In Vitro Mutagenesis of Escherichia coli Citrate Synthase to Clarify the Locations of Ligand Binding Sites*

Deborah H. Anderson and Harry W. Duckworth
From the Department of Chemistry, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2

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In vitro mutagenesis techniques have been used to investigate two structure-function questions relating to the allosteric citrate synthase of Escherichia coli. The first question concerns the binding site of \( \alpha \)-keto-glutarate, which is a structural analogue of the substrate oxaloacetate and yet has been suggested to be an allosteric inhibitor of the enzyme. Using oligonucleotide-directed mutagenesis of the cloned \( E. \text{coli} \) citrate synthase gene, we prepared missense mutants, designated CS226H-Q and CS229H-Q, in which histidine residues at positions 226 and 229, respectively, were replaced by glutamine. In the homologous pig heart citrate synthase it is known (Wiegand, 1986; Annu. Rev. Biophys. Chem. 15, 97–117) that the equivalent of His-229 helps to bind oxaloacetate, while the equivalent of His-226 is nearby. Kinetic and ligand binding measurements showed that CS226H-Q had a reduced affinity for oxaloacetate and \( \alpha \)-ketoglutarate, while CS229H-Q bound oxaloacetate even less effectively, and was not inhibited by \( \alpha \)-ketoglutarate at all under our conditions. This parallel loss of binding affinities for oxaloacetate and \( \alpha \)-ketoglutarate, in two mutants altered in residues at the active site of \( E. \text{coli} \) citrate synthase, strongly suggests that inhibition of this enzyme by \( \alpha \)-ketoglutarate is not allosteric but occurs by competitive inhibition at the active site. The second question investigated was whether the known inhibition by acetyl-CoA of binding of NADH, an allosteric inhibitor of \( E. \text{coli} \) citrate synthase, occurs heterotropically, as an indirect result of acetyl-CoA binding at the active site, or directly, by competition at the allosteric NADH binding site. Using existing restriction sites in the cloned \( E. \text{coli} \) citrate synthase gene, we prepared a deletion mutant which lacked 24 amino acids near what is predicted to the acetyl-CoA-binding portion of the active site. The mutant protein was inactive, and acetyl-CoA did not bind to the active site but still inhibited NADH binding. Thus acetyl-CoA can interact with both the allosteric and the active sites of this enzyme.

All citrate synthases which have been carefully investigated are composed of a single type of polypeptide chain of about 430 amino acids, corresponding to a molecular weight of about 48,000 g/mol (Sreer, 1972; Tong and Duckworth, 1975; Suissa et al., 1984; Rosenkranz et al., 1986; Wood et al., 1987; Donald and Duckworth, 1987). In most organisms, native citrate synthase is a dimer (Sreer, 1972; Weitzman and Danson, 1976). In Gram-negative bacteria, on the other hand, the enzymes are larger and form aggregates among which hexamers are prominent (Tong and Duckworth, 1975; Weitzman and Danson, 1976). These "large" citrate synthases are subject to strong and specific allosteric inhibition by NADH (Weitzman, 1966a; 1966b; Weitzman and Jones, 1968; Weitzman and Danson, 1976). In addition to this well established inhibition effect, the Krebs cycle intermediate \( \alpha \)-ketoglutarate inhibits some Gram-negative citrate synthases. Since this inhibition may be abolished by treatments (high pH, high salt concentration) which do not eliminate activity, it has been suggested that this \( \alpha \)-ketoglutarate inhibition is allosteric as well (Wright et al., 1967; Wright and Samwal, 1971). It is far from certain, however, that \( \alpha \)-ketoglutarate binds to a separate allosteric site, and the structural similarity between this molecule and the citrate synthase substrate oxaloacetate suggests the possibility that \( \alpha \)-ketoglutarate inhibition is simply a special kind of interaction at the active site (Talgoy and Duckworth, 1979).

A number of adenine nucleotides inhibit the binding of NADH to \( E. \text{coli} \) citrate synthase, the best studied of the Gram-negative enzymes; one of these is the substrate, acetyl-CoA (Duckworth and Tong, 1976). This inhibition by acetyl-CoA was first explained as a heterotropic interaction between the allosteric sites, where NADH binds, and the active sites, where acetyl-CoA must bind (Duckworth and Tong, 1976). This explanation was called into question, however, by the later finding that both NADH and acetyl-CoA, besides various noninhibitory adenylates, will protect a cysteine of citrate synthase from reaction with 5,5'-dithio-bis-(2-nitrobenzoic acid). Except for this acetyl-CoA effect, all the evidence indicated that this cysteine is in or near the NADH-binding site (Talgoy and Duckworth, 1979). Some adenylates which bind to this site actually activate the enzyme, and if acetyl-CoA bound at the allosteric site (as well as the active site) it might also act as an allosteric activator. This effect, rather than a classical homotropic interaction among active sites, could also explain the sigmoid saturation curve which acetyl-CoA exhibits under some conditions (Faloona and Sreer, 1969).

To clarify the first point, we have used the techniques of site-directed in vitro mutagenesis of the cloned \( E. \text{coli} \) citrate synthase gene to construct two active site mutants which, on the basis of the known structure of pig heart citrate synthase (Remington et al., 1982; Wiegand and Remington, 1986) should be partially defective in binding of oxaloacetate. To investigate the question of whether acetyl-CoA can bind to the NADH-binding site, we used existing restriction sites in the \( E. \text{coli} \) citrate synthase gene to delete 24 amino acids from the sequence. This deletion is predicted to be near enough to the active site to eliminate acetyl-CoA binding there.
**Experimental Procedures**

**Bacterial Strains and Plasmids**—Wild type and mutant versions of the *E. coli* citrate synthase gene *gltA* were expressed in the plasmid pESgltA, in which the clone previously used to obtain large amounts of wild type enzyme (Duckworth and Bell, 1982), as follows. The 733-base pair HindIII-BamHI fragment of pHSGtA, which contains most of the natural *gltA* promoter but none of the citrate synthase coding sequence, was cloned into M13mp19, and a synthetic oligonucleotide (5'-GAGGAGATTCGCGAGC-3') was used in the procedure of Zoller and Smith (1983) to insert a new EcoRI site into this fragment by mutating T-319 in the sequence of Ner et al. (1983) to A. In the work-up of the reaction, JM103 cells were used and the sucrose gradient step was omitted. Mutants were screened by digesting the replicative form of the resulting phage DNA with EcoRI. The new, shorter EcoRI-BamHI fragment was then cut out, isolated by agarose gel electrophoresis, and ligated with the gel-purified large fragment from an EcoRI-BamHI digest of pHSGtA. This new plasmid, pESgltA (6233 base pairs) is 24 base pairs smaller than pHSGtA but still expresses wild type and mutant versions of the citrate synthase gene efficiently and lacks certain restriction sites (for Gla, HindIII, KpnI, and HpaI) which were present in the non-coding part of pHSGtA itself.

Mutant plasmids were expressed in the *E. coli* host strain MOB154, and mutant proteins were purified from extracts of this strain harboring the appropriate mutant plasmid. MOB154, a gift from Dr. D. O. Wood (University of Alabama, Mobile), has a stable mutation in the gene for the DNA gyrase subunit essential for the expression of the synthetic *gltA* gene, which was used in both enzyme assay and immunological methods (data not shown). It is a recA derivative of MOB147 (described in Wood et al., 1983).

**Construction of Mutants by in Vitro Techniques**—Two mutants used in this study were constructed using the approach of the Zoller and Smith (1983). The first, which specifies the mutant protein CS229-4, was made using as template an M13mp8 clone of the BamHI-Sall fragment of pHSGtA, which contains the entire citrate synthase coding region. An oligonucleotide (5'-GGTGACAATCCGAGCATGTC-3') complementary to bases 977-993 of the coding strand, but with C instead of G at the position complementary to base 996 (numbering is according to Ner et al., 1983), was complementary to the natural *gltA* concentration of the synthetase enzyme produced by both synthetic and natural methods. Absence of any detectable protein was determined by both enzyme assay and immunological methods.

The second mutant, designated CS229-2, was constructed using the Eckstein (Taylor et al., 1985a, 1985b) version of the Zoller and Smith (1983) approach, with a kit supplied by Amersham (Canada) Ltd. The oligonucleotide (5'-CTTCTGGCTCTTGCTGCAG-3'), complementary to bases 987-1003 of the coding strand but with C instead of A at position 995, was synthesized at the Institute of Cell Biology, University of Manitoba, using an Applied Biosystems Inc. 380B DNA Synthesizer and phosphoramidite chemistry. Eleven templates were used and screened by primer extension in the presence of dideoxy-C, using synthetic oligonucleotide 1078-1097 (complementary to a 17-base sequence of the *gltA* gene between bases 1078 and 1094) as primer. One mutant was found. Its sequence, from the HpaI site to the end of the coding region, was verified as above, and the HpaI-Sall fragment containing the mutation was cloned into pESgltA for expression.

The third mutant described in this study, designated CSA264-287 because the codons for amino acids 264 through 287 have been deleted, was created by purifying the BamHI-Sall fragment of pHSGtA by agarose gel electrophoresis, digesting it with FspI and XmnI, and re-ligating the blunt ends. Recutting the ligation product with BamHI and Sall, as well as with XmnI to select against reassembly at type sequence, and ligating the resulting fragment to the gel-purified large fragment from BamHI-Sall-digested pHSGtA gave the mutant gene in expressible form. This construction was verified by DNA sequencing of the appropriate Sau3AI fragment cloned into M13mp18.

Our rescreening of the above-mentioned portions of the *gltA* gene gave results which agreed completely with the sequence published by Ner et al. (1983) except that base 1170 in their sequence, which they reported as thymine we consistently found to be guanine. This means that the wild type acid 288 in the amino acid sequence is valine rather than phenylalanine. This is not inconsistent with partial information available for this region by protein chemistry methods (Bhayaana and Duckworth, 1984 and Footnote 1).

**Collection and Analysis of Kinetic Data**—All kinetic studies were performed using the methods of Sanger et al. (1983) at 20 °C. Standard buffer was 0.02 M Tris-Cl, pH 7.8, containing 1 mM disodium EDTA. The routine citrate synthase assay solution contained 0.1 mM each of acetyl-CoA and oxaloacetate, in standard buffer containing 3.1 M KCl. For studies of NADH or α-ketoglutarate inhibition the KCl was omitted. The full steady state kinetic studies on the wild type and nonsense mutants were performed in the presence of 0.1 M KCl. All data from these studies were initially plotted in double-reciprocal form, points which showed a systematic deviation from the Ordered Bisubstrate equation (Cleland, 1963) were discarded, and the remaining data were fitted to that equation by the GENLSS program of DeTar (1972). This program finds best values for all four parameters of the Ordered Bisubstrate equation (K_m, K_a, K_b, and K_o; for definitions of these parameters see “Results”) and assigns an error to each which is approximately equivalent to standard error, for the exact definition of the error see DeTar (1972). For all three enzymes, acetyl-CoA inhibition was zero at 0.5 mM acetyl-CoA, but the method is a rapid and sensitive way of measuring relative strengths of binding and shapes of binding curves for a variety of ligands and conditions. Data, in the form of changes in fluorescence, ΔF_iso as a function of ligand concentration, [L], were initially plotted as Scatchard plots (K/*AF, I, /AF, versus AF, iso), and data sets which gave linear plots were then fitted to the equation for a rectangular hyperbola, using the GENLSS program of DeTar (1972).

Data sets whose Scatchard plots were concave down, indicating sigmoid saturation, were fitted to the equation

\[
\log \frac{K}{[L]} = -\log K + \frac{1}{n} \log [L] 
\]

where \( K \) is the value of \( \Delta F_{iso} \) which would be observed at saturating [L], and \( n \) is a constant whose square root gives the value of [L] needed to achieve (AF, iso = A/F_, iso) = A/2. There is no profound justification for fitting sigmoid saturation curves to this equation (it is simply the Hill (1910) equation with \( n = 2 \)), but it described almost all the sigmoid data well and therefore was a useful way to estimate parameters which empirically described the data, with their errors.

**Routine Procedures—NADH binding was measured using the fluorescence enhancement technique, in standard buffer except for the studies of pH dependence, for which 0.015 M sodium phosphate was used for pH values of 7.4 or lower and 0.02 M Tris-Cl for values of 7.4 or higher; a measurement was made with each buffer at pH 7.4 to test for buffer dependence (Duckworth and Tong, 1976).**

Most enzymes were purified according to Duckworth and Bell (1982), except that the DNase treatment and ammonium sulfate precipitation steps were omitted. Sedimentation equilibrium measurements of molecular weight were as described previously (Tong and Duckworth, 1975). Circular dichroism spectra were determined with a JASCO Model J-500 spectropolarimeter, and K helix content was calculated according to Chen et al. (1972).

**Reagents**—The following reagents were purchased from Boehringer Mannheim (Canada) Ltd: 

- DNA ligase, DNA polymerase I Klenow fragment, and some restriction enzymes. The restriction enzymes FspI and XmnI were obtained from New England Biotabs. 
- Poly-nucleotide kinase and some restriction enzymes were from Pharmacia (Canada) Ltd. The radioactive nucleotides, ([α-32P]dCTP and [α-32P]dATP.

1. V. Bhayana, unpublished results.
2. The abbreviations used are: ANS, 1-anilino-8-naphthalenesulfonate; SDS, sodium dodecyl sulfate.
RESULTS

Steady State Kinetic Studies of Citrate Synthase Mutants CS226H"Q and CS229"Q—As is explained in the "Discussion," the catalytic mechanism of citrate synthase is probably Ordered Bisubstrate, with oxaloacetate binding first. The steady state data obtained for wild type E. coli enzyme and the two missense mutants conformed in general to the rate equation for this mechanism (Cleland, 1963), with the exceptions noted under "Experimental Procedures." Least squares fitting of the three sets of data to the Ordered Bisubstrate equation gave the parameters in Table I.

The CS226H"Q protein behaved like the wild type enzyme during purification and gave a single band of molecular weight about 48,000 g/mol on an SDS gel. Its specific activity in the standard assay (0.1 M KCl, 0.1 mM each of acetyl-CoA and oxaloacetate, pH 7.8) was 17 units/mg, compared with 96 units/mg for the wild type enzyme. Its sensitivity to activation by KCl and the amount of KCl needed to achieve half-maximal activation were similar, although not identical, to wild type values (Table I). CS226H"Q showed normal sensitivity to NADH inhibition (measured kinetically; data not shown), and its NADH binding was normal (Table I). It was, however, less sensitive to inhibition by α-ketoglutarate: the $k_d$ for α-ketoglutarate as a competitive inhibitor with respect to oxaloacetate was 840 μM for the CS226H"Q and 93 μM for the wild type citrate synthase (Table I). $K_{d,OA}$, the true $K_M$ for oxaloacetate (determined by extrapolation to saturating acetyl-CoA), was the same for CS226H"Q as for the wild type, but $K_{d,AC}$, the dissociation constant for the enzyme-oxaloacetate complex in the absence of the second substrate, was about four times higher for the mutant enzyme (Table I).

Thus, both oxaloacetate and α-ketoglutarate interactions with the enzyme were weakened by the mutation of His-226 to glutamine.

Mutant protein CS229"Q was also active and could be purified like the wild type enzyme; the purified enzyme again gave a single band, of molecular weight about 48,000 g/mol, on an SDS gel. The specific activity of this enzyme was only 0.14 units/mg, about 0.4% of the wild type value, in the standard assay. The parameters for activation by KCl were similar to those for wild type enzyme, and most of the reduced activity could be traced to the fact that the mutant enzyme had a substantially lower affinity for oxaloacetate; $K_{d,OA}$ was some 18 times and $K_{d,AC}$ more than 50 times, the wild type values (Table I). The $k_d$ for this mutant was about one-tenth that for the wild type enzyme. Most strikingly, α-ketoglutarate did not inhibit CS229"Q at all, at concentrations as high as 0.8 mM. The data actually showed a slight activation by α-ketoglutarate, which may perhaps be attributed to the increase in ionic strength when buffered α-ketoglutarate was added to the assay solutions. Inhibition of CS229"Q by NADH and binding of that nucleotide (Table I) were normal.

Measurements of Ligand Binding to Citrate Synthase Mutant Forms by ANS Displacement—Because of the instability of oxaloacetate at pH values above 7, conventional methods of measuring binding of this ligand to citrate synthase and its mutants were not attempted, but the ANS displacement method of Talgoy and Duckworth (1979) did allow indirect measurements of binding constants. As was found previously for the wild type enzyme, acetyl-CoA and coenzyme A each caused a saturable decrease in the fluorescence of ANS-citrate synthase mixtures, from which values of $L_{0,5}$ (the concentration of ligand needed to achieve half the maximal decrease, were calculated (Table II). These $L_{0,5}$ values were decreased by 0.1 M KCl, a known activator of E. coli citrate synthase which has previously been shown to shift the substrate saturation curve for acetyl-CoA to lower concentration (Faloona and Sere, 1969). Oxaloacetate (0.2 mM) also decreased the $L_{0,5}$ value for coenzyme A, in the case of the wild type enzyme and CS226"Q, and the combination of oxaloacetate and KCl decreased the parameter still further (Table II). In the case of CS229"Q, 0.2 mM oxaloacetate had no effect on the $L_{0,5}$ value for coenzyme A, in the presence or the absence of 0.1 M KCl, but this is not surprising in light of the weak affinity of this mutant for oxaloacetate, already demonstrated by the steady state kinetic studies above.

Again as found previously (Talgoy and Duckworth, 1979), oxaloacetate alone did not cause a decrease in the fluorescence of ANS-citrate synthase mixtures, but did so if 0.2 mM coenzyme A was also present, because of the fact that oxaloacetate tightens coenzyme A binding. From this indirect effect, we could measure oxaloacetate saturation of wild type enzyme and CS226"Q, in the presence and absence of α-ketoglutarate. As expected, α-ketoglutarate was a competitive inhibitor of the oxaloacetate effect, and apparent dissociation constants for oxaloacetate and α-ketoglutarate, in the presence of 0.2 mM coenzyme A, were calculated (Table II). Binding of oxaloacetate to CS229"Q in the presence of 0.2 mM coenzyme A was so weak that no change in fluorescence was detected unless very large amounts of oxaloacetate were added. Such concentrations, 5 mM and above, caused a net increase in fluorescence which did not become saturated over the concentration range tested, and we regard the effect as an unexplained artifact.

Citrone Synthase Mutant CSA(264-287)—This mutant lacks 24 amino acids including the residue His-264, which is homologous to pig heart citrate synthase residue His-274,
Ligand Binding Sites in E. coli Citrate Synthase

TABLE II
Parameters for ligand binding to wild type citrate synthase and mutants CS226H4 and CS229H4 as measured by ANS displacement

For statistical treatment of data, see "Experimental Procedures." Abbreviations used are AcCoA, acetyl-CoA; α-KG, α-ketoglutarate; OAA, oxaloacetate. Quantities in brackets are the fractions of initial fluorescence of ANS-citrate synthase complex quenched by saturating amounts of ligand; in all cases the uncertainties are no more than ±0.02.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild type</th>
<th>CS226H4</th>
<th>CS229H4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No KCl</td>
<td>0.1 M KCl</td>
<td>No KCl</td>
</tr>
<tr>
<td>L0.5, AcCoA, μM</td>
<td>260 ± 40</td>
<td>60 ± 3</td>
<td>410 ± 45</td>
</tr>
<tr>
<td>L0.5, CoA, μM</td>
<td>(**)</td>
<td>(0.64)</td>
<td>(0.69)</td>
</tr>
<tr>
<td>L0.5, CoA, μM*</td>
<td>270 ± 40</td>
<td>63 ± 3</td>
<td>380 ± 30</td>
</tr>
<tr>
<td>L0.5, CoA, μM*</td>
<td>(**)</td>
<td>(0.57)</td>
<td>(0.68)</td>
</tr>
<tr>
<td>K0.5, OAA, μM</td>
<td>78 ± 3</td>
<td>47 ± 5</td>
<td>137 ± 8</td>
</tr>
<tr>
<td>K0.5, α-KG, μM</td>
<td>(0.78)</td>
<td>(0.38)</td>
<td>(0.69)</td>
</tr>
<tr>
<td>K0.5, α-KG, μM</td>
<td>25 ± 1</td>
<td>ND</td>
<td>63 ± 10</td>
</tr>
<tr>
<td>K0.5, α-KG, μM</td>
<td>(0.54)</td>
<td>(0.67)</td>
<td></td>
</tr>
</tbody>
</table>

* Fraction of initial fluorescence quenched at saturation could not be estimated precisely by curve-fitting and was assumed to be 0.68 (as found for both mutants) for the purpose of estimating L0.5.
* Measured in the presence of 0.2 mM oxaloacetate.
* Measured in the presence of 0.2 mM coenzyme A.
* ND = not determined.
* Measured in the presence of 0.2 mM coenzyme A; calculated from the effect on K0.5 for oxaloacetate. For the wild type enzyme, 0.4 mM α-ketoglutarate was present; and for the two mutants, 5 mM.

Vitaly involved in catalysis at the active site (Wiegand and Remington, 1986). CSA(264–287) showed no activity in crude extracts or after purification. It did cross-react, however, with rabbit antiserum raised against wild type E. coli citrate synthase and thus could be purified like the wild type enzyme, locating citrate synthase protein in fractions by the Ouchterlony double diffusion method. Since this mutant protein tended to tail severely during passage through the Sephadex G-200 column, the final step in the purification procedure, individual fractions from this column were also monitored by SDS-polyacrylamide gel electrophoresis and the purest fractions pooled. This mutant protein has been prepared three times, and each final preparation consisted of one major band of apparent molecular weight about 45,000 g/mol (expected subunit molecular weight: 45,343 g/mol), plus five minor bands. We estimate that these preparations were about 80% pure.

High speed sedimentation equilibrium measurements on purified CSA(264–287) indicated that its weight-average molecular weight under native conditions was similar to that of the wild type enzyme, a finding which shows that deletion of 24 amino acids does not prevent aggregation of the subunits (data not shown). The tailing in the Sephadex G-200 gel filtration, however, suggests that this aggregation is not completely normal. The part of the sequence deleted is predicted, from homology of E. coli citrate synthase to the pig heart enzyme, to include the N helix plus the NO corner and a few residues of the O helix (see "Discussion"). When circular dichroism was used to compare the secondary structure of CSA(264–287) with that of the wild type enzyme, the α-helix content of the deletion mutant, as calculated from the ellipticity at 222 nm (Chen et al., 1972), was lower by about 9% (Fig. 1).

Since CSA(264–287) was inactive, no kinetic studies were possible, but NADH binding was measured by the fluorescence enhancement method. CSA(264–287), like wild type citrate synthase, bound NADH in a pH-dependent manner (Fig. 2). The dissociation constant was smallest at low pH, and the number of sites occupied at saturation decreased as pH was raised. In the case of the wild type enzyme the change in number of sites with pH is not an artifact of the binding

![Fig. 1. Circular dichroism spectra of wild type citrate synthase and deletion mutant CSA(264–287), in standard buffer. A, buffer blank; B, CSA(264–287), 0.25 mg/ml; C, wild type, 0.24 mg/ml.](image1)

![Fig. 2. The pH dependence of binding parameters for the NADH-citrate synthase complex. A, pKD versus pH; B, number of binding sites per subunit. Data are shown for wild type enzyme (●) and CSA(264–287) (○).](image2)
method used (Duckworth and Tong, 1976); it may be the result of the changing degree of aggregation of subunits as pH is changed (Tong and Duckworth, 1975). Less NADH was bound to CSA(264-287) than to the other citrate synthases, and the binding was tighter throughout the pH range (Fig. 2), but detection of strong, pH-dependent binding of NADH indicates that the basic features of the sites are essentially normal.

Both the substrate, acetyl-CoA, and the activator, KCl, weakened NADH binding, in the case of the deletion mutant as with the wild type enzyme (Fig. 3). The inactive CSA(264-287) appeared less sensitive to the effect of KCl, by this criterion, but the difference may not be significant. Acetyl-CoA was equally effective with both proteins.

**Binding of Coenzyme A Derivatives to CSA(264-287)**—The binding of acetyl-CoA and coenzyme A to CSA(264-287) was measured by the ANS displacement method. In contrast to the situation with the wild type enzyme and the missense mutants CS226H and CS229H, the deletion mutant showed relatively tight binding of both coenzyme A ligands, with an $K_D$ value of about 70 μM, which was slightly weakened by 0.1 M KCl. The total amount of fluorescence decrease induced by these ligands was much smaller than that observed with the other proteins. Oxaloacetate, 0.2 mM, had no effect on coenzyme A saturation (Fig. 4). A further difference between CSA(264-287) and the other proteins was the effect of KCl alone on the fluorescence of the ANS-citrate synthase complex. In the case of wild type citrate synthase and the two missense mutants, the fluorescence obtained for the same citrate synthase-ANS mixture was about 50% as great in the presence of 0.1 M KCl as in its absence (Fig. 4A). With CSA(264-287), 0.1 M KCl increased this fluorescence about 25%; the total fluorescence seen with the deletion protein, in the absence of KCl, was about the same as for the wild type and missense mutant citrate synthases (Fig. 4B).

**DISCUSSION**

The steady state kinetic data for wild type citrate synthase and the two missense mutants described here have been interpreted in terms of the Ordered Bisubstrate mechanism and the equation which it predicts (Cleland, 1963). In the case of pig heart citrate synthase, there is good kinetic evidence that the reaction is Ordered, with oxaloacetate binding first (Johansson and Pettersson, 1974); this conclusion is borne out by x-ray crystallographic observations (Wiegand and Remington, 1986). In the case of E. coli citrate synthase, oxaloacetate improves binding of the acetyl-CoA analogue coenzyme A (Talgoy and Duckworth, 1979 and Table 1), and citrate, the product, is a competitive inhibitor with respect to oxaloacetate (Wright and Sanwal, 1971); both facts indicate that with this enzyme, also, oxaloacetate binds first under catalytic conditions. Curvature of double-reciprocal plots of velocity at acetyl-CoA concentrations below 0.1 mM, in the case of the mutant enzymes, shows that the Ordered Bisubstrate equation of Cleland (1963) does not describe the data throughout the concentration range. Substrate inhibition was also detected at acetyl-CoA concentrations above 0.5 mM, for mutant and wild type enzymes. The equation was adequate at intermediate substrate concentrations studied, however, and values for the four parameters which the equation contains, for each of the three enzymes, are shown in Table 1.

**The Missense Mutations at the Active Site**—The two His→Gln mutations described in this paper were chosen because the histidines involved had originally been implicated in the binding of citrate and probably oxaloacetate to the active site of pig heart citrate synthase (Remington et al., 1982) and because the amino acid substituted, glutamine, could probably play a partial role in binding as an alternative to histidine. In one study, for instance, it was found that replacing His-48 in *Bacillus stearothermophilus* tyrosyl-tRNA synthetase by glutamine increased $K_M$ for ATP by a factor of 13 and decreased $K_S$ by 7% of the wild type value; if asparagine was introduced at this position instead, little or no change in the kinetics was found (Lowe et al., 1985). Refinement of the pig heart citrate synthase structure has led Wiegand and Remington (1986) to conclude that His-235 (the pig heart equivalent of E. coli His-226) is not directly involved in citrate binding, while His-238 (the equivalent of E. coli His-229) is believed to form either a hydrogen bond or an ion pair with a carboxylate anion of citrate and oxaloacetate. In view of the high degree of amino acid sequence homology between the *E. coli* and pig heart sequences, particularly around the active site, similar roles should be played by His-226 and His-229 in the E. coli enzyme.

Although almost all the kinetic parameters in Table 1 were changed to some extent by the mutations in CS226H and CS229H, the only large effects were on constants which measure the affinity of the enzyme for oxaloacetate and α-ketoglutarate. In the case of CS226H, the estimated value for $K_{OAA}$, which is interpreted as the dissociation constant for the binary enzyme-oxaloacetate complex, was four times that for the wild type enzyme, while the $K_a$ for α-ketoglutarate
inhibition was nine times that for the wild type; the binding strengths for these two α-keto acids both decreased. With CS229H4Q, the K_{DAA} was more than 50 times that for wild type. We could not detect α-ketoglutarate inhibition in this mutant, but if K_{D} for this inhibition increased in proportion the value would be about 5 mM, well above the lower limit of 2 mM which we estimated for this parameter (Table I).

These kinetic measurements were confirmed in large part by static measurements made with the citrate synthase-ANS complex (Table II). The K_{D} values for oxaloacetate reported in Table II are not strictly comparable to the K_{DAA} values in Table I, since those in Table II were measured from the ability of oxaloacetate to enhance the binding of coenzyme A and therefore were measured in the presence of 0.2 mM coenzyme A. Binding of oxaloacetate or α-ketoglutarate to CS229H4Q was too weak to measure by this method, but even this result shows that the binding of the α-keto acids to this mutant is very weak, as expected from the kinetic data.

In the kinetic equation for the Ordered Bisubstrate mechanism, the second order rate constant for formation of the catalytically productive enzyme-oxaloacetate complex, k_{o}, is given by k_{cat/K_{DAA}} while the rate constant for the dissociation of this complex, k_{d}, is given by (k_{cat}/K_{DAA})/K_{DAA} (Cleland, 1983). The values calculated for k_{o} from the data in Table I were 3.1 × 10^6, 5.8 × 10^6, and 2.0 × 10^6 M^{-1}s^{-1} for wild type, CS229H4Q, and CS229H4Q, respectively, while the values of k_{d} were 100, 810, and 36 s^{-1}, respectively. These calculations have rather large errors, but they suggest that the CS229H4Q mutant binds oxaloacetate less strongly than wild type enzyme because the complex forms at a normal rate but dissociates more easily. More striking, the CS229H4Q mutant forms the enzyme-oxaloacetate complex far more slowly than the wild type and CS229H4Q enzymes do. The very weak binding of oxaloacetate to CS229H4Q, relative to the wild type and CS229H4Q enzymes (see K_{DAA} values in Table I), would appear to arise entirely from the low rate of formation of this complex. These findings are consistent with the conclusion of the crystallographic studies on pig heart citrate synthase, that His-238 (the equivalent of His-229 in the E. coli enzyme) is more important in oxaloacetate binding than His-235 (the equivalent of E. coli His-226). Thus, the CS229H4Q mutant showed only a slight effect on oxaloacetate binding, perhaps attributable to removal of a positive charge from the neighborhood of a ligand carbohydrate; α-ketoglutarate binding was somewhat more strongly affected by this change. Mutation of histidine to glutamine in CS229H4Q, on the other hand, had a profound effect on α-keto acid binding; the rate of formation of the complex with oxaloacetate was slower by 2 orders of magnitude, and binding of α-ketoglutarate was not detectable at all. Removal of one of the normal points of attachment of oxaloacetate in the active site might also be expected to affect the catalytic rate to some extent, since the full distortion of substrate in the direction of the transition state should be harder than usual, and indeed a 10-fold decrease in k_{o} was measured with CS229H4Q (Table I).

The principal reason for making and studying these two mutants was to test whether factors which weaken oxaloacetate binding would weak binding of the inhibitor α-ketoglutarate in a parallel fashion. This is clearly the case, and therefore it seems that α-ketoglutarate exerts its inhibition on E. coli citrate synthase not through a specific allosteric site but by a special kind of interaction at the active site. Its binding to the active site can occur only in the absence of KCl (which desensitizes the enzyme to α-ketoglutarate) (Wright et al., 1967), presumably because KCl induces a conformational adjustment at the active site which makes the site discriminate among α-keto acids more precisely. This adjustment would also be the reason why KCl substantially activates E. coli citrate synthase (Table I).

The Deletion Mutant—From the alignment of the amino acid sequence of E. coli citrate synthase with that of the pig heart enzyme (Bhayana and Duckworth, 1984), it may be concluded that the sequence deleted in CSA(264-287) corresponds to the N helix in the structure of Remington et al. (1982), the NO corner, and a few residues of the O helix. Even if exactly the same secondary structure is not assumed by this portion of the E. coli enzyme, the deletion is very close to the acetyl-CoA binding portion of the active site (Remington et al., 1982), and the mutant protein should be seriously affected in its interaction with acetyl-CoA. As noted under “Results,” CSA(264–287) was inactive but could be purified by the method used for the wild type and missense mutant enzymes, using an immunological method to assay fractions. The preparations, though not completely pure, were still suitable for the experiments which we wished to perform. NADH binding was retained in this mutant, and the K_{D} values obtained for this process were actually smaller (that is, the binding was tighter) than those measured with the wild type enzyme (Fig. 2). The number of binding sites was low, a fact which we attribute to greater and perhaps less organized aggregation of the subunits of the deletion protein, which could have the result of “burying” binding sites. Still, the number of sites did increase as pH was lowered, in a manner qualitatively like the effect on the wild type enzyme. This binding was also weakened by KCl or acetyl-CoA, just as is the case with the wild type enzyme.

The fact that acetyl-CoA inhibits NADH binding is most readily explained by assuming that acetyl-CoA binds at the allosteric site, since the deletion almost certainly would destroy normal binding at the active site. To confirm this, we used the method of ANS displacement to measure the effects of KCl and oxaloacetate on coenzyme A binding to the deletion mutant CSA(264–287). The interaction of ANS with this protein was different from that with the wild type and missense mutants, in that 0.1 M KCl increased the fluorescence of the complex by about 25%, whereas it decreased the fluorescence of the other complexes by about 50% (Fig. 4). This difference, which must have arisen from rearrangements in the protein as a result of the deletion, was not really a complication, since changes in fluorescence could still be measured and used to monitor ligand binding. Acetyl-CoA or coenzyme A displaced very little ANS from: the deletion protein, and this weak effect was not enhanced by oxaloacetate (Fig. 4). The ability of coenzyme A binding to improve in the presence of oxaloacetate, in the wild type and missense mutant proteins (Table II), is an indication that this binding occurs at the active site. Because coenzyme A binding to CSA(264–287) was unresponsive to oxaloacetate, then, we conclude that coenzyme A derivatives do not bind to the damaged active site of this mutant and attribute the small amount of ANS displacement from the deletion mutant to binding of these derivatives at the allosteric NADH binding site. Binding of acetyl-CoA to the NADH site could allow this ligand to activate acetyl-CoA binding at the active site, as ADPribose, 5'AMP, and NADPH seem to do (Talloy and Duckworth, 1979). Such an effect would contribute to the sigmoid saturation of E. coli citrate synthase which acetyl-CoA shows at low salt concentration (Falona and Sere, 1969).

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Ligand Binding Sites in *E. coli* Citrate Synthase

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