Probing the Roles of Key Residues in the Unique Regulatory NADH Binding Site of Type II Citrate Synthase of *Escherichia coli*§

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The citrate synthase of *Escherichia coli* is an example of a Type II citrate synthase, a hexamer that is subject to allosteric inhibition by NADH. In previous crystallographic work, we defined the NADH binding sites, identifying nine amino acids whose side chains were proposed to make hydrogen bonds with the NADH molecule. Here, we describe the functional properties of nine sequence variants, in which these have been replaced by nonbonding residues. All of the variants show some changes in NADH binding and inhibition and small but significant changes in kinetic parameters for catalysis. In three cases, Y145A, R163L, and K167A, NADH inhibition has become extremely weak. We have used mass spectrometry, under non-denaturing conditions, to show that two of these, R163L and K167A, do not form hexamers in response to NADH binding, unlike the wild type enzyme. One variant, R109L, shows tighter NADH binding. We have crystallized this variant and determined its structure, with and without bound NADH. Unexpectedly, the greatest structural changes in the R109L variant are in two regions outside the NADH binding site, both of which, in wild type citrate synthase, have unusually high mobilities as measured by crystallographic thermal factors. In the R109L variant, both regions (residues 360–311 and 316–342) are much less mobile and have rearranged significantly. We argue that these two regions are elements in the path of communication between the NADH binding sites and the active sites and are centrally involved in the regulatory conformational change in *E. coli* citrate synthase.

The Type II citrate synthases (CS)† of Gram-negative bacteria, such as *Escherichia coli*, are hexamers of identical subunits, which are strongly and specifically inhibited by the biological reducing agent, NADH, binding at a location distinct from the active site (1, 2). These properties distinguish them clearly from the Type I citrate synthases found in Gram-positive bacteria, archaea, and eukaryotes, which are homodimers without regulatory properties. Type I and Type II CS subunits all have the same overall fold and very similar active sites, so that, as a first approximation, a Type II hexamer may be regarded as a trimer of Type I dimers (3). From this we have argued that NADH inhibition in the Type II enzymes is an evolutionary add-on; Type I dimers were altered by a series of mutations to generate NADH sites and new contact surfaces so that hexamers could form. Our previous structural work on *E. coli* CS has shown that the six NADH binding sites are remote from the active sites and are located near the dimer-dimer interfaces, with some residues contributed to each site by two subunits on either side of the interface (4). This finding explained why NADH binding induces a dimer to hexamer shift in the dimer-hexamer equilibrium shown by *E. coli* CS at moderate concentrations (1–20 μM) (5).

With the structure of the CS-NADH complex in hand, we can describe in detail the interactions between the protein and the nucleotide (4). The binding site is a shallow cationic pocket, whose average surface charge probably explains why the oxidized form of the nucleotide, NAD⁺, does not bind at a significant strength. Within this pocket, nine amino acid side chains are in positions to make hydrogen bonds to NADH. In this paper, we describe the functional properties of a series of nine new CS variants, in which the nine putative hydrogen-bonding residues in the NADH site, identified through the structural studies, have been replaced by nonbonding residues. In addition, one particularly intriguing variant has been crystallized in the presence and absence of NADH, and the three-dimensional structures have been determined using x-ray diffraction methods. The information obtained from these structures permits us to draw plausible conclusions about the nature of the regulatory conformational change induced by NADH and how binding of this nucleotide impacts on the distant active site region.

EXPERIMENTAL PROCEDURES

Preparation and Characterization of Variant Proteins—Variant CS proteins were prepared by site-directed mutagenesis, using the QuikChange™ kit from Stratagene. The plasmid template used for this work, pCCgltA, contained the *E. coli* CS gene, *gltA*, cloned into the EcoRI and SalI sites of the vector pUC18; this template was prepared by Charlton Cooper. Mutagenic oligonucleotides were purchased from Sigma. Following the mutagenesis procedure and transformation, two or three colonies were picked, plasmid DNA was prepared, and the presence of the desired mutation was established by DNA sequencing (Plant Biotechnology Institute, Saskatoon, Canada). The mutant genes were expressed, and the variant proteins were purified, as previously described (6). Protein purity was assessed by SDS-PAGE and by electrospray time-of-flight mass spectrometry of the denatured (monomeric) protein in 10% aqueous acetic acid.
NADH binding was measured by fluorescence enhancement (7), and kinetic measurements were made as previously described (8). NADH inhibition was measured in dilute buffer (20 mM Tris-Cl, pH 7.8, containing 1 mM EDTA) with subsaturating amounts of acetyl-CoA and OAA (100 μM each) and no added KCl. KCl, NaCl, and some other salts are powerful activators of the enzyme (1, 9). Therefore, it was necessary to correct NADH inhibition data in light of the fact that the NADH used was a disodium salt and that the amounts of sodium ions introduced along with NADH were sometimes high enough to cause partial activation of the enzyme. Thus, the net effect of the NADH additions was a combination of inhibition (by NADH itself) and activation (by sodium ions introduced along with it). The activation effect is negligible in the case of wild type E. coli CS, since very little NADH (~20 μM) is needed for assay purposes. With several of the variant proteins in this study, however, NADH inhibition was so weak that the sodium activation effect was dominant, unless corrected for. All of the variants showed the same hyperbolic concentration dependence of activation on sodium ions, which, for [Na+] < 5 mM may be approximated by the equation,

$$v_{max} = v_0 + 1.42[Na^+]$$  (Eq. 1)

where [Na+] is millimolar. We used this formula to correct all data points downward, and the resulting saturation curves for NADH were readily analyzed to yield inhibition parameters.

Catalytic properties of the CS variants were assessed in two ways. Rate data were collected over a range of concentrations of the two substrates, acetyl-CoA and OAA, in buffer containing 0.1M KCl, in which wild type E. coli CS binds the ordered bisubstrate steady-state mechanism with OAA binding first. These data were fitted to the appropriate steady-state equation as described (8). To assess cooperativity of the acetyl-CoA saturation curve, which wild type CS shows only at low salt concentration, rate data were also collected in the absence of KCl, over a wide range of acetyl-CoA concentrations, in the presence of 100 μM OAA. These data were fitted to the Hill equation,

$$v/v_{max} = [(acetyl-CoA)^n]/(S_{acetyl-CoA}^n + [acetyl-CoA]^n)$$  (Eq. 2)

where n represents the Hill parameter, and $S_{acetyl-CoA}$ is the concentration of acetyl-CoA at which n = 0.5v_{max}. All kinetic data were analyzed using the nonlinear least-squares fitting function of Kaleidagraph (Abelback Software), and the errors quoted are those assigned by the program.

Mass Spectrometry—Mass spectra of wild type and variant CS were obtained by electrospray time-of-flight mass spectrometry or nanospray ionization/time-of-flight mass measurement, using the in-house built instrument TOF III in the Department of Physics, University of Manitoba. This instrument has been described (10). Protein samples were transferred into the desired buffer by dialysis using “waterbugs” (11) or by repeated washing through Centriprep-50 cartridges (Amicon). For studies of CS in the “native” state, ammonium bicarbonate buffers, ranging from 5 to 100 mM, were used, and protein concentrations were fitted to the range 1–10 μM (CS subunit concentration). For mass spectrometry to study the CS dimer-hexamer equilibrium, SF6 was the collision gas, and the declustering voltage was 200–350 V, adjusted as required to minimize the presence of adducts associated with the protein ions.

Structural Analyses—The R109L variant of E. coli CS was crystallized using the hanging drop vapor diffusion method from 2.0 to 2.2 M ammonium sulfate, 2% (v/v) PEG 400, and 0.1 M Na-Hepes at pH 6.0. The NADH-bound form of R109L CS was obtained by soaking a variant crystal in a solution containing 1.22 mM NADH, 2.8 M ammonium sulfate, 2% (v/v) polyethylene glycol 400, and 0.1 M Na-Hepes at pH 6.0 for 3 h.

Diffraction data from R109L variant and R109L-NADH complex crystals were collected at 100 K on a Rigaku R-AXIS IIC imaging plate area detector system equipped with Osmic mirrors and using Cu Kα radiation supplied by a Rigaku RU300 rotating anode generator operating at 50 kV and 100 mA. In total, 99 frames were collected from a crystal of the R109L variant (1.0° oscillation, 8-min exposures) and 190 frames from a crystal of the R109L-NADH complex (0.9° oscillation, 5-min exposures). Intensity data were integrated, scaled, and reduced to structure factor amplitudes with the HKL (12) and CCP4 (13) program suites.

The space group and unit cell parameters obtained (Table I) indicated that crystals of both the R109L variant and the R109L-NADH complex were isomorphous with those of wild type E. coli CS (3). This permitted the wild type structure (with residue 109 truncated to an alanine) to be used as the starting refinement model for the R109L variant protein. The final structure determined for the R109L variant protein was then used as the starting refinement model for the R109L-NADH complex. As part of both structure determinations, it was necessary to apply a detwinning approach (perfect merohedral) in conjunction with positional and thermal factor refinement (14, 15). During this process, the complete polypeptide chain of each refinement model was examined periodically with XDS (16). The location and difference electron density maps. When necessary, manual adjustments were made to the refinement models using O (16). The location and preliminary placement of bound NADH in the R109L-NADH complex was determined on the basis of a difference electron density map calculated once the protein portion of this complex had been refined. Additional refinement of both NADH and protein atomic positions, as well as thermal factors, was then carried out to convergence. For both the R109L and R109L-NADH structures, solvent peaks were identified by a final series of difference electron density maps, and the validity of these was monitored on the basis of hydrogen bonding potential to protein or NADH atoms and the refinement of a thermal factor of <75 Å2. An assessment of the final refined structures obtained indicates that both of these have excellent polypeptide chain geometry (Table I).

### TABLE I

| Structure determination parameters | R109L structure | R109L + NADH structure |
|-----------------------------------|-----------------|------------------------|
| Data collection                   |                 |                        |
| Space group                       | R3              | R3                     |
| Unit cell dimensions (Å)          | 165.1           | 165.0                  |
| Number of unique reflections      | 155.2           | 157.8                  |
| Mean I/σ²                         | 29.4 (10.9)     | 22.1 (7.3)             |
| Multiplicity                      | 4.6 (2.3)       | 4.2 (1.9)              |
| Merging R-factor (%)              | 9.4 (16.1)      | 8.3 (12.2)             |
| Maximum resolution (Å)            | 2.2             | 2.2                    |
| Structure refinement              |                 |                        |
| No. of reflections                | 71,007          | 74,765                 |
| Resolution range (Å)              | 10-2.2          | 10-2.2                 |
| Completeness within range (%)     | 88 (78)         | 92 (73)                |
| No. of protein atoms              | 6726            | 6726                   |
| No. of solvent atoms              | 511             | 583                    |
| No. of ligand atoms               | 88              |                        |
| Average thermal factors (Å²)      |                 |                        |
| Protein atoms                     | 24.3            | 26.3                   |
| Solvent atoms                     | 37.8            | 35.6                   |
| Ligand atoms                      | 19.2/33.6       |                        |
| Final R-factor/R-free (%)         | 16.1/21.0       | 16.2/21.9              |
| Final structure stereochemistry    |                 |                        |
| Root mean square                  | 0.008           | 0.008                  |
| Bonds (Å)                         | 1.7             | 1.6                    |
| Angles (degrees)                  |                 |                        |

a Values in parentheses refer to the highest resolution shell (2.24-2.20 Å).

b For the R109L-NADH complex structure, the two values indicated correspond to the NADH molecules bound to the A and B subunits, respectively.

For the R-free test, 5% of the diffraction data were kept aside.
than does the wild type CS, with $K_d$ values 2.5–6.4 times the wild type CS value. Effects on the strength of NADH inhibition were much more dramatic (Table II), with three variants showing $K_i$ values more than 100 times the wild type value, a far stronger effect than would be anticipated from the weaker binding. These three variants, Y145A, R163L, and K167A, all involve residues that are believed to take part in a complex hydrogen bonding network with the pyrophosphate moiety of NADH (4).

Table III shows the effects of these amino acid substitutions on the kinetics of catalysis in the absence of NADH (i.e. on properties directly related to the active site). The kinetics of each variant are different. All turnover numbers are within 50% of the wild type value (except for Tyr45, which is known to be impure), but there are surprising changes to the affinity for substrate. The most striking effect is on $K_d$: a parameter interpreted as the dissociation constant of the CS-dimer complex, which must form before the second substrate, acetyl-CoA, can bind (8). In structural studies on vertebrate CS, it has been found that OAA binding induces a significant tightening of the active site (17, 18). The data in Table III show that formation of this complex is more difficult as a result of any change to hydrogen-bonding residues in the NADH site.

**Mass Spectrometry of CS Variants**—Given that the NADH binding sites of E. coli CS are located at the interfaces between dimers, it was possible that amino acid substitutions in these sites would have effects on the dimer-hexamer equilibrium shown by wild type CS at low salt concentrations. This equilibrium has been successfully studied by electrospray ionization time-of-flight mass spectrometry, using 5 mM ammonium bicarbonate (pH 7.5) as solvent (5). In the present work, we have made use of nanospray ionization in place of the conventional electrospray, a change that allows studies at much higher salt concentrations. This improved mass spectrometry approach has been used to monitor the quaternary structures of certain of the CS variants in solution.

In Fig. 1 we show an experiment that became possible only when nanospray ionization was available, a study of the effect of salt concentration on the position of the wild type CS dimer-hexamer equilibrium. As is usual with electrospray/nanospray ionization, each protein species is represented by a charge envelope, consisting of a small number of peaks of the same mass but different charges. Fig. IA shows the mass spectrum of wild type E. coli CS (5 μM subunit concentration) in 5 mM NH$_4$HCO$_3$ buffer. Three charge envelopes, representing dimers ($M_r = 95878 ± 224$ Da), tetramers ($M_r = 19,1692 ± 416$) and hexamers ($M_r = 287,523 ± 504$), are visible. This result is similar to that obtained previously by Ayed et al. (5) using electrospray ionization, except that tetramers are somewhat more prominent in the new sample. B–D of Fig. 1 show that, as NH$_4$HCO$_3$ concentrations are increased, there is a decided shift of almost all the protein to the charge envelope for hexamer. Whereas electrospray ionization did not permit observation of protein ions at salt concentrations above 10 mM, nanospray allows clean spectra to be obtained at 100 mM NH$_4$HCO$_3$. Over this same concentration range, enzyme activity increases substantially (data not shown). Salt activation of E. coli CS is well known and has been traced to an increase in affinity for acetyl-CoA (9). The experiment in Fig. 1 now shows that this activation is accompanied by a stabilization of the CS hexamer. In light of our earlier finding that NADH, the inhibitor, also stabilizes a hexameric form (5), it is now clear that both the active (R state) and inactive (T state) conformations of E. coli CS are hexamers.

Some of the variant proteins listed in Tables II and III were examined by nanospray/time-of-flight mass spectrometry. At low salt (5 mM NH$_4$HCO$_3$), the R109L variant (4.2 μM subunits) showed charge envelopes representing dimer, tetramer, and hexamer, with dimer predominating; increasing the salt to 100 mM shifted about half of the protein to hexamer (data not shown). The Q182A variant was also more shifted to dimer than wild type (data not shown). The cases of the R163L and K167A variants were more interesting, since these two, although they still bind NADH reasonably well, show extremely weak NADH inhibition (Table II). As already noted, the third variant in this category, Y145A, was not pure enough for mass spectrometry experiments. In Fig. 2, we show that, in contrast to wild type CS, the R163L and K167A variants show no tendency to form more hexamer in the presence of NADH. Fig. 2, A and B, a control experiment, shows the effect of near-saturating (20 μM) NADH on the dimer-hexamer equilibrium of wild type CS. There is a marked shift toward hexamer, and the hexamer mass increases by slightly over 4000 Da, or about 6 NADH units ($M_r$/NADH = 665). This shift of the wild type CS dimer-hexamer equilibrium toward hexamer, as NADH binds, was documented previously in more detail, in the electrospray ionization studies of Ayed et al. (5). C and D of Fig. 2 show that the R163L variant does not respond to NADH in the same way, although at least two NADH molecules bind per dimer (Fig. 2D, inset), and at least four bind per hexamer. E and F of Fig. 2 show the same experiment for the K167A variant. This protein shows severe disruption of the normal subunit structure; it is almost entirely tetrameric, with traces of dimer. NADH binds to both the dimer and the tetramer (Fig. 2F, inset, shows
In the presence of 0.1 m KCl, the kinetics of E. coli citrate synthase conform to the ordered bisubstrate mechanism, and saturation by both substrates is hyperbolic (noncooperative). In the absence of KCl, saturation by acetyl-CoA becomes sigmoid. See Ref. 8.

| Variant | 0.1 m KCl | No KCl |
|---------|-----------|--------|
|         | h<sub>cat</sub> | K<sub>cat</sub> | K<sub>cat</sub> | K<sub>cat</sub> | K<sub>cat</sub> | s<sub>n,AcCoA</sub> | Hill no. | V<sub>max</sub> |
|         | s<sup>-1</sup> | µM | µM | µM | µM | | | units/mg |
| Wild type | 81 ± 6 | 26 ± 5 | 33 ± 7 | 120 ± 20 | 700 ± 30 | 1.55 ± 0.04 | 78 ± 2 |
| R109L | 121 ± 5 | 7 ± 4 | 98 ± 24 | 69 ± 24 | 1200 ± 200 | 1.68 ± 0.14 | 68 ± 7 |
| H110A | 67 ± 5 | 3 ± 15 | 460 ± 210 | 49 ± 17 | 1800 ± 200 | [1.0] | 97 ± 7 |
| T111A | 54 ± 4 | 18 ± 20 | 360 ± 140 | 79 ± 21 | 1700 ± 700 | 1.56 ± 0.25 | 77 ± 25 |
| Y145A | 36 ± 2<sup>a</sup> | 51 ± 17 | 110 ± 30 | 230 ± 30 | 1600 ± 500 | 1.22 ± 0.10 | 5.3 ± 1.1<sup>b</sup> |
| R163L | 108 ± 4 | 5 ± 4 | 55 ± 13 | 80 ± 8 | 2900 ± 200 | [1.0] | 112 ± 5 |
| K167A | 84 ± 6 | 37 ± 19 | 130 ± 30 | 220 ± 40 | 2300 ± 1200 | 1.17 ± 0.14 | 3.3 ± 1.9 |
| Q182A | 95 ± 2 | 10 ± 3 | 93 ± 16 | 56 ± 5 | 980 ± 110 | 1.96 ± 0.20 | 5.8 ± 0.5 |
| N189A | 124 ± 4 | 17 ± 6 | 150 ± 30 | 69 ± 8 | 2300 ± 700 | 1.18 ± 0.09 | 89 ± 18 |
| T204A | 118 ± 7 | 4 ± 11 | 120 ± 30 | 210 ± 30 | 910 ± 50 | 1.95 ± 0.10 | 54 ± 2 |

<sup>a</sup> Because our preparations of Y145A were estimated to be only about 50% pure (see “Experimental Procedures”), the values reported here for h<sub>cat</sub> and V<sub>max</sub> are systematically too low by about a factor of 2.

Fig. 1. Mass spectra of E. coli citrate synthase, 5 µM (subunit concentration), as a function of concentration of the buffer salt, NH<sub>4</sub>HCO<sub>3</sub>, as follows: 5 mM (A), 10 mM (B), 20 mM (C), and 50 mM (D). A declustering voltage of 200 V was used for all spectra. Charge envelopes correspond to dimers, tetramers, and hexamers are centered around m/z = 5200, 6900, and 8800, respectively.

Evidence for binding of at least three NADH units to the tetramer, but again the dinucleotide makes no difference to the mix of oligomeric forms present. Thus, the data in Fig. 2 show that NADH binding has become decoupled from the ability to induce a dimer to hexamer shift, just as it has become almost decoupled from the ability to inhibit enzyme activity.

**Structural Consequences of the R109L Replacement**—The R109L variant is unique among the variants in Table II, in that its ability to bind NADH and to respond to NADH inhibition is actually stronger than what is observed with wild type CS. The turnover number of this variant and its affinity for both substrates are also increased (Table III). These results suggest that Arg<sup>109</sup> has an important role in the regulatory mechanism of CS.

Our earlier structure determination of an E. coli CS-NADH complex showed that Arg<sup>109</sup> is centrally positioned at the entrance to the NADH binding site (Fig. 3), although its interaction with NADH is limited to the dihydronicotinamide end (4). Based on this observation, we proposed that the positively charged side chain of Arg<sup>109</sup> acts to allow selective binding of the neutral NADH and to screen out binding of the positively charged NAD<sup>+</sup>. Our new finding, that NADH binding and inhibition are stronger in the R109L variant, now suggests that Arg<sup>109</sup> also modulates the sensitivity of CS to the reduced pyridine nucleotide pool; without Arg<sup>109</sup>, this enzyme. Thus, the observed changes in kinetic properties actually stronger than what is observed with wild type CS. The turnover number of this variant and its affinity for both substrates are also increased (Table III). These results suggest that Arg<sup>109</sup> has an important role in the regulatory mechanism of CS.

The CS R109L variant crystallized under the same conditions as wild type enzyme, and the crystals diffracted equally well. Substitution of Arg<sup>109</sup> with a leucine causes minimal perturbation of the neighboring polypeptide chain (Figs. 3 and 4). This is the case for both the A and B subunits of each of the three dimers that make up the overall hexameric structure of this enzyme. Thus, the observed changes in kinetic properties observed for the R109L variant seem to be primarily dependent upon the removal of the positive charge at the extremity of the Arg<sup>109</sup> side chain. As illustrated in Fig. 4, loss of this positive charge alters the electrostatic character at the entrance way to the NADH binding site.

It is also apparent from Figs. 3 and 4 that binding of NADH to the R109L variant is much the same as binding to the wild type enzyme. The Leu<sup>109</sup> side chain has shifted from the position of the Arg<sup>109</sup> side chain that it replaces, however. This allows the imidazole ring of His<sup>110</sup> to assume more of a stacking orientation relative to the dihydronicotinamide ring, which, for its part, is bound much further into the pocket than it is in the complex with the CS F383A variant studied previously (4). These adjustments probably account for the tighter binding of NADH to the R109L variant (Table II).
FIG. 2. Mass spectra of *E. coli* citrate synthase, wild type (*A* and *B*), and the R163L (*C* and *D*) and K167A (*E* and *F*) variants. All spectra are of 10 μM CS (subunit concentration), dissolved in 20 mM NH₄HCO₃. Charge envelopes corresponding to dimers, tetramers, and hexamers are as in Fig. 1. In *B*, *D*, and *F*, 20 μM NADH was added. In the spectrum shown in *B*, individual NADH complexes are not resolved, but the charge envelope has shifted upward by an amount corresponding to the mass of six NADH per hexamer. The *inset* in *D* is a more dispersed plot of the +18 charge state of the dimer, showing the appearance of complexes containing one and two NADH per dimer (the peaks are spaced at intervals of 665/18, where 665 Da is the molecular mass of NADH). The *insets* in *E* and *F* are more dispersed plots of the +27 charge state of the tetramer, showing the appearance of complexes containing one, two, three, and four NADH per tetramer (spaced at intervals of 665/27) when NADH is present.
A second and surprising result from our R109L variant structural studies is that the largest structural changes are observed to occur remote from the position of residue 109 and the NADH binding site. This phenomenon is illustrated in Figs. 5 and 6, which show comparisons of main chain thermal factor values (Fig. 5) and positional displacements (Fig. 6) between the wild type and R109L variant enzymes. In particular, for the R109L variant, Fig. 5 documents a remarkable reduction in the thermal factors for two segments of polypeptide chain composed of residues 260–311 and 316–340. In wild type CS, both of these segments show significant flexibility with thermal factors as much as 10 times the average of the rest of the polypeptide chain (3). The same high mobility was observed for the active site variant F383A, in both the presence and absence of NADH (4).

Coincident with the polypeptide chain mobility changes observed for the R109L variant are accompanying shifts of the

![A. Subunit A: Wild-type and R109L variant](image1)

![B. Subunit B: Wild-type and R109L variant](image2)

![C. Superpositioning of NADH's in the A and B subunits of the R109L variant](image3)
were prepared with the assistance of GRASP (27). The rest of the protein (i.e. alternative conformations that are much closer in mobility to causing two very mobile regions of wild type CS to settle into calming influence on the polypeptide chain conformation of CS, replacement of Arg109 by a neutral leucine side chain has a Together, the data presented in Figs. 5 and 6 suggest that involves residues 262–298 and to a lesser extent in residues 316–342 in the R109L variant of Type II E. coli citrate synthase.

regions in wild type CS are able to sample a number of conformations, the R109L CS variant samples only one).

**DISCUSSION**

That E. coli CS and the CS from other Gram-negative bacteria are probably allosteric enzymes was recognized almost 40 years ago, and the main facts indicating this have been in the literature for a long time (1, 2). This kind of CS, which we now designate Type II, is largely hexameric, in contrast to the dimeric Type I CS found in other organisms, and it is subject to strong and specific inhibition by NADH. Weitzman’s group assembled several pieces of evidence to show that the NADH binding sites are distinct from the active sites. Thus, NADH inhibition of E. coli CS is weakened by conditions, such as high salt or alkaline pH, which favor activity (1, 2). Treatment of this enzyme with the sulfydryl reagent, Ellman’s reagent, abolishes NADH inhibition but has only a partial effect on activity (19). To this we added information on the nucleotide specificity of the NADH site (20), and identified the reactive cysteine as Cys206, which appeared, from modeling studies, to be remote from the active sites (21). The distinctness of the NADH binding sites from the active sites has now been established by our recent structural studies on a CS-NADH complex (4).

Several pieces of kinetic evidence suggest that E. coli CS exists in two conformational states, one of which (the R state) binds acetyl-CoA selectively, whereas the other (the T state) binds NADH selectively. NADH inhibition is competitive with respect to acetyl-CoA and noncompetitive with respect to the other substrate, OAA (22). NADH binding is competitively inhibited by acetyl-CoA (7). NADH inhibition is strongest in low ionic strength buffers and is weakened by the addition of KCl and other salts (1, 2). In dilute buffers, acetyl-CoA saturation is sigmoid, but KCl converts the curve to hyperbolic (9). These effects of KCl suggest that it is acting as an allosteric activator, shifting the T-R equilibrium toward R state. Qualitatively, these observations are consistent with the classical allosteric theory of Monod et al. (23). This theory predicts, however, that NADH saturation curves will become sigmoid in the presence of sufficient acetyl-CoA and/or KCl. This is not the case with E. coli CS; although both ligands weaken NADH binding, the shape of the binding curve always remains hyperbolic (7). ² Despite this inconsistency with a formal theory, we feel it is still useful to use the R state/T state terminology, and our recent structural work on the CS-NADH complex shows

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² L. Donald and H. Duckworth, unpublished results.
that a conformational state that is incapable of binding acetyl-CoA does exist.

In this paper, we present information about what parts of the structure are involved in the communication between the NADH sites and the active sites. Our conclusions fulfill well short of a detailed mechanism for the action of NADH on Type II CS, but with the new information, more incisive investigations of this topic will now be possible.

The kinetic effects of the other mutations in the NADH binding sites, as detailed in Tables II and III, are complex and cannot yet be rationalized in detail. Given that some variants have significantly altered subunit interactions, it may be that each will have to be considered as an individual case. Shifts in affinity for substrates and in sensitivity to NADH inhibition cannot be accounted for simply by changes in the ratio of T to R state (calculations not shown). One striking effect, seen with all variants, is the increase in the steady state kinetic parameter $K_{i,OAA}$ relative to the wild type value (Table III). In the ordered bisubstrate catalytic mechanism obeyed by E. coli CS in the presence of KCl, this parameter is interpreted as the dissociation constant for the CS-OAA complex, which must form first, before the second substrate, acetyl-CoA, binds and the catalytic complex forms (8). It now appears that OAA binding is a significantly harder step in the variants with amino acid replacements at the NADH binding sites.

Implications of the Structural Results for the CS R109L Variant—Two polypeptide chain segments are affected in the R109L variant protein. Interestingly, one of these, residues 262–298, is a part of the active site region. In wild type E. coli CS, this large segment of polypeptide chain is loosely folded and located on the outer surface of the enzyme (3). Surprisingly, these studies also showed that although residues in this segment are needed to form the binding site for the substrate acetyl-CoA, their observed fold would not allow for this. We therefore proposed that refolding of this segment of polypeptide chain is part of the regulatory mechanism of Type II CS, although at that time there was no apparent structural link from this region of the enzyme to the distant NADH binding site.

We now have direct evidence for this linkage from our new structure for the R109L variant. In this protein, residues 262–298 refold into a conformation that appears to be much of the way toward that required for a functional acetyl-CoA binding site. The evidence for this is a comparison with the structure of this region as it appears in the simpler, unregulated Type I CS enzymes (3, 24). The structural element that provides the link between the R109L replacement and residues 262–298 appears to be the second polypeptide chain segment most perturbed in the R109L CS variant. Residues 316–342 also demonstrate a marked decrease in flexibility, although this is smaller than that experienced by residues 262–298 (Fig. 5). In both the wild type and R109L variant CS structures, residues 316–342 form a helix-turn-helix arrangement that can be described as a “helical linker.” The more rigid structure presented by these residues seems to provide a more suitable packing surface for residues 262–298, which in the refolded state are found closely associated with these residues.

Examination of the hexameric structure of E. coli CS as a whole shows that the helical linker region (residues 316–342) structurally links the refolded active site region polypeptide chain segment (residues 262–298) to an NADH binding site. However, the NADH binding site involved is not from the same subunit, instead being located across the dimer-dimer interface on an adjacent subunit. This can be clearly seen in the RasMol view in Fig. 7A, which is also shown schematically in Fig. 7B. The details of the interactions between the NADH binding site, the “helical linker,” and the acetyl-CoA binding loop remain the subject of further experimentation, but our current analysis suggests that one reason why the Type II CS system must be a hexamer is to facilitate the transmission of a signal between the NADH binding sites and the active sites.

In a previous discussion of the rearrangements that must occur in the mobile segment of our wild type E. coli CS structure (residues 262–298), in order to form an acetyl-CoA binding site and catalytic center, we drew attention to the fact that this rearrangement has some formal similarities to a well studied
domain closure process in Type I CS (3). The subunits of CS, whether Type I or Type II, are organized into two domains, and in vertebrate Type I CS it has been shown that these rotate relative to each other as part of substrate binding (17, 25). In the most recent investigation of the domain closure in Type I CS, by molecular dynamics simulation, Roccatano et al. (26) concluded that the most important part of the process is a helicalike bending motion centered at residues His<sup>275</sup>, Gly<sup>276</sup>. These residues have exact homologues in E. coli CS (where they are numbered His<sup>284</sup>, Gly<sup>285</sup>), and they are located within the mobile loop region whose motion and position are dramatically altered in the R109L variant. Once again, we have evidence that the regulatory conformational change in Type II CS resembles the domain closure in the Type I enzyme, although in a more extreme form.

Collectively, the kinetic and structural descriptions of the R109L CS variant, presented in this paper, provide a basis for understanding how the R-T state conversions in wild type Type II CS might occur (Fig. 7). This is particularly valuable, since it has thus far not been possible to characterize an R state enzyme structurally and all of the structures of E. coli CS studied to date have been in the T state. In the T (inactive) state of the wild type enzyme, we believe, Arg<sup>109</sup> is positioned so as to cause high thermal motion in the helical linker region. This translates into refolding of polypeptide chain in the active site so that the enzyme is no longer able to bind the substrate acetyl-CoA. In this scenario, NADH serves to stabilize the hexameric structure of the enzyme in this T state conformation. NADH binding does not directly induce the mobility and refolding events, since the NADH complex with T state-shifted F383A CS has the same structure as the uncomplexed protein (4).

Similarly, we now find that the presence of NADH has little effect on the segment mobility and overall folding of the R109L CS protein (Figs. 5 and 6).

In the wild type enzyme, switching to the R (active) state would involve the release of NADH and movement of Arg<sup>109</sup> to alleviate its charge-charge conflict with residues in the adjacent helical linker region. The subsequent rigidity of the helical linker segment would then promote refolding of residues 262–298 to form the acetyl-CoA binding site required for catalysis to occur. In this way, the helical linker feature of the enzyme provides a convenient mechanism for the transmission of the regulatory signal between the NADH and active sites, which are in relatively close proximity across the dimer-dimer interface in the hexameric form of the enzyme. In a general sense, this mechanistic proposal nicely fits the results that have been obtained in structural studies of the wild type enzyme and those of the F383A and R109L variants, but clearly additional kinetic and structural studies will be essential to attain a comprehensive understanding of how this process occurs.

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