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Enzyme–substrate complexes of allosteric citrate synthase: Evidence for a novel intermediate in substrate binding

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ABSTRACT

The citrate synthase (CS) of *Escherichia coli* is an allosteric hexameric enzyme specifically inhibited by NADH. The crystal structure of wild type (WT) *E. coli* CS, determined by us previously, has no substrates bound, and part of the active site is in a highly mobile region that is shifted from the position needed for catalysis. The CS of *Acetobacter aceti* has a similar structure, but has been successfully crystallized with bound substrates: both oxaloacetic acid (OAA) and an analog of acetyl coenzyme A (AcCoA). We engineered a variant of *E. coli* CS wherein five amino acids in the mobile region have been replaced by those in the *A. aceti* sequence. The purified enzyme shows unusual kinetics with a low affinity for both substrates. Although the crystal structure without ligands is very similar to that of the WT enzyme (except in the mutated region), complexes are formed with both substrates and the allosteric inhibitor NADH. The complex with OAA in the active site identifies a novel OAA-binding residue, Arg306, which has no functional counterpart in other known CS-OAA complexes. This structure may represent an intermediate in a multi-step substrate binding process where Arg306 changes roles from OAA binding to AcCoA binding. The second complex has the substrate analog, S-carboxymethyl-coenzyme A, in the allosteric NADH-binding site and the AcCoA site is not formed. Additional CS variants unable to bind adenylates at the allosteric site show that this second complex is not a factor in positive allosteric activation of AcCoA binding.

1. Introduction

The citrate synthase (CS) of most Gram-negative bacteria is an allosteric hexamer of identical subunits, activated by KCl and similar salts, and strongly and specifically inhibited by the biological reducing agent, NADH [1]. We have reported structures of two CS from *Escherichia coli*: the wild-type (WT) without bound ligands [2], and a mutant, (Phe383Ala), with NADH bound in the allosteric site [3]. Both structures show a 3-fold axis of symmetry effectively forming a trimer of dimers. Within each dimer unit, an approximate 2-fold axis of symmetry is present, leading to the close, but not exact, equivalence of monomers within each dimer. Each pair of monomers has extensive interactions, but there are relatively few interactions between different dimers. The dimer–dimer interactions are concentrated in a thin cylindrical region surrounding the 3-fold axis of the hexamer; this is the region in which NADH binds, so that the monomers contributing to the NADH binding site are from different dimers [3,4]. In contrast, the six substrate binding sites of *E. coli* CS are located in large open spaces within dimers, allowing ready access by substrate molecules. The structures for both WT and the Phe383Ala mutant must represent the T state or inactive conformation of the enzyme, not only because the NADH binding site is present, but also because some parts of the presumed substrate binding sites would have to refold substantially for catalysis to be possible. For example, most of the binding site for oxaloacetate (OAA) is appropriately aligned but His264, a residue crucially involved in enzyme activity, is about 11 Å away from a catalytically useful position. Residues 267–297, which form a large part of the binding site for the second substrate, acetyl-coenzyme A (AcCoA), are in a disordered state [2], referred to here as the "mobile loop" (Fig. 1A).

Although the CS from the Gram-negative bacterium *Acetobacter aceti* is also a hexamer, it is insensitive to NADH, most likely because amino acids critical for NADH binding are missing [5]. The *A. aceti* CS has ~64% sequence identity with the *E. coli* enzyme, the overall folding pattern is similar, and most of the *E. coli* and *A. aceti* CS structures, including the relative positions of the six subunits, can be superimposed. Nonetheless, the region corresponding to the "mobile loop" of *E. coli* CS has more order in the *A. aceti* CS structure (Fig. 1B). In contrast to the high mobility of residues 267–297 of *E. coli* CS, the comparable region in *A. aceti* CS has two alpha-helices, with a well-defined corner at residues 287–291 (Fig. 1B). The turn between helices N and O appears to be stabilized by hydrogen bonds between Asn291 and both of Lys288 and

Abbreviations: CS, citrate synthase; WT, wild-type; OAA, oxaloacetate; AcCoA, acetyl coenzyme A; NADH, nicotinamide adenine dinucleotide; CMCoA, S-carboxymethyl-CoA; CMX, carboxymethyldethia-CoA; 5AA-CS, variant *E. coli* CS protein with multiple substitutions in the mobile loop region

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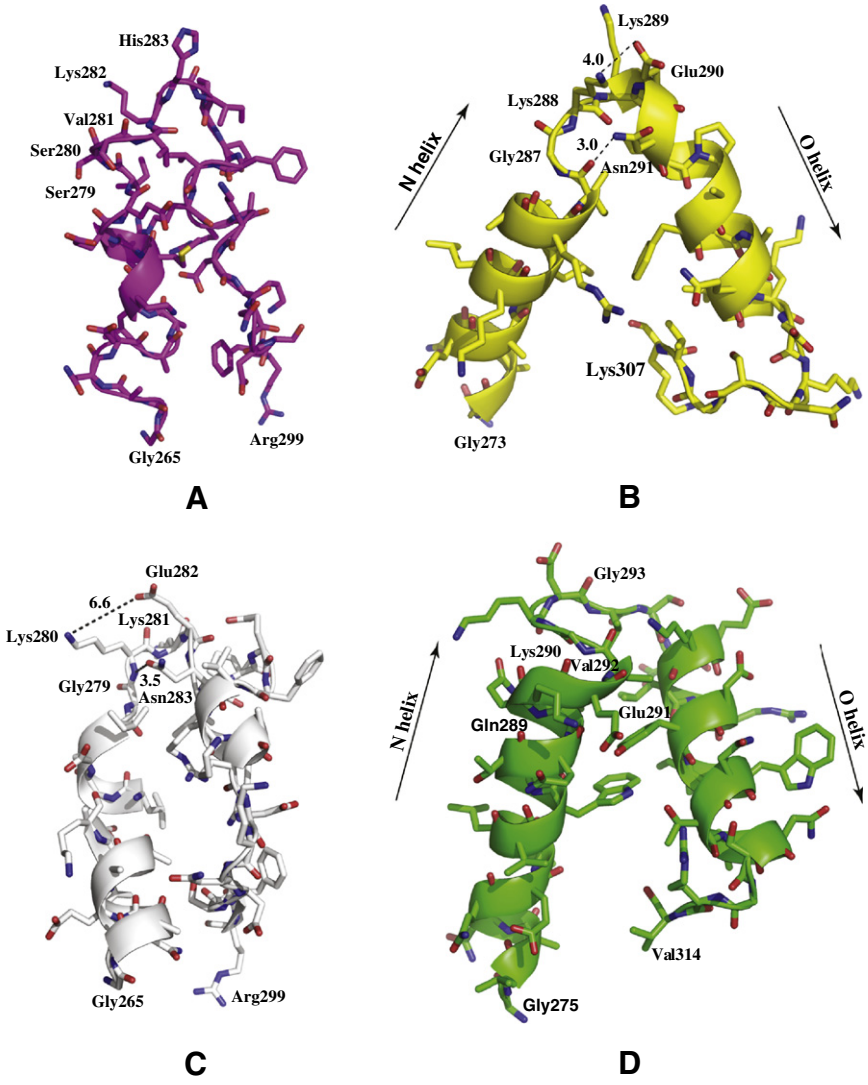

Fig. 1. The "mobile loop" region of *E. coli* CS, compared to the equivalent regions from *A. aceti* and pig heart CS. A. *E. coli* WT CS (residues 265–299 from PDB ID: 1K3P); B. *A. aceti* CS (residues 273–307 from PDB ID: 2H12); C. *E. coli* 5AA-CS variant (residues 265–299 from PDB ID: 4JAD). D. Pig heart CS (residues 275–314 from PDB ID: 4CTS).

Lys289; Asn291 further forms an N-cap with helix O. Unlike the allosteric *E. coli* enzyme, the *A. aceti* CS forms stable structures with both substrates (OAA and carboxymethyldethia-coenzyme A (CMX)) bound in the active site. The *A. aceti* enzyme–substrate complexes are similar to those of vertebrate and archaeal CS [6–8].

In an effort to introduce more stability into the mobile loop region of *E. coli* CS, and to facilitate crystallographic studies with bound substrates, we have engineered the amino acid sequence of the *A. aceti* turn between helices N and O into the equivalent part of the *E. coli* CS sequence. Reported here are the kinetic, crystallographic, and mass spectrometric analyses of this engineered protein. These data gave hints of rearrangements that must take place during substrate binding. To probe further, three additional single-site mutant proteins were made and studied. These allowed postulation of an additional conformational state during the events that change *E. coli* CS from T-state to R-state in a classic allosteric interaction.

2. Methods and materials

2.1. Mutagenesis, purification and kinetic studies

The single amino acid substitutions Gly181Glu, Thr204Arg, and Arg306Leu, were prepared by site-directed mutagenesis as recommended in the QuikChange™ kit (Stratagene). The mobile loop change SerSerValLysHis to GlyLysLysGluGln (residues 279–283) required nine base changes (shown in upper case), so we designed two 54 base oligonucleotides, 5′gctgaaaatg ctggaagaaa tcGgcAAAAA AGaaAacatt ccggaatttg ttcg 3′ and 5′cgaacaaatt ccggaatgtT ttCTTTTTg cCgatttctt ccagcatttt cagc 3′, and modified the Stratagene-recommended protocol by reducing the annealing temperature to 45 °C. PCR products were transformed into our *gltA*$^{-}$ strain MOB154 (a *recA* derivative of MOB147) [9]. One colony from each of the four mutagenesis experiments was chosen for purification. Mass spectrometry analysis was used to confirm the mass and integrity of each intact protein, and to verify each of the mutations. Kinetic measurements on the purified proteins, and NADH binding studies, were carried out following previously described protocols [4,10,11]. For the kinetic measurements, the freed thiol group reacts with Ellman's reagent, and the resulting liberation of a yellow thiolate anion is monitored at 412 nm [12,13]. All samples of AcCoA contain a few percent of free coenzyme A that seriously interferes with measurements of CS activity at concentrations of AcCoA greater than 2 mM. Accordingly, no kinetic measurements were done at substrate levels greater than this. S-Carboxymethyl Coenzyme A (CMCoA) was synthesized from Coenzyme A (Calbiochem) as described [14,15].