 1990). Because the enzymes from pig heart and *E. coli* have conserved active-site residues they should operate via similar catalytic mechanisms. If this assumption is accepted then the mechanism proposed by these authors is not supported by our results.

EXPERIMENTAL

Materials

Chemicals were obtained from Sigma Chemical Co. [³H]Acetic anhydride and [1-¹⁴C]acetic anhydride were obtained from Amersham International, and radioactive acetyl-CoA was prepared as described previously (Handford *et al.*, 1988). Citryl-CoA was prepared and purified as described by Lohlein & Eggerer (1980).

Site-directed mutagenesis

A 2.1 kb *SacI*–*SalI* fragment from plasmid pDB2 (Ner *et al.*, 1983) was subcloned into bacteriophage M13 mp19 before oligonucleotide-mediated mutagenesis (Zoller & 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 (Asp-362 to Glu-362), 5'-CCGAACGTCGAATTCTACTCT-3'; mp2 (Asp-362 to Asn-362), 5'-CCGAACGTCGAATTCTACTCT-3'; mp3 (Asp-362 to Gly-362), 5'-ACGTCGGTTTCTACTCT-3'. In each case the

Abbreviations used: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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mutated genes were resequenced with the use of specific primers before transfer of the coding region, as an *SacI*-*Bgl*II fragment, to pDB2. Plasmids specifying the mutant and non-mutant forms of citrate synthase were then introduced into the recombination-deficient *E. coli* strain DEK15, which carries a deletion in *gltA*, the structural gene for citrate synthase (Walsh & Koshland, 1985).

Purification of wild-type and mutant citrate synthases

A 2-litre volume of L-broth containing ampicillin (25 µg/ml, final concentration) was inoculated with 1 ml of an overnight culture of *E. coli* DEK15 containing pDB2 and incubated with forced aeration at 37 °C for 24 h. Bacteria were isolated and the enzyme was purified as described previously (Handford *et al.*, 1988) except that cells were broken by using a French press. Enzyme fractions eluted after DEAE-cellulose ion-exchange chromatography were further purified by f.p.l.c. on a Pharmacia Mono-Q column.

Enzyme assays

The DTNB assay of citrate synthase (Srere, 1963) and its partial reactions (enolization, citryl-CoA hydrolysis and citryl-CoA cleavage) have been described previously (Handford *et al.*, 1988). All spectrophotometric assays were performed at room temperature in a total volume of 0.8 ml with a Hitachi U2000 spectrophotometer. In enolization experiments incubations were performed for between 7 and 20 h with between 1.25 and 2.25 mg of enzyme protein. Enolization rates were determined as d.p.m. of tritium released/h per mg of protein.

In order to determine the deprotonation of [³H]acetyl-CoA during the course of the overall reaction, normal enzyme assays (0.8 ml) were monitored at 412 nm. The reaction was stopped with 0.05 ml of 1 M-KOH and 0.01 ml of 1 M-sodium acetate and freeze-dried. The radioactivity in 0.1 ml of water was determined. Control incubations were performed without enzyme, and tritium released was subtracted from enzyme assays.

Citrate was purified from incubations on a Dowex-1 (formate form) column eluted with a formic acid gradient. Citric acid was eluted with 4 M-formic acid.

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. Catalytically inactive proteins were treated identically with the wild-type enzyme and the corresponding fractions were taken during purification. The wild-type and mutant proteins ran as a single band with an *M_r* of 50000 on SDS/PAGE.

Catalytic activity of wild-type and Asp-362 mutant forms of *E. coli* citrate synthase

The catalytic activities of the wild-type and mutant forms of citrate synthase are summarized in Table 1. Only the D362E mutant protein showed significant overall activity, being about 1 % of the wild-type value, whereas essentially no activity could be detected with the D362N and D362G mutants within the limits of detection of the assay method. When the activity of citryl-CoA hydrolysis was assessed, the D362E mutation showed a rate of about 2 % of the wild-type value.

The crucial observation concerned the measurement of citryl-CoA cleavage back to oxaloacetate and acetyl-CoA. In this assay the rates of the wild-type and D362E mutant enzymes were essentially identical. By contrast, no significant activity could be detected in the assay of citryl-CoA hydrolysis or cleavage with either the D362N or the D362G mutant proteins. The lack of activity of the glycine mutant confirms our previous observation on this mutant, where the protein was unable to bind carboxymethyl-CoA (Handford *et al.*, 1988).

Measurement of the enolization reaction catalysed by wild-type and mutant citrate synthases

The first stage in the condensation reaction catalysed by citrate synthase is the presumptive enolization of acetyl-CoA, and the method of assaying this step has been to monitor the deprotonation of [³H]acetyl-CoA in the presence of (*S*)-malate (Eggerer, 1965). Although the rate of the exchange reaction is 1.4×10^5 -fold slower than the rate of the overall reaction (Singh *et al.*, 1970) and requires the 'incorrect' stereoisomer of malate as a presumptive analogue of oxaloacetate, its occurrence has been taken as evidence that the first step in the citrate synthase-catalysed transformation involves a deprotonation reaction. Table 1 shows that the D362E mutation showed greatly decreased rates of exchange in this assay, with inhibition being to a similar extent to that seen for the overall and hydrolysis steps.

Measurement of tritium loss from [³H]acetyl-CoA during the overall reaction catalysed by citrate synthase

The demonstration that the hydrolytic stage in the overall reaction was decreased to about 2 % in the D362E mutant enzyme whereas the citryl-CoA lyase reaction was unaffected prompted the measurement of the release of tritium from [³H]acetyl-CoA in the presence of oxaloacetate catalysed by the wild-type and mutant enzymes. Increased tritium loss over and above that compulsorily required in the overall reaction may be expected during the reversal of the reaction, i.e.:

when the hydrolysis of citryl-CoA into citrate is blocked by the D362E mutation.

Tritium loss was measured under normal assay conditions and involved stopping the reaction after approx. 10 % formation of products (Table 2, Expts. 1-3). It should be noted that in this experiment the amounts of protein used have been adjusted so that the overall rates in the wild-type and mutant incubations are very similar. When tritium loss to the medium from the [³H]acetyl-CoA was measured the D362E mutant protein showed about a 5-fold greater loss of tritium compared with the wild-type protein (Table 2). Thus in the condensation reaction in the D362E mutant a high degree of exchange of the tritium from [³H]acetyl-CoA occurs before product formation as a result of the hydrolysis step being about 98 % inhibited. This represents a new catalytic feature of the mutant enzyme. The pattern of tritium loss was maintained when the reaction was allowed to go to completion in the presence of limiting acetyl-CoA (Table 2, Expt. 4), providing further evidence that the loss of tritium was an integral part of the overall reaction.

In order to confirm the high loss of tritium from acetyl-CoA on conversion into citrate, the experiment was repeated in the presence of [¹⁻¹⁴C]acetyl-CoA (³H/¹⁴C ratio 3.00) and the citrate was isolated after the reaction was allowed to proceed to completion in the presence of excess oxaloacetate. The ³H/¹⁴C ratios for citrate derived from wild-type and mutant enzyme incubations were 2.64 and 1.65 respectively. This result confirms the greatly enhanced loss of tritium from acetyl-CoA with the mutant enzyme. In particular the results demonstrate that in

Table 1. Catalytic activities of *E. coli* citrate synthases

Experimental details are given in the text.

Protein	Overall activity (units/mg)	Enolization of acetyl-CoA (% of wild-type rate)	Citryl-CoA hydrolysis (units/mg)*	Citryl-CoA cleavage (units/mg)*
Wild-type (Asp-362)	81.1 ± 0.14	100	8.82 ± 0.65	3.10 ± 0.59
Glu-362	0.65 ± 0.06	2.3	0.20 ± 0.03	3.19 ± 0.48
Asn-362	< 0.001†	0	< 0.001	< 0.001
Gly-362	< 0.001†	—	< 0.001	< 0.001

* Assays were performed at 50 μ M-citryl-CoA and initial rates were determined.

† Assays were performed at 625 μ M-acetyl-CoA and 125 μ M-oxaloacetate and initial rates were determined.

Table 2. Deprotonation of [3 H]acetyl-CoA during catalysis by wild-type and Glu-362 *E. coli* citrate synthases

All assays were performed in 0.1 M-Tris/HCl buffer, pH 8, in a final volume of 0.8 ml. In Expts. 1–5 the amounts of wild-type and mutant enzymes were adjusted to give essentially identical initial rates of product formation. Expts. 1–3: incubations were performed with 145 μ M-acetyl-CoA and 100 μ M-oxaloacetate. Expt. 4: incubations were performed with 72.5 μ M-acetyl-CoA and 1 mM-oxaloacetate and allowed to proceed to completion. Expt. 5: incubations were performed with 109 μ M-acetyl-CoA (labelled with both 3 H and 14 C) and 1 mM-oxaloacetate and allowed to proceed to completion. Expt. 6: incubations were performed with 6.7 μ g of wild-type and 7.0 μ g of mutant enzyme; product formation was too low to measure with the mutant enzyme.

		Incubation time (min)	Product formed (nmol)	Tritium released (ng-atoms)	Hydrogen lost (g-atoms/mol of product)
Expt. 1	Wild-type	1	7.2	2.90	0.40
	Glu-362	1	7.0	17.4	2.49
Expt. 2	Wild-type	1.5	9.8	2.65	0.27
	Glu-362	1.5	10.1	20.7	2.05
Expt. 3	Wild-type	2.5	10.5	3.66	0.35
	Glu-362	2.5	10.6	21.0	1.98
Expt. 4	Wild-type	30	51	17.35	0.34
	Glu-362	30	55	77.7	1.41
Expt. 5	Wild-type	30	68	14.1	0.26
	Glu-362	30	70	66.0	1.19
Expt. 6	Wild-type	0.4	20	6.3	0.32
	Glu-362	0.4	—	0.3	—

excess of one tritium atom is lost per molecule of citrate that is formed. With the wild-type enzyme of tritium product isotope effect of about 3 is indicated for the overall reaction, assuming there is no exchange of the hydrogen atoms of acetyl-CoA.

When the tritium released to the medium in this double-labelled experiment was also measured (Table 2, Expt. 5) the result mirrored that for the tritium retained in the citrate. Thus again there was in excess of one atom of tritium per molecule lost to the medium and over 90 % of the tritium could be accounted for either in citrate or in the medium for both for wild-type and mutant experiments.

In order to obtain a more direct comparison of rate of tritium loss from acetyl-CoA for both wild-type and mutant enzymes, the initial rate of tritium loss was compared per mg of enzyme protein. When this was calculated for Expts. 1–3 in Table 2 the mean values for wild-type and mutant were 2462 and 103 g-atoms of tritium released/min per mg of enzyme respectively. That is to say the mutant rate of tritium loss from acetyl-CoA was 4.2 % of that of the wild-type enzyme, and this compares with the value of 0.8 % when the overall rate of the mutant is compared with that of the wild-type enzyme. The results from Expt. 6 in Table 2 in which similar amounts of enzyme are used confirms this value of 4.2 %.

If citryl-CoA is an obligatory intermediate during normal

enzyme catalysis then the rate of formation of citryl-CoA with the D362E mutant should be the same as for the wild-type because the rates of the reverse reaction, citryl-CoA lyase, are identical for the two enzymes. However, since citryl-CoA is not a free intermediate in the reaction, tritium loss as a result of reversible formation of citryl-CoA depends, in the first instance, on the tritium released in the active site being exchangeable with the medium. If there were no exchange, then tritium loss would always parallel release of the final products and hence only reflect the rate of the overall reaction. The enhanced tritium loss with the D362E mutant indicates that partial exchange of the tritium can occur on citryl-CoA formation, and this process is greatly facilitated when citryl-CoA hydrolysis is inhibited while citryl-CoA formation is unaffected. Because there are no data on the rates of exchange of enzyme-bound tritium with the medium, it is not possible to quantify the rate of formation of enzyme-bound citryl-CoA from the tritium-exchange data.

DTNB-reactivity of wild-type and mutant forms of *E. coli* citrate synthase

It might be expected that other minimal modifications of the aspartic acid residue would also result in a mutant enzyme with significant citryl-CoA lyase activity. A clue to the lack of this activity demonstrated by, in particular, the D362N mutant came

Scheme 2. Condensation of acetyl-CoA with oxaloacetate involving Asp-362 and His-264 in proton removal from acetyl-CoA

X = His-305, Y = His-264 and Z = Asp-362 in the *E. coli* enzyme. X = His-320, Y = His-274 and Z = Asp-375 in the pig heart enzyme.

for which the crystal structure is available, these residues are His-274, His-320 and Asp-375, and the corresponding residues in the enzyme from *E. coli* are His-264, His-305 and Asp-362. Since alignment of the two sequences shows that virtually every residue that is thought to lie in the vicinity of the active site is conserved, it is therefore assumed that both enzymes operate by a similar mechanism. However, it should be borne in mind that in the absence of a crystal structure for the *E. coli* enzyme the possibility remains that it operates by a different mechanism. Although the role of His-320 has been clearly identified from the crystal structure of the pig heart enzyme as the group responsible for protonating the carbonyl group of oxaloacetate during the condensation reaction, the role of the other groups in catalysis, particularly Asp-375, remains elusive.

We have previously identified the involvement of the aspartic acid residue in the binding of acetyl-CoA by mutation of this residue in the *E. coli* enzyme to a glycine (D362G) (Handford *et al.*, 1988). However, such a drastic mutation failed to demonstrate any differential effect on the various partial reactions of citrate synthase. Moreover, subsequent studies indicated that the *gltA* gene is only partially deleted in *E. coli* W620, the host strain originally employed (Handford *et al.*, 1988). Thus the possibility of recombination between the plasmid-borne mutant *gltA* gene and the chromosomal *gltA'* gene, to yield residual wild-type citrate synthase activity, could not be discounted.

In this paper we describe the more subtle alterations of Asp-

362 to Glu-362 and Asn-362 in an *recA*⁻ derivative, strain DEK15, as well as repeating analysis of the Gly-362 mutation in this strain. The cumulative results from mutagenesis argue very strongly for a primary catalytic role of the active-site aspartic acid residue in the hydrolysis reaction (Scheme 1). It would also appear that binding of acetyl-CoA in the mutant, as judged by a lower *K_m*, is facilitated, presumably as the result of a subtle change in conformation of the acetyl-CoA-binding domain. The overall mechanism proposed for the condensation reaction in Scheme 1 involves His-264 in a catalytic role as the base that deprotonates acetyl-CoA. A concerted mechanism may be possible for enzymes catalysing a Claisen condensation (Clark *et al.*, 1988), and it should be noted that all these reactions proceed with inversion of stereochemistry (Hanson & Rose, 1975; Floss & Tsai, 1979). However, this type of mechanism has been excluded in the case of the analogous reaction catalysed by malate synthase (Clark *et al.*, 1988), where a stepwise mechanism involving enolate formation is proposed.

The results involving analysis of the partial reactions run contrary to the recent proposals by Karpusas *et al.* (1990) based on examination of the crystal structure of an enzyme-substrate-inhibitor complex involving the presumptive transition-state inhibitor carboxymethyl-CoA. They proposed a mechanism for the pig heart enzyme involving Asp-375 (pig heart numbering) as the base in the initial enolization of acetyl-CoA required for the condensation reaction (Scheme 2) while His-274 protonated the

carbonyl oxygen atom of the thioester. In addition, mutation studies with the pig heart enzyme in which the effects of the mutation on the overall reaction were investigated (Alter *et al.*, 1990) were also used to support a mechanism directly involving the aspartic acid residue as the base in the enolization step (Scheme 2). In an alternative mechanism (Wilde *et al.*, 1990) the role of these two groups has been reversed, with the aspartic acid residue facilitating enolization by protonation of the carbonyl oxygen atom of the thioester.

N.m.r. and Fourier-transform i.r. studies on the pig heart enzyme have identified considerable polarization of the carbonyl group of enzyme-bound oxaloacetate in the absence of the second substrate (Kurz *et al.*, 1985; Kurz & Drysdale, 1987). Such a polarization with the development of positive charge on the carbonyl carbon atom of oxaloacetate would stabilize the developing carbanion character of the acetyl-CoA and facilitate deprotonation of acetyl-CoA by His-264 and formation of a new carbon-carbon bond with oxaloacetate. The formal reversal of this process would allow cleavage of citryl-CoA back to substrates (Scheme 1). The n.m.r. studies (Kurz *et al.*, 1985) clearly demonstrated further polarization of the carbonyl carbon atom of oxaloacetate in the presence of carboxymethyl-CoA, and they proposed that this enhanced polarization resulted from stabilization of the positive charge on the carbon atom by the negatively charged carboxylate group of carboxymethyl-CoA. Scheme 1 supports this proposal and the enolate structure envisaged by these workers (Kurz & Drysdale, 1987). Thus the carboxymethyl-CoA may be a transition-state analogue of the developing carbanion character of acetyl-CoA inherent in Scheme 1.

Two mechanisms are possible to involve this aspartate in the hydrolysis step. (1) The aspartic acid residue can carry out a nucleophilic attack on the thioester carbonyl group of citryl-CoA to produce a covalent enzyme-bound citric anhydride intermediate that must subsequently be cleaved by a deprotonated water molecule to release products. (2) The aspartate acting as a base will directly deprotonate an active-site water molecule to allow OH⁻ ion cleavage of the thioester to release products.

As yet no evidence is available to support either of these alternatives. However, the availability of the D362E mutant citrate synthase in which the final hydrolysis is greatly inhibited may assist to resolve these two possibilities.

Wiegand & Remington (1986) highlighted the movement of this aspartic acid residue in the pig heart crystal structure as the protein conformation changed to its closed hydrolysis form. In that paper they suggested that the aspartic acid residue was involved in the hydrolysis reaction and suggested that movement of the aspartic acid residue prevented the enzyme from hydrolysing acetyl-CoA before condensation was complete. Our results indicate that the primary role of this residue is in hydrolysis and add support to the concept that conformational changes during catalysis prevent premature thioester hydrolysis.

In summary, we have established that the active-site aspartic

acid residue is involved in the hydrolysis step of the reaction catalysed by *E. coli* citrate synthase, a conclusion that is consistent with the speculations by Wiegand & Remington (1986) and Wilde *et al.* (1990). We suggest that the general mechanism should be applicable to other enzymes that catalyse this type of reaction.

We thank Professor D. Koshland for kindly supplying *E. coli* strain DEK15, and we are particularly grateful to Professor M. Akhtar for many helpful discussions. A Science and Engineering Research Council postgraduate studentship to W.J.M. is acknowledged.

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