# Two Distinct Proton Donors at the Active Site of *Escherichia coli* 2,4-Dienoyl-CoA Reductase Are Responsible for the Formation of Different Products†

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ABSTRACT: NADPH-dependent 2,4-dienoyl-CoA reductase (DCR) is one of the auxiliary enzymes required for the $\beta$-oxidation of unsaturated fatty acids. Mutants of *Escherichia coli* DCR were generated by site-directed mutagenesis to explore the molecular mechanism of this enzyme. The Tyr166Phe mutant, which was expected to be inactive due to the loss of its putative proton donor residue, exhibited 27% of the wild-type activity. However, the product of the reduction was 3-enoyl-CoA instead of 2-enoyl-CoA, the normal product. Glu164 seems to function as proton donor in the Tyr166Phe mutant, because the Tyr166Phe/ Glu164Gln double mutant was inactive whereas the Glu164Ala mutant exhibited low but significant activity. His252 is important for the efficient operation of Tyr166 because a His252Ala mutation by itself reduced the activity of DCR by 3 orders of magnitude, whereas the Tyr166Phe/His252Ala double mutation exhibited 4.4% of the wild-type activity. This data supports a mechanism that has Tyr166 with the assistance of His252 acting as proton donor in the wild-type enzyme to produce 2-enoyl-CoA, whereas Glu164 serves as the proton donor in the absence of Tyr166 to yield 3-enoyl-CoA. A Cys337Ala mutation, which resulted in the loss of most of the iron and acid-labile sulfur, decreased the reductase activity more than 1000-fold. This observation agrees with the proposed operation of an intramolecular electron transport chain that is essential for the effective catalysis of *E. coli* DCR.

Both saturated and unsaturated fatty acids are degraded by $\beta$-oxidation. However, the breakdown of unsaturated fatty acids requires auxiliary enzymes in addition to the enzymes necessary for the $\beta$-oxidation of saturated fatty acids (reviewed in ref *1*). One of the auxiliary enzymes is 2,4-dienoyl-CoA reductase (DCR;[1] EC 1.3.1.34) that catalyzes the reduction of double bonds at even-numbered positions (even-numbered double bonds) of fatty acids (*2*). Even-numbered double bonds either are present in unsaturated fatty acids, like the position 12 double bond of linoleic acid, or are formed from odd-numbered double bonds by positional isomerization. $\beta$-Oxidation of fatty acids with even-numbered double bonds yields 2,4-dienoyl-CoA intermediates that are reduced by nicotinamide adenine dinucleotide phosphate (NADPH) -dependent DCRs to yield 3-*trans*-enoyl-CoAs in eukaryotic organisms and 2-*trans*-enoyl-CoAs in bacteria (*3*). Even though the mammalian and *E. coli* DCRs yield different products, they are functionally similar in that they act almost equally well on cis and trans double bonds and are effective catalysts at low micromolar concentrations of their preferred 2,4-dienoyl-CoA substrates (*3*−*5*). Yet the mammalian and *E. coli* DCRs are structurally unrelated proteins. The mitochondrial reductase is a 124 kDa homotetrameric protein without cofactors that belongs to the short-chain dehydrogenase/reductase superfamily (*3*, *6*). In contrast, the *E. coli* enzyme is a 72 kDa monomeric protein that contains 1 mol each of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and a 4Fe-4S cluster (*3*, *7*, *8*). The binding sites for NADPH and 2,4-dienoyl-CoA are well separated in *E. coli* DCR so that direct hydride transfer from NADPH to substrate does not seem possible (*8*). An electron transport chain, consisting of FMN, a 4Fe-4S cluster, and FAD, is thought to facilitate the transfer of electrons from NADPH to carbon atom 5 of the 2,4-dienoyl-CoA substrate (*7*, *8*). Tyr 166 and His 252 have been proposed to form a catalytic dyad that donates a proton to carbon atom 4 of the substrate, thereby completing the reduction of the C4−C5 double bound (*8*).

This study was undertaken to verify the proposed catalytic mechanism of *E. coli* DCR and to prove the essential function of an intramolecular electron transport chain in the reduction catalyzed by this monomeric protein. The surprising conclusion is that this reductase has a cryptic alternate proton donor that functions only in the absence of the primary proton donor

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[1] Abbreviations: DCR, 2,4-dienoyl-CoA reductase; NTA−$Ni^{2+}$−agarose, nitrilotriacetic acid−$Ni^{2+}$−agarose; NBT, *p*-nitroblue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt; IPTG, isopropyl $\beta$-D-thiogalactopyranoside; enoyl-CoA isomerase, $\Delta^3$,$\Delta^2$-enoyl-CoA isomerase; crotonase, enoyl-CoA hydratase; HPLC, high-performance liquid chromatography; CD, circular dichroism; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBST, Tris-buffered saline with Tween.

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to catalyze the formation of 3-enoyl-CoA instead of 2-enoyl-CoA.

## EXPERIMENTAL PROCEDURES

*Materials*. Quikchange II XL site-directed mutagenesis kit, Quikchange multisite-directed mutagenesis kit, and BL21 competent cells were purchased from Stratagene. QIAprep spin miniprep kit for purification of plasmid DNA, QIAquick PCR purification kit, QIAquick gel extraction kit, and RGS-His antibody, nitrilotriacetic acid−$Ni^{2+}$−agarose (NTA−$Ni^{2+}$−agarose), and vector pQE-80L were bought from Qiagen. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. *Sac*I, *Hin*dIII, *Bam*HI, Taq DNA polymerase, deoxynucleotide solution mix, $T_4$ DNA ligase, and 2-long DNA ladder (0.1−10) were obtained from New England Biolabs Inc. High-strength analytical-grade agarose, protein assay dye reagent, 10% and 4−20% polyacrylamide ready gels, Trans-Blot transfer medium pure nitrocellulose membrane, goat anti-rabbit IgG (H+L)-AP conjugate, alkaline phosphatase (AP) color development reagent, nitroblue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine (BCIP) were purchased from Bio-Rad Laboratories. Gt × Ms IgG AP conjugate was purchased from Chemicon International. Rabbit antiserum against *E. coli* 2,4-dienoyl-CoA reductase was produced by Pocono Rabbit Farms and Laboratory (Canadensis, PA). Ultrafree-4 centrifugal filter units and syringe-driven filter units (0.22 $\mu$m) were from Millipore. Imidazole was purchased from Fischer Scientific. Standard cellulose dialysis tubing was obtained from Thomas Scientific. 2-*trans*,4-*trans*-Decadienal and 4-*cis*-decenal were bought from Aldrich. CoA-SH, NADPH, and isopropyl $\beta$-D-thiogalactoside (IPTG) were obtained from Life Science Resources. Sep-Pak $C_{18}$ cartridges used for concentrating acyl-CoAs and $\mu$Bondapak $C_{18}$ columns (30 cm × 3.9 mm) were purchased from Waters Associates. Fatty acid-free bovine serum albumin and all other standard biochemicals were purchased from Sigma. Recombinant rat liver $\Delta^3,\Delta^2$-enoyl-CoA isomerase (enoyl-CoA isomerase) (*9*) and bovine liver enoyl-CoA hydratase (crotonase) (*10*) were prepared as described.

*Preparation, Purification, and High Performance Liquid Chromatographic Analysis of Acyl-CoAs*. 4-*cis*-Decenoic acid and 2-*trans*,4-*trans*-decadienoic acid were synthesized from 4-*cis*-decenal and 2-*trans*,4-*trans*-decadienal, respectively, by oxidation with $Ag_2O$ according to the method of Thomason and Kubler (*11*). Coenzyme A derivatives of the two acids were prepared by the mixed anhydride method as described by Fong and Schulz (*12*). 4-*cis*-Decenoyl-CoA was converted to 2-*trans*,4-*cis*-decadienoyl-CoA by acyl-CoA oxidase in the presence of catalase according to the method of Yang et al. (*13*). 5-Phenyl-2,4-pentadienoyl-CoA was prepared as described (*14*). All acyl-CoAs were purified by HPLC as follows. Before samples were subjected to HPLC, the pH of the solutions was adjusted to ~2 with HCl and then readjusted to ~5 with KOH. Reaction mixtures were filtered through 0.22-$\mu$m membranes. The filtrates were injected into a Waters $\mu$Bondapak $C_{18}$ reverse-phase column (30 cm × 3.9 mm) attached to a Waters gradient HPLC system. The absorbance of the effluent was monitored at 254 nm. Separation was achieved by linearly increasing the acetonitrile content of the 50 mM $(NH_4)_2KP_i$ elution buffer (pH 5.5) from 20% to 50% in 30 min at a flow rate of 1.5 mL/min. Concentrations of substrates were determined by measuring CoA-SH according to the method of Ellman (*15*) after cleavage of the thioester bond with 1 M $NH_2OH$ at pH 7.0.

*Cloning and Site-Directed Mutagenesis of fadH*. The *fadH* gene encoding *E. coli* DCR had been cloned into the *Bam*HI/*Hin*dIII sites of the vector pND-1 to yield the expression plasmid designated pNDH (*16*). However, another *Bam*HI restriction site exists in the *fadH* gene. To remove it, plasmid pNDH was used as the template with a pair of primers consisting of primer I with a *Sac*I cleavage site (5′-TGA-CTGAGCTCAGCTACCCGTCGCTGTTCGC-3′) and primer II with a *Hin*dIII site (5′-GTGACAAGCTTCCGGCTGCT-TCAAATAACGAT-3′) to replace the *Bam*HI/*Hin*dIII sites with *Sac*I/*Hin*dIII sites. This construct was used to amplify the complete *fadH* gene. Amplification was for 30 cycles under the following conditions: 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 3 min. Thereafter, the reaction mixture was kept at 72 °C for 10 min. The PCR-amplified DNA fragments were purified by use of a QIAquick PCR purification kit. The purified PCR product was digested with *Sac*I plus *Hin*dIII and subcloned into the *Sac*I/*Hin*dIII sites of the vector pQE-80L by $T_4$ DNA ligase at 16 °C overnight to form a new expression plasmid designated pQE-80LH. The segment of vector pQE-80LH containing the $His_6$ form of the *fadH* gene was verified by DNA sequence analysis (data not shown). *E. coli* BL21 cells were transformed with the new expression vector pQE-80LH, which has an insert coding for the $His_6$ form of the wild-type DCR or its mutants, according to the method of Chung et al. (*17*). The transformants were grown in LB medium at 37 °C with vigorous shaking (~220 rpm) until the $OD_{600}$ was ~0.7. At that time, 0.13 mM riboflavin and 0.8 mM isopropyl $\beta$-D-thiogalactopyranoside (IPTG) were added. The cells were induced by IPTG for 4.5 h at 37 °C under shaking at 220 rpm. Then the cells were harvested by centrifugation at 4000*g* at 4 °C for 20 min and stored at −80 °C.

For site-directed mutagenesis, nine pairs of mutagenic primers were designed according to primer design guidelines of Stratagene and synthesized by Integrated DNA Technologies, Inc. (Table 1). The new expression vector pQE-80LH that contains an insert encoding the $His_6$-form of the *E. coli* DCR was used as DNA template. Site-directed mutagenesis was performed by use of Quikchange II XL site-directed mutagenesis kit and Quikchange multisite-directed mutagenesis kit from Stratagene, following the manufacturer's instructions. The mutations were confirmed by sequencing the segment of vector pQE-80LH that encodes the $His_6$-form of the mutant DCRs.

*Purification of $His_6$-Tagged Wild-Type and Mutant E. coli DCRs*. The induced cells were resuspended in 50 mM $KP_i$ (pH 8.0) containing 500 mM sodium chloride, 10 mM imidazole, 1 mM benzamidine hydrochloride, 1 mM phenylmethanesulfonyl fluoride, and 10 mM 2-mercaptoethanol and then lysed by sonication (15 × 10 s). The lysate was centrifuged at 26000*g* for 30 min at 4 °C to obtain an extract of soluble proteins. An appropriate amount of 50% NTA−$Ni^{2+}$−agarose slurry was added to this extract, and the mixture was shaken at 4 °C for 1 h and then loaded onto a column with a capped outlet. After the bottom cap was removed and the column flowthrough was collected, an imidazole step gradient (10, 20, and 30 mM) was sequentially applied to the NTA−$Ni^{2+}$−agarose column. After a wash,

Table 1

| mutation | primer | sequence |
|---|---|---|
| Tyr166 → Phe | sense | 5′-GGTTCCGAAGGG**TTT**TTGATCAACGAATTTCTG-3′ |
| | antisense | 5′-CAGAAATTCGTTGATCAA**AAA**CCCTTCGGAACC-3′ |
| His252 → Phe | sense | 5′-GGCATTGGCTGG**TTT**GAAGCACGTATTCCG-3′ |
| | antisense | 5′-CGGAATACGTGCTTC**AAA**CCAGCCAATGCC-3′ |
| His252 → Ala | sense | 5′-GGCATTGGCTGG**GCT**GAAGCACGTATTCCG-3′ |
| | antisense | 5′-CGGAATACGTGCTTC**AGC**CCAGCCAATGCC-3′ |
| Cys337 → Ala | sense | 5′-GAGATCAACACTTGTATTGGC**GCC**AATCAGGCC-3′ |
| | antisense | 5′-GGCCTGATT**GGC**GCCAATACAAGTGTTGATCTC-3′ |
| Glu164 → Gln | sense | 5′-GTGATGGGTTCC**CAA**GGGTATTTGATCAACG-3′ |
| | antisense | 5′-CGTTGATCAAATACCC**TTG**GGAACCCATCAC-3′ |
| Glu164 → Gln/Tyr166 → Phe | sense | 5′-GTGATGGGTTCC**CAA**GGG**TTT**TTGATCAACG-3′ |
| | antisense | 5′-CGTTGATCAA**AAA**CCC**TTG**GGAACCCATCAC-3′ |
| Glu164 → Ala | sense | 5′-GTGATGGGTTCC**GCA**GGGTATTTGATCAACG-3′ |
| | antisense | 5′-CGTTGATCAAATACCC**TGC**GGAACCCATCAC-3′ |
| Cys334 → Ala/Cys337 → Ala | sense | 5′-GAGATCAACACT**GCT**ATTGGC**GCC**AATCAGGCC-3′ |
| | antisense | 5′-GGCCTGATT**GGC**GCCAAT**AGC**AGTGTTGATCTC-3′ |
| Tyr166 → Phe/His252 → Ala | sense | 5′-GAGGTGATGGGTTCCGAAGGG**TTT**TTGATCAACGAA-TTTCTGACG-3′ |
| | antisense | 5′-AACACCGGCATTGGCTGG**GCT**GAAGCACGTATTCCG-ACCATTGCC-3′ |

the $His_6$-tagged wild-type or mutant *E. coli* DCR was eluted by washing the column with 50 mM $KP_i$ (pH 8.0) containing 300 mM sodium chloride, 300 mM imidazole, 10 mM 2-mercaptoethanol, and 20% glycerol.

*Enzyme Assays, Protein Measurements, and Chemical Analyses*. The activity of DCR was determined spectrophotometrically by measuring the oxidation of NADPH at 340 nm as described by Kunau and Dommes (*2*). The assay mixture contained 50 mM $KP_i$ (pH 7.4), 0.1 mM NADPH, 25 $\mu$M 2-*trans*,4-*trans*/*cis*-decadienoyl-CoA, and purified wild-type/mutant $His_6$-tagged *E. coli* DCR to achieve an absorbance change of 0.02−0.06/min. When less active mutant DCR was assayed, the amount of enzyme was increased to obtain an absorbance change of at least 0.01/min. The kinetic parameters of wild-type and mutant DCRs were determined at a fixed NADPH concentration of 0.1 mM that was saturating with all DCR forms subjected to kinetic analyses. Kinetic data were analyzed by nonlinear curve-fitting with the Sigma Plot program. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 $\mu$mol of substrate to product in 1 min. NADPH oxidase activity of *E. coli* DCR was measured spectrophotometrically at 340 nm. The assay mixture contained 50 mM $KP_i$ (pH 7.4), 0.1 mM NADPH, and purified wild-type/mutant *E. coli* DCR. Enoyl-CoA hydratase activity of *E. coli* DCR was determined spectrophotometrically at 263 nm. The assay mixture contained 50 mM $KP_i$ (pH 7.4), 25 $\mu$M 2-decenoylCoA, and purified DCR. Protein concentrations were assayed as described by Bradford (*18*) with the Bio-Rad protein assay dye reagent. Ultraviolet circular dichroism (CD) spectra of the wild-type and mutant DCRs were recorded with a Jasco spectropolarimeter. A negative band was observed between 210 and 250 nm with a minimum at 220 nm. The CD spectrum of a mutant was considered similar to that of wild-type protein when its band was centered at 220 nm and its ellipticity was ±20% of the wild-type ellipticity. The amounts of iron and acid-labile sulfide in wild-type and C337A mutant *E. coli* DCR were determined as described by Vanoni et al. (*19*). and Brumby et al. (*20*), respectively.

*SDS−PAGE and Western Blotting*. Proteins were separated by SDS−PAGE on 10% ready gels at pH 8.3 and were either stained with Coomassie brilliant blue R or used for Western blotting. After SDS−PAGE, the proteins were transferred from the gel to a nitrocellulose membrane by use of a TransBlot SD semidry electrophoretic transfer cell system. The transferred membrane was soaked in Tris-buffered saline (TBS) containing 5% nonfat milk overnight at 4 °C or for 1 h at 37 °C. The membrane was washed three times with TBS and then incubated with antiserum raised against *E. coli* DCR (dilution 1:500) under gentle shaking for 1 h at room temperature. The membrane was washed three times with Tris-buffered saline with Tween (TBST) and then incubated with the second antibody [goat anti-rabbit IgG(H+L)−AP conjugate]. After that, the membrane was washed three times with TBST and then incubated with color development solution containing NBT and BCIP until bands appeared on the membrane.

## RESULTS

*Cloning and Expression of the E. coli fadH Gene That Encodes 2,4-Dienoyl-CoA Reductase*. For the efficient and rapid purification of recombinant *E. coli* DCR, DNA encoding the mature enzyme (*16*) was inserted into plasmid pQE-80L to produce $His_6$-DCR modified at its N-terminus. Expression of $His_6$-DCR also facilitated its separation from DCR that is expressed by the *fadH* gene of the *E. coli* chromosome. This approach eliminated the possible contamination of mutant $His_6$-DCR by small amounts of wild-type DCR. Recombinant $His_6$-DCR was obtained in highly purified form after chromatography on a NTA−$Ni^{2+}$−agarose column (see Figure 1). The specific activity of this preparation was 7.5 units/mg with 2-*trans*,4-*trans*-decadienoyl-CoA as substrate (Table 2) and thus was almost the same as the activity of 7.3 units/mg determined for recombinant DCR without the $His_6$ attachment that had been used to solve the crystal structure of this protein (*8*).

*Evaluation of the Proposed Reaction Mechanism by Use of DCR Mutants*. The crystal structure of the ternary complex consisting of *E. coli* DCR, $NADP^+$, and fatty acyl-CoA substrate provided the information necessary to propose a reaction mechanism for this reductase. According to the mechanism, hydride transfer from $FMNH_2$ to carbon atom 5 and proton transfer from Tyr166 to carbon atom 4 bring about the reduction of the 4,5 double bond of the 2,4-dienoyl-CoA substrate (*8*). The active-site residues and their proposed

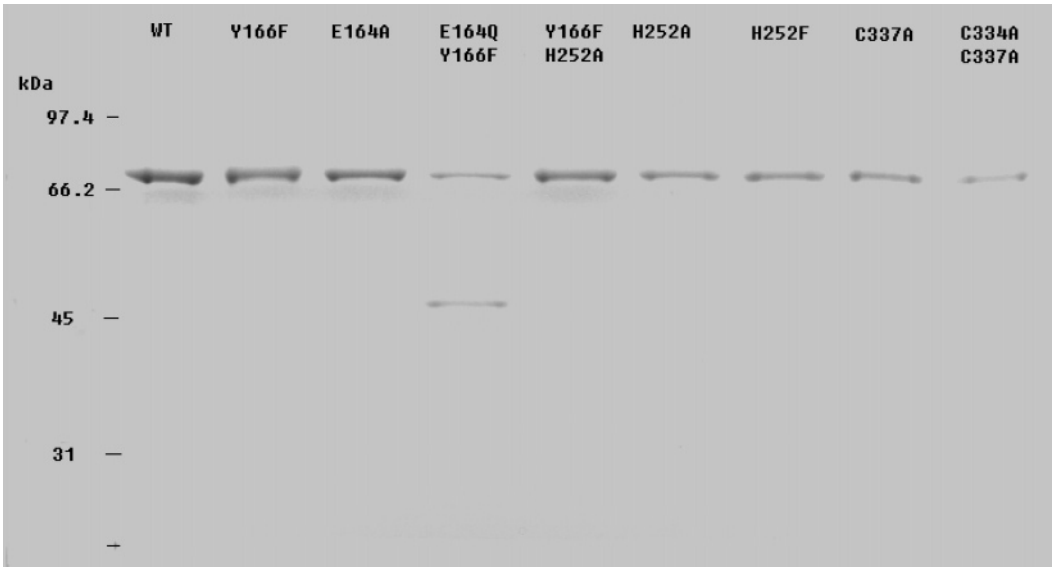



FIGURE 1: SDS−PAGE analysis of purified N-terminal $His_6$-tagged wild-type and mutant 2,4-dienoyl-CoA reductases from *E. coli*.

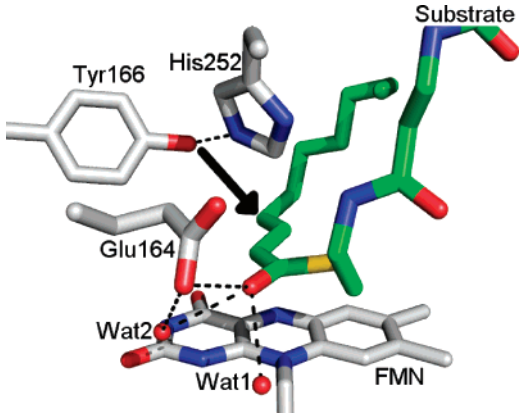
Table 2: Activities and Reaction Products of Wild-Type and Mutant *E. coli* 2,4-Dienoyl-CoA Reductases

| DCR | product[a] | specific activity[b] (units/mg) | relative activity (%) |
|---|---|---|---|
| wild type | 2-decenoyl-CoA | 7.54 ± 0.19 | 100 |
| Tyr166Phe | 3-decenoyl-CoA | 2.02 ± 0.08 | 27 |
| Glu164Ala | 2-decenoyl-CoA | 0.095 ± 0.01 | 1.3 |
| Glu164Gln/ Tyr166Phe | none | 0[c] | 0 |
| His252Phe | none | 0[c] | 0 |
| His252Ala | 3-decenoyl-CoA + 2-decenoyl-CoA | 0.007 ± 0.000 06 | 0.09 |
| His252Ala/ Tyr166Phe | 3-decenoyl-CoA | 0.33 ± 0.007 | 4.4 |
| Cys337Ala | 2-decenoyl-CoA | 0.0067 ± 0.0001 | 0.09 |
| Cys334Ala/ Cys337Ala | 2-decenoyl-CoA | 0.0015 ± 0.0002 | 0.02 |

[a] Identified by HPLC. [b] With 2-*trans*,4-*trans*-decadienoyl-CoA as substrate. [c] The limit of detecting enzymatic activity was 0.0008 units/mg.

participations in the proton transfer are shown in Scheme 1. To test the proposed mechanism, Tyr-166 of DCR was replaced by a phenylalanine residue via site-directed mutagenesis for a near-isosteric substitution. The mutant protein was obtained in highly purified form (see Figure 1) and its CD spectrum was similar to that of wild-type DCR (data not shown). The Tyr166Phe mutant exhibited 27% of wild-type activity (see Table 2) and had a slightly lower $K_m$ for 2-*trans*,4-*trans*-decadienoyl-CoA of 0.44 $\mu$M versus 0.8 $\mu$M obtained with the wild-type DCR (see Table 3). The high activity of the Tyr166Phe mutant did not seem to agree with the proposal that only Tyr166 functions as proton donor. Hence another residue might be responsible for the proton transfer, or Tyr166 may be the proton donor in wild-type DCR while a second residue may function in that capacity in the Tyr166Phe mutant. In an attempt to gain an understanding of the reactions catalyzed by wild-type DCR and the Tyr166Phe mutant, their reaction products were analyzed by HPLC. As shown in Figure 2, wild-type DCR reduced 2-*trans*,4-*trans*-decadienoyl-CoA (Figure 2A) to 2-decenoyl-CoA (Figure 2B), which was identified by its hydration to 3-hydroxydecanoyl-CoA in the presence of crotonase (Figure 2C). However, the Tyr166Phe mutant formed a product other

Scheme 1: Ball-and-Stick Diagram Outlining the Relative Positions of the Three Residues Proposed to be Involved in Proton Transfer during Reduction of 2,4-Decadienoyl-CoA at the Active Site of *E. coli* DCR[a]




[a] The diagram was generated from the coordinates of the ternary complex of DCR with $NADP^+$ and the product analogue [2-*trans*,4-*trans*-decadienoyl-CoA modified by mercaptoethanol at the C5 position of the substrate by the enzyme (*8*, PDB Code, 1PS9)]. For clarity, the mercaptoethanol moiety of the product analogue modified in situ is not shown. Tyr166, which is properly oriented by forming a hydrogen bond with His252, transfers a proton to the C4 atom of the substrate in wild-type DCR. The black arrow indicates the proton transfer, and the dotted lines show potential hydrogen bonds.

Table 3: Kinetic Parameters of Wild-Type and Mutant *E. coli* 2,4-Dienoyl-CoA Reductases

| DCR | $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $\mu M^{-1}$) |
|---|---|---|---|
| Wild-type | 0.80 ± 0.35 | 9.50 ± 0.40 | 11.90 ± 0.49 |
| Tyr166Phe | 0.44 ± 0.25 | 2.50 ± 0.08 | 5.74 ± 0.18 |
| Glu164Ala | 5.34 ± 1.30 | 0.15 ± 0.01 | 0.028 ± 0.002 |
| His252Ala | 5.48 ± 0.53 | 0.011 ± 0.0004 | 0.002 ± 0.0001 |
| His252Ala/ Tyr166Phe | 2.40 ± 0.46 | 0.44 ± 0.019 | 0.183 ± 0.008 |
| Cys337Ala | 1.41 ± 0.17 | 0.008 ± 0.0004 | 0.006 ± 0.0003 |

than 2-decenoyl-CoA as indicated by its elution time from HPLC (compare Figure 2B with Figure 2D). When this reaction mixture was treated with enoyl-CoA isomerase, the reaction product was converted to 2-decenoyl-CoA (see Figure 2E), which was hydrated by crotonase to 3-hydroxy-

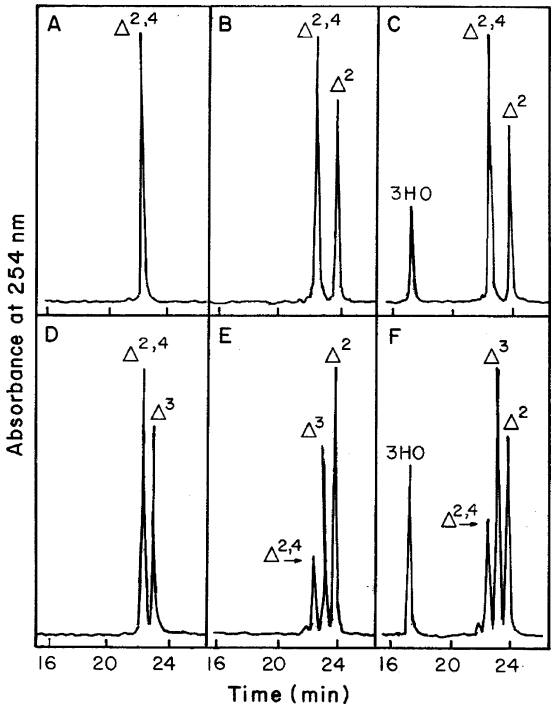



FIGURE 2: HPLC analysis of reaction products formed by wild-type *E. coli* DCR and the Tyr166Phe mutant. (A) HPLC-purified 2-*trans*,4-*trans*-decadienoyl-CoA. (B) 2-*trans*, 4-*trans*-Decadienoyl-CoA after incubation for 3 min with NADPH and 1 $\mu$g of wild-type DCR. (C) Same as in panel B plus crotonase for the final 30 s of the incubation period. (D) 2-*trans*,4-*trans*-Decadienoyl-CoA after incubation for 3 min with NADPH and 12.6 $\mu$g of Tyr166Phe DCR in the absence or presence of crotonase. (E) Same as in panel D in the absence of crotonase plus $\Delta^3$,$\Delta^2$-enoyl-CoA isomerase for the final 30 s of the incubation period. (F) Same as for panel E plus crotonase for the final 30 s of the incubation period. Abbreviations: $\Delta^{2,4}$, 2-*trans*,4-*trans*-decadienoyl-CoA; $\Delta^3$, 3-decenoyl-CoA; $\Delta^2$, 2-decenoyl-CoA; 3HO, 3-hydroxydecanoyl-CoA.

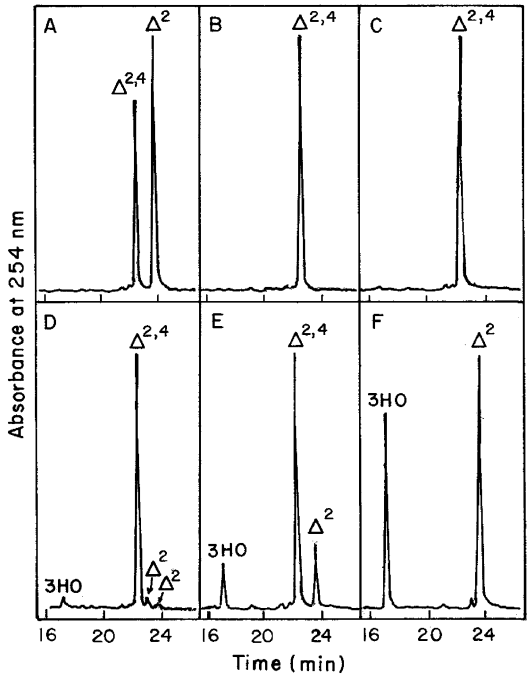



FIGURE 3: HPLC analysis of the reaction products formed by mutants of *E. coli* DCR. (A) 2-*trans*,4-*trans*-Decadienoyl-CoA after incubation for 3 min with NADPH and 26 $\mu$g of Glu164Ala DCR. (B) 2-*trans*,4-*trans*-Decadienoyl-CoA after incubation for 3 min with NADPH and 396 $\mu$g of Glu164Gln/Tyr166Phe DCR. (C) 2-*trans*,4-*trans*-Decadienoyl-CoA after incubation for 3 min with NADPH and 302 $\mu$g of His252Phe DCR. (D) 2-*trans*,4-*trans*-Decadienoyl-CoA after incubation for 30 min with NADPH and 895 $\mu$g of His252Ala DCR. (E) 2-*trans*,4-*trans*-Decadienoyl-CoA after incubation for 5 min with NADPH and 300 $\mu$g of Cys337Ala DCR. (F) 2-Decenoyl-CoA after incubation for 5 min with 319 $\mu$g of Cys337Ala DCR. Abbreviations: $\Delta^{2,4}$, 2-*trans*,4-*trans*-decadienoyl-CoA; $\Delta^3$, 3-decenoyl-CoA; $\Delta^2$, 2-decenoyl-CoA; 3HO, 3-hydroxydecanoyl-CoA.

decanoyl-CoA (see Figure 2F), whereas the initial reaction product was not hydrated by crotonase (Figure 2D). The conclusion of this set of experiments is that Tyr166Phe DCR catalyzes the reduction of 2-*trans*,4-*trans*-decadienoyl-CoA to 3-decenoyl-CoA instead of 2-decenoyl-CoA, which is the reaction product of wild-type reductase. Thus, a residue other than Tyr166 seems to serve as proton donor in the Tyr166Phe mutant and deliver a proton to carbon atom 2 of the substrate, thereby completing the 1,4-reduction of the diene and producing 3-decenoyl-CoA. A possible residue is Glu164, which is located at the active site close to the thioester function of the substrate. The function of Glu164 was evaluated by replacing it with an alanine or glutamine residue via site-directed mutagenesis. The purified Glu164Ala mutant (see Figure 1), which had a CD spectrum similar to that of wild-type DCR (data not shown), exhibited 1.3% of the activity detected with wild-type DCR (see Table 2). Although the mutation caused the $K_m$ value for 2-*trans*,4-*trans*-decadienoyl-CoA to increase from 0.8 $\mu$M to 5.34 $\mu$M, its main effect was a 63-fold reduction of the $k_{cat}$ value (see Table 3). Product analysis revealed that this enzyme catalyzes the formation of 2-decenoyl-CoA, the product of the wild-type enzyme (see Figure 3A). Replacement of Glu164 and Tyr166 with Gln and Phe, respectively, yielded an inactive DCR (see Table 2 and Figure 3B), in agreement with the notion that only Tyr166 or Glu164 can serve as proton donor. However, Glu164 seems only to participate in the proton transfer when Tyr166 is replaced by a nonfunctional residue like phenylalanine, because no 3-decenoyl-CoA was detected with wild-type reductase. Of all DCR mutants generated, the Tyr166Phe/Glu164Gln double mutant was the least stable enzyme as it was partially processed to a smaller protein (see Figure 1) that was recognized by anti-DCR antiserum (data not shown). However, approximately half of the Tyr166Phe/Glu164Gln DCR existed as the full-length protein and its CD spectrum in the 200−250 nm region was similar to those of other DCRs. The inactivity of the Tyr166Phe/Glu164Gln mutant supports the conclusion that either Tyr166 or Glu164 functions as proton donor. If only Tyr166 functions as a proton donor in wild-type DCR, what explains the 80-fold decrease in activity upon mutation of the Glu164 residue? The answer may be the loss of the suggested hydrogen bond between Glu164 and the thioester carbonyl oxygen of the substrate acyl chain (*8*). This hydrogen bond is thought to cause a polarization and optimal alignment of the conjugated dienone system (*7*, *8*), a situation that would make carbon atom 5 more electrophilic and more susceptible to attack by the hydride ion of $FMNH_2$. To test this idea, UV spectra of the DCR substrate 5-phenyl-2,4-pentadienoyl-CoA bound to wild-type DCR and to the Tyr166Phe and Glu164Ala mutants were recorded and compared with the spectrum of unbound substrate. As shown in Figure 4, binding of the substrate to wild-type DCR resulted in a red shift of close to 30 nm, whereas the Glu164Ala and

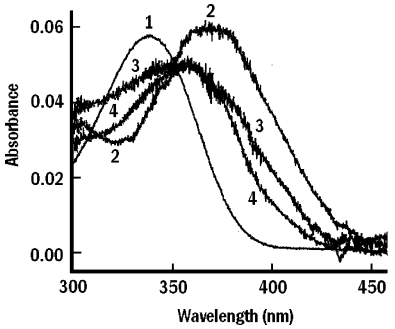



FIGURE 4: Spectral shifts of 5-phenyl-2,4-pentadienoyl-CoA induced by wild-type or mutant *E. coli* 2,4-dienoyl-CoA reductase. All solutions used for spectral analyses contained 0.91 $\mu$M 5-phenyl-2,4-pentadienoyl-CoA in 0.1 M $KP_i$ (pH 7.4) plus (spectrum 1) no addition, (spectrum 2) 36 $\mu$M wild-type DCR, (spectrum 3) 36 $\mu$M Tyr166Phe DCR, or (spectrum 4) 36 $\mu$M Glu164Ala DCR.

Table 4: NADPH Oxidase Activities of Wild-Type and Mutant 2,4-Dienoyl-CoA Reductases from *E. coli*

| DCR | specific activity (milliunits/mg) |
|---|---|
| wild type | 19.7 ± 1 |
| Tyr166Phe | 24.5 ± 2.2 |
| His252Ala | 18.0 ± 0.4 |
| Glu164Ala | 129.4 ± 1.6 |
| Glu164Gln | 130.4 ± 0.5 |

Tyr166Phe mutants caused smaller red shifts of approximately 18 and 12 nm, respectively. The reduced red shift detected with the Glu164Ala mutant may be due to the loss of the hydrogen bond between Glu164 and the carbonyl oxygen of the thioester group. If so, the conjugated dienone system would be less polarized and therefore less reactive in a nucleophilic attack by the hydride ion of $FMNH_2$ at carbon atom 5. This situation may explain the lower activity observed with the Glu164Ala mutant as compared to wild-type DCR. Another property of DCR that was changed by the mutation of the Glu164 residue was the NADPH oxidase activity detected with wild-type DCR. As shown in Table 4, this activity was increased more than 6-fold from 20 to130 milliunits/mg when Glu164 was replaced by an alanine or glutamine residue. In contrast, mutations of His252 and Tyr166 did not affect the oxidase activity. It is possible that loss of the hydrogen bond between Glu164 and the carbonyl oxygen of the thioester induces a structural change in DCR, which makes it easier for molecular oxygen to enter the active site.

Histidine 252 was proposed to stabilize the phenolate ion that is formed as the result of the proton transfer from Tyr166 to carbon atom 4 of the substrate (*8*). To evaluate the role played by His252 in the reduction of the double bond, the His252Phe mutant of DCR was generated, purified (see Figure 1), and assayed. Its CD spectrum was similar to that of wild-type DCR but the mutant was inactive ($\leq$0.015% of the wild-type activity; see Table 2). This finding agrees with the proposed essential function of His252 in the protonation event. When His252 was replaced by alanine, an activity was detected that was 1000 times lower than the activity of wild-type DCR (see Table 2). Surprisingly, this mutant catalyzed the reduction of 2-*trans*,4-*trans*-decadienoyl-CoA to both 3-decenoyl-CoA and 2-decenoyl-CoA (Figure 3D). Thus, in the absence of His252, Tyr166 and Glu164 seem to compete as proton donors but at rates close to the detection limit of the DCR assay. An attempt was made to determine if His252 is involved in the proton transfer from Glu164 to the substrate by generating and analyzing the Tyr166Phe/His252Ala double mutant of DCR. The purified mutant protein (see Figure 1) had a CD spectrum similar to that of wild-type DCR (data not shown) and a $K_m$ value for the substrate that was only 3 times higher than the $K_m$ obtained with wild-type DCR (Table 3). As expected of a Tyr166Phe mutant, the reaction product was 3-decenoyl-CoA (data not shown). The mutant DCR was surprisingly active as evidenced by a rate that was a sixth of the rate of Tyr166Phe DCR and 4.4% of the wild-type rate (see Table 2). Together, the data obtained with His252 mutants support the notion that His252 is very important for the effective protonation of carbon atom 4 by Tyr 166 while its contribution is less significant when, in the absence of Tyr166, Glu164 protonates carbon atom 2 of the substrate.

Table 5: Content of Iron and Acid-Labile Sulfur in Wild-Type and Mutant Dienoyl-CoA Reductases from *E. coli*

| DCR | iron/protein[a] | sulfur/protein[a] | Fe/S[a] | [4Fe-4S]/protein[a] |
|---|---|---|---|---|
| wild type | 3.9 | 3.9 | 1 | 1 |
| Cys337Ala | 0.54 | 0.53 | 1 | 0.13 |

[a] Molar ratio.

*Assessment of the Putative Electron Transport Chain.* The presence of 1 mol each of FMN, FAD, and a 4Fe-4S cluster in the monomeric *E. coli* DCR (*7*) and the identification of their intramolecular locations (*8*) led to the proposal that these cofactors form an electron transport chain that facilitates the flow of electrons from NADPH to the 2,4-dienoyl-CoA substrate. Because the 4Fe-4S cluster is thought to participate in the electron transfer from FAD to FMN, its absence should inhibit this process or reduce its rate and consequently inactivate the reductase or at least greatly reduce its activity. To test this prediction, Cys337, which is one of four cysteine residues that anchor the 4Fe-4S cluster, was mutated in the hope of generating a DCR form without the 4Fe-4S cluster. The purified Cys337Ala mutant behaved like wild-type DCR on SDS−PAGE and its CD spectrum was similar to that of wild-type DCR (data not shown). The Cys337Ala mutant and wild-type DCR were assayed for iron and acid-labile sulfur, which were found to be present in equimolar amounts in both proteins (Table 5). However, the mutant protein contained only 13% of the amount of iron and acid-labile sulfur detected in wild-type DCR that carried one 4Fe-4S cluster. The Cys337Ala mutation caused a more than 1000-fold decrease of the reductase activity (see Table 2) without having a significant effect on the $K_m$ for the 2-*trans*,4-*trans*-decadienoyl-CoA substrate (see Table 3). The latter observation suggests that the affinity of the substrate for the active site was not significantly affected by this mutation. When two of the four cysteine residues that anchor the 4Fe-4S cluster (Cys334 and Cys337) were mutated to alanine, the activity was reduced to 0.02% of the wild-type activity (see Table 2) without having a significant effect on the CD spectrum (data not shown). These dramatic activity losses agree with the hypothesis that the 4Fe-4S cluster is an essential component of the electron transport chain, which is required for the efficient reduction of the 2,4-dienoyl-CoA substrate. The reduction of 2-*trans*,4-*trans*-decadienoyl-CoA catalyzed by Cys337Ala DCR yielded 2-decenoyl-CoA, the

product formed by the wild-type enzyme (see Figure 3E). Surprisingly, the enzyme also catalyzed the formation of 3-hydroxydecanoyl-CoA even though no crotonase was present in the incubation mixture. This observation prompted us to determine whether Cys337Ala DCR has enoyl-CoA hydratase activity. As illustrated in Figure 3F, this DCR mutant catalyzed the hydration of 2-*trans*-decenoyl-CoA to 3-hydroxydecanoyl-CoA. When the wild-type and Cys337Ala DCRs were assayed for enoyl-CoA hydratase activity with 2-decenoyl-CoA as substrate, a specific activity of 2.5 milliunits/mg of protein was observed with the wild-type enzyme in contrast to 12.8 milliunits/mg of protein determined with the mutant. Thus, DCR has a low endogenous enoyl-CoA hydratase activity that is increased 5-fold by the Cys337Ala mutation. It is not surprising that the hydratase activity of wild-type DCR had previously not been detected because it is 3000 times lower than its reductase activity. In contrast, the reductase and hydratase activities of Cys337Ala DCR are of similar magnitude, and therefore the product analysis revealed the formation of both 2-enoyl-CoA and 3-hydroxyacyl-CoA.

## DISCUSSION

The crystal structure of *E. coli* DCR with bound $NADP^+$ and substrate revealed the structural details necessary to propose a molecular mechanism for the double-bond reduction catalyzed by this enzyme (*8*). According to the proposal (see Figure 5A; adapted from ref *8*), a hydride ion is transferred from $FMNH_2$ to carbon atom 5 of the substrate's acyl chain while the phenolic hydroxyl group of Tyr166, which is 3.5 and 4.1 Å away from carbon atoms 4 and 2, respectively, donates a proton to carbon atom 4. This proposal was confirmed, but only after the operation of a second reduction mechanism was revealed and analyzed in a mutant form of DCR. According to the proposed mechanism, replacement of Tyr166 by phenylalanine should yield an inactive enzyme. Surprisingly, the Tyr166Phe mutant actively catalyzed the reduction of 2,4-decadienoyl-CoA. However, it did not form the expected product, 2-decenoyl-CoA, but instead generated 3-decenoyl-CoA, the product of eukaryotic 2,4-dienoyl-CoA reductases (*1*). Thus, the Tyr166Phe mutant had lost the capacity to protonate carbon atom 4, a step that is necessary to complete the reduction of the 4,5 double bond, but had gained the ability to transfer a proton to carbon atom 2 for completion of a 1,4 reduction of the conjugated diene. Both Glu164 and His252 are potential proton donors in the Tyr166Phe mutant because of their protic properties and their proximity to the active site (*8*). We first investigated the role of Glu164 in catalysis. Glutamate and aspartate residues often serve as proton acceptors and proton donors, as illustrated by the mechanisms of $\Delta^{3,5},\Delta^{2,4}$-dienoyl-CoA isomerase (*21*, *22*), where the two residues simultaneously add and abstract a proton from the substrate. The idea of Glu164 serving as a proton donor in Tyr166Phe DCR is supported by the observations that the Tyr166Phe/Glu164Gln DCR double mutant was completely inactive, whereas the Tyr166Phe/His252Ala mutant retained a significant level of DCR activity. These observations rule out His252 as a proton donor and leave Glu164 as the only residue at the active site that could serve as a proton donor in the Tyr166Phe/His252Ala mutant. The function of Glu164 as a proton donor is also supported by distances of 3.1 and 4.1 Å between the carboxylate oxygen and carbon atoms 2 and 4, respectively, after allowing for a minor rotation at the C$\beta$ position of the Glu164 side chain. This situation favors the transfer of a proton from Glu164 to carbon atom 2 of the substrate. In wild-type DCR, Glu164 was observed to be 3.3 Å away from the oxygen of the thioester group and therefore was proposed to form a hydrogen bond to the thioester oxygen (*8*). The existence of such hydrogen bond is also supported by an observed spectral red shift of 30 nm for 5-phenyl-2,4-pentadienoylCoA bound to wild-type DCR. That red shift was reduced to 18 nm when the substrate was bound to Glu164Ala DCR. Thus Glu164 contributes to the polarization of the phenyl dienone of the substrate but is not responsible for the whole effect. Two water molecules, which are 2.8 and 2.9 Å away from the carbonyl oxygen of the thioester (*8*) and therefore are thought to participate in hydrogen bonds, might be responsible for the partial polarization of the phenyl dienone chromophore of the substrate. The partial polarization of the substrate's chromophore agrees with the observation that the Glu164Ala mutant retains a reduced but significant activity level compared to wild-type DCR. It was noted that Tyr166Phe DCR caused a red shift of only 12 nm in the spectrum of 5-phenyl-2,4-pentadienoyl-CoA. This limited polarization of the phenyl dienone system may be the consequence of Glu164 no longer participating in a hydrogen bond with the carbonyl oxygen of the thioester. Instead Glu164 may form a hydrogen bond with the imidazole of His252 that is available for hydrogen bonding because of the absence of Tyr166. If so, His252 would be expected to enhance the proton transfer from Glu164 to the substrate and thereby contribute to the catalytic efficiency of the Tyr166Phe mutant. This prediction is supported by the activity loss that was observed when His252 was replaced by an alanine residue in the Tyr166Phe mutant. A pivotal function of His252 in wild-type DCR was proposed (*8*) on the basis of its predicted hydrogen bond with Tyr166, which would stabilize the phenolate ion that forms during proton transfer. This proposal is supported by the observed inactivity of the His252Phe mutant. The residual activity detected in the His252Ala mutant could be the consequence of a water molecule residing in the space that was occupied by the imidazole group of His252 in wild-type DCR. The observed formation of 2-decenoyl-CoA and 3-decenoyl-CoA by His252Ala DCR suggests that, in the absence of His252, both Tyr166 and Glu164 have the opportunity to participate in the proton transfer, although at very low rates. The conclusion is that proton transfer requires or is enhanced by hydrogen bonding between the proton donor group and the imidazole of His252. Thus when Glu164 is hydrogen-bonded to the oxygen of the thioester in wild-type DCR, it is inactive as a proton donor, whereas it is active when it is hydrogen-bonded with His252 in the Tyr166Phe mutant. Possible evidence for a structural change associated with the loss of the hydrogen bond between Glu164 and the oxygen of the thioester was the increased NADPH oxidase activity that was observed when Glu164 was replaced by an alanine or glutamine residue.

This study also addressed the question as to whether the three cofactors of DCR—namely, FAD, a 4Fe-4S cluster, and FMN—form a functional electron transport chain that facilitates the flow of electrons from NADPH to the substrate. The mutation of one of the cysteine residues that anchor the

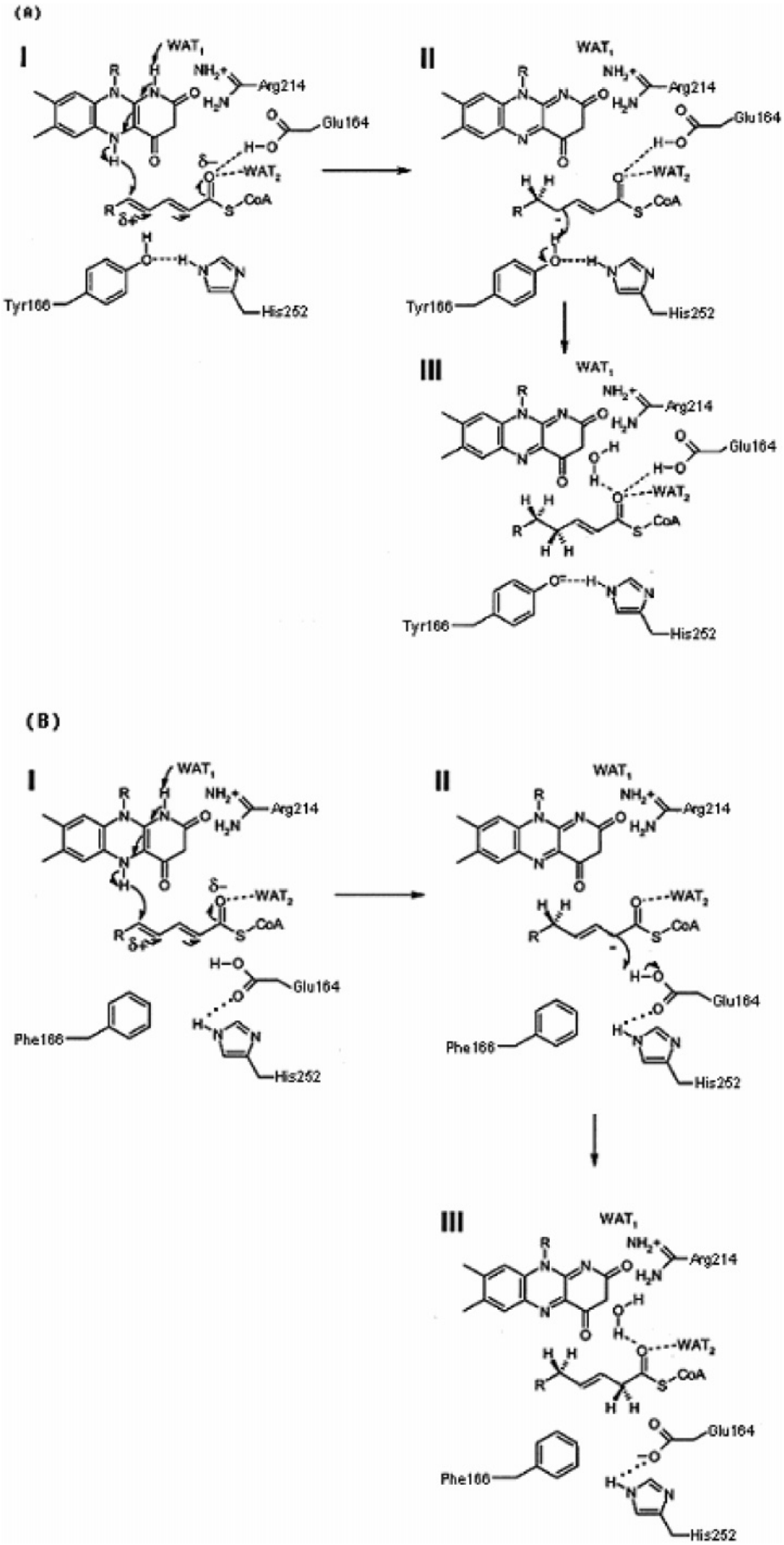



FIGURE 5: Proposed reaction mechanisms of *E. coli* (A) wild-type DCR (adapted with permission from Figure 6 of ref *8*; copyright 2003 American Society for Biochemistry and Molecular Biology) and (B) Tyr166Phe DCR.

4Fe-4S cluster resulted in the loss of most, but not all, of the iron and acid-labile sulfur and greatly reduced the activity of the enzyme. The presence of low but equimolar amounts of iron and sulfur in Cys337Ala DCR agrees best with the existence of a 4Fe-4S cluster in a small percentage of DCR molecules. Such a 4Fe-4S cluster, which would be suspended by only three cysteine residues due to the absence of a residue that could substitute for Cys337, might be less active in transferring electrons than a cluster in wild-type DCR. This idea is supported by the observation that the Cys337Ala mutation caused a 1000-fold decrease in reductase activity, whereas the cluster concentration was decreased less than 10-fold. The lower activity of a cluster in the Cys337Ala mutant might be caused by its distorted orientation. Another possibility is that DCR with a 4Fe-4S cluster anchored by only three cysteine residues is inactive but that electrons are transferred, albeit very slowly, directly from FAD to FMN. Transfer of electrons over long distances up to 14 Å can occur in native proteins (*23*), but it is uncertain whether it could take place, even at very slow rates, in DCR, where FAD and FMN are separated by 15.5 Å. Either way, the data agree with the notion that an intramolecular electron transport chain that includes the 4Fe-4S cluster is important for efficient electron flow from NADPH to the substrate.

In conclusion, this study confirms the proposed mechanism of reduction by wild-type *E. coli* DCR (*8*). As shown in Figure 5A, the 4,5 double bond of the substrate is reduced by transfer of a hydride ion from $FMNH_2$ to carbon atom 5, while Tyr166 donates a proton to carbon atom 4. The hydride transfer is assisted by Glu164 and two water molecules that contribute to the polarization and optimal alignment of the conjugated dienone system via hydrogen bonds, while the protonation is activated by His252 due to its hydrogen bonding with Tyr166. The identification of a second mechanism of reduction catalyzed by Tyr166Phe DCR was totally unanticipated. The hydride transfer is most likely not affected by the mutation, but a different amino acid residue, apparently Glu164, donates a proton to carbon atom 2, thereby yielding 3-enoyl-CoA instead of 2-enoyl-CoA, the normal product of *E. coli* DCR (see Figure 5B). His252 enhances the proton-transfer possibly by hydrogen bonding with Glu164. It is possible that hydrogen-bonding of His252 with Tyr166 in wild-type DCR or with Glu164 in the Tyr166Phe mutant determines which of the two mechanisms is operative and which product is formed.

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