Δ^{3,5},Δ^{2,4}-Dienoyl-CoA Isomerase Is a Multifunctional Isomerase
A STRUCTURAL AND MECHANISTIC STUDY*

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Δ^{3,5},Δ^{2,4}-Dienoyl-CoA isomerase (DI), an auxiliary enzyme of unsaturated fatty acid β-oxidation, was purified from rat mitochondria and peroxisomes and subjected to N-terminal sequencing to facilitate a mechanistic study of this enzyme. The mature mitochondrial DI from rat heart was lacking its 34 N-terminal amino acid residues that have the properties of a mitochondrial targeting sequence. The peroxisomal isomerase was identified as a product of the same gene with a truncated and ragged N terminus. Expression of the cDNA coding for the mature mitochondrial DI in Escherichia coli yielded an enzyme preparation that was as active as the native DI. Because the recombinant DI also exhibited Δ^{3,5,7},Δ^{2,4,6}-triene isomerase (TI) activity, both isomerases reside on the same protein. Mutations of any of the 3 acidic amino acid residues located at the active site (Modis, Y., Filippula, S. A., Novikov, D. K., Norledge, B., Hiltunen, J. K., and Wierenga, R. K. (1998) Structure 6, 957-970) caused activity losses. In contrast to only a 10-fold decrease in activity upon replacement of Asp^{176} by Ala, substitutions of Asp^{204} by Asn and of Glu^{196} by Gln resulted in 10^5-fold lower activities. Such activity losses are consistent with the direct involvement of these latter two residues in the proposed proton transfers at carbons 2 and 6 or 8 of the substrates. Probing of the wild-type and mutants forms of the enzyme with 2,5-octadienoyl-CoA as substrate revealed low Δ^{2,4}-enoyl-CoA isomerase and Δ^{3,5,7}-enoyl-CoA isomerase activities catalyzed by Glu^{196} and Asp^{204}, respectively. Altogether, these data reveal that positional isomerizations of the diene and triene are facilitated by simultaneous proton transfers involving Glu^{196} and Asp^{204}, whereas each residue alone can catalyze, albeit less efficiently, a monoene isomerization.

The degradation of unsaturated fatty acids by β-oxidation requires several auxiliary enzymes in addition to the enzymes necessary for the breakdown of saturated fatty acids (for a review see Ref. 1). One of the auxiliary enzymes is Δ^{3,5},Δ^{2,4}-dienoyl-CoA isomerase (dienoyl-CoA isomerase), which catalyzes the isomerization of 3,5-dienoyl-CoA to 2,4-dienoyl-CoA (2, 3). During the β-oxidation of unsaturated fatty acids with odd-numbered double bonds, 5-enoyl-CoA intermediates can be converted to 3,5-dienoyl-CoA by the sequential actions of acyl-CoA dehydrogenase and Δ^{2,4}-enoyl-CoA isomerase (EC 5.3.3.8) (enoyl-CoA isomerase). The further degradation of 3,5-dienoyl-CoA requires its isomerization to 2,4-dienoyl-CoA, because the latter compound can be reduced by 2,4-dienoyl-CoA reductase (EC 1.3.1.34) to 3-enoyl-CoA. The isomerization of 3-enoyl-CoA to 2-enoyl-CoA by enoyl-CoA isomerase completes the reductase-dependent sequence of reactions during the β-oxidation of unsaturated fatty acids with odd-numbered double bonds.

Dienoyl-CoA isomerase was first identified in rat liver mitochondria but later was also detected in rat liver peroxisomes (4, 5). The molecular characterization of this enzyme revealed the amino acid sequence of the unprocessed subunit, which has a peroxisomal targeting signal, type 1, at the C terminus and an N-terminal sequence that is consistent with the targeting of this protein to mitochondria (6). This situation is suggestive of a dual subcellular localization and agrees with the previously observed kinetic and immunological similarities of the mitochondrial and peroxisomal forms of this enzyme (5). The crystal structure of a recombinant form of dienoyl-CoA isomerase consisting of subunits without the 53 N-terminal amino acid residues was obtained at 1.5-Å resolution (7). This study confirmed the proposed hexameric structure of the enzyme (6) and revealed the active site as a deeply buried hydrophobic pocket with three acidic residues, Asp^{176}, Glu^{196}, and Asp^{204}. The latter two of these residues were predicted to catalyze proton transfers at carbons 2 and 6, respectively, of the 3,5-dienoyl-CoA substrate (6). Surprisingly Δ^{3,5,7},Δ^{2,4,6}-triene isomerase (triene-CoA isomerase), an enzyme involved in the degradation of unsaturated fatty acids with conjugated double bonds, was found to be a component enzyme of dienoyl-CoA isomerase (8).

The association of dienoyl-CoA isomerase and trienoyl-CoA isomerase with the same protein prompted this study aimed at elucidating the mechanisms of action of these enzymes. This goal necessitated the characterization of the highly active, mature forms of these enzymes present in mitochondria and peroxisomes.

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2 The abbreviations used are: dienoyl-CoA isomerase or 3,5–2,4-dienoyl-CoA isomerase, Δ^{3,5,7},Δ^{2,4,6}-dienoyl-CoA isomerase; enoyl-CoA isomerase, Δ^{3,5,7},Δ^{2,4,6}-enoyl-CoA isomerase; CD, circular dichroism; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; trienoyl-CoA isomerase, Δ^{3,5,7},Δ^{2,4,6}-triene isomerase.
**EXPERIMENTAL PROCEDURES**

**Materials—**A PCR Advantage kit and a Transformer site-directed mutagenesis kit were purchased from CLONTECH. PGEM-T Easy was obtained from Promega. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. Restriction endonucleases, T4 polynucleotide kinase, T4 ligase, and T4 DNA polymerase were supplied by New England BioLabs. Nycodenz, CAAH, NADPH, Polybuffer Exchanger 94, and Polybuffer 94 were obtained from Pharmacia and Upjohn, respectively. C. elegans and Drosophila melanogaster were obtained from the Center for Biological Research, National Institutes of Health. Most standard biochemicals were purchased from Sigma Chemical Co.

**Hydroxyapatite Column—** The column was equilibrated with buffer A. The column was washed with buffer A and was applied to a hydroxyapatite column (1.5 × 22 cm) previously equilibrated with buffer A. The column was washed with buffer A containing 0.5 mM KCl and then was developed with a gradient made up of 160 ml of buffer A and 160 ml of buffer B containing 0.8 mM potassium Pi (pH 6.3). Fractions of 3 ml each were collected, and the fractions containing the dienoyl-CoA isomerase activity were combined and concentrated in an Amicon concentrator with a YM-10 membrane. After dialysis overnight against 25 mM ethanolamine-acetic acid (pH 9.4) containing 5 mM mercaptoethanol, 0.5 mM EDTA, 0.5 mM PMFS, and 20% glycerol (buffer B), the sample was applied to a chromatofocusing column (1 × 15 cm) containing Polybuffer Exchanger 94 equilibrated with buffer B. The column was extensively washed with buffer B and then developed with 12 column volumes of Polybuffer 96 adjusted to pH 6.0 with acetic acid. Fractions of 3 ml each were collected and assayed for dienoyl-CoA isomerase. The active fractions were combined and concentrated with a Millipore centrifugal filter device.

**SDS-PAGE and Immunoblotting—** Aliquots of purified dienoyl-CoA isomerase were treated with SDS sample buffer and subjected to SDS-PAGE on gradient (4–20%) gels (12). Proteins were transferred to a polyvinylidene difluoride membrane by semi-dry blotting (13), and proteins remaining on the gel were visualized by staining with Coomassie Blue. The membrane was incubated for 1 h with a 500-fold-diluted rabbit antiserum or with monospecific antibodies (1 μg/ml) prepared from the serum raised against rat liver dienoyl-CoA isomerase. After incubating the membrane with goat anti-rabbit IgG conjugated with alkaline phosphatase, it was developed with a staining mixture containing the alkaline phosphatase substrate until the antigen bands were visualized (14).

**Analysis of Protein Sequence—** N-terminal amino acid sequencing was performed by Stephen Bobin at the Dartmouth College Molecular Biology Core Facility. The N-terminal sequence of the full-length dienoyl-CoA isomerase was analyzed with the program HelicalWheel to draw a helical wheel as described previously (15).

**Cloning and Expression of Dienoyl-CoA Isomerase—** Rat heart Marathan-Ready cDNA (CLONTECH) was used as the template for cloning the cDNA of the full-length dienoyl-CoA isomerase by touch-down PCR according to the protocol of CLONTECH. The primers were 5'-CAGATGCTCCATATGGCTACCGCGATGACAGTTTCCA-3’ and 5'-CGATAGCTTATCGAGGCTTGGAAGAGGTGATGC-3’.

**Site-directed Mutagenesis of Dienoyl-CoA Isomerase—** Site-directed mutagenesis was carried out by use of a Transformer site-directed mutagenesis kit (CLONTECH) following the manufacturer’s instructions. A synthetic oligonucleotide, 5’-ATGCCTTCAATAGATGTTAAGAAAAGGA-3’, designed to eliminate a SspI site, was used as the selection primer. The following synthetic oligonucleotides were used as the mutagenic primers.

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was transformed into *E. coli* BL21(DE3)pLysS, and the desired mutant was selected for the absence of the *Streptococcus* restriction site. The point mutation was confirmed by sequencing of the respective mutant strain. Expression of the mutant enzymes was achieved by the procedure used for expressing the wild-type dienoyl-CoA isomerase.

**Purification of Recombinant Wild-type and Mutant Dienoyl-CoA Isomerase**—The frozen pellet from ~350 ml of cell culture was suspended in 10 ml of 10 mM potassium *Pi* (pH 8.8) containing 5 mM mercaptoethanol, 1 mM EDTA, 1 mM benzamidine, and 0.5 mM PMSF (buffer A) and sonicated 12 times for 20 s each. The resultant suspension was centrifuged at 100,000×*g* for 30 min. The supernatant was loaded onto a Q-Sepharose column (1.5×4 cm) previously equilibrated with buffer A. The column was extensively washed with buffer A and then eluted with a gradient made up of 120 ml of buffer A and 120 ml of buffer A containing 0.4 M KCl. The active fractions were combined and concentrated in an Amicon concentrator with a PX-10 membrane. The concentrate was diluted 10-fold with 10 mM potassium *Pi* (pH 6.0) containing 1 mM EDTA, 5 mM mercaptoethanol, and 20% glycerol (buffer B) and applied to an S-Sepharose column (1.5×4 cm) previously equilibrated with buffer B. After washing extensively with buffer B, the column was developed with a gradient made up of 30 ml of buffer B and 30 ml of buffer B containing 0.4 M KCl. The active fractions were combined and concentrated.

**CD Spectra of Wild-type and Mutant Dienoyl-CoA Isomerases**—Far-UV CD scans were acquired between 190 and 250 nm with an AVantes spectrophotometer equipped with temperature control. Two average scans were acquired at 20 °C for each sample. The scans were normalized for protein concentration and corrected for the influence of the buffer.

**RESULTS**

**Molecular Characterization of the Mitochondrial and Peroxisomal Forms of Dienoyl-CoA Isomerase**—For a planned mechanistic study of rat dienoyl-CoA isomerase, milligram quantities of highly active enzyme were required. Although a recombinant form of this enzyme, lacking its 53 N-terminal amino acid residues, has been described (6), its activity was much lower than that of the native enzyme and too low for the contemplated mechanistic study. Attempts to express the full-length isomerase-cDNA were unsuccessful. Hence, we embarked on the molecular characterization of the native mitochondrial and peroxisomal dienoyl-CoA isomerases with the aim of producing a recombinant form of the highly active mature enzyme.

N-terminal sequencing of the purified rat liver dienoyl-CoA isomerase revealed the presence of several polypeptides in agreement with the observation of at least three closely spaced bands when the same preparation was subjected to SDS-PAGE (Fig. 1A, lanes 2 and 5). Because this preparation may have been a mixture of the mitochondrial and peroxisomal forms of dienoyl-CoA isomerase, the enzyme was also purified from rat hearts, which contain few peroxisomes. SDS-PAGE and immunoblotting of the heart preparation led to the identification of the dienoyl-CoA isomerase, which seemed to be slightly larger than the liver forms of the enzyme (Fig. 1A, A and B, lanes 2 and 3). When the heart dienoyl-CoA isomerase was subjected to N-terminal sequencing, a unique sequence was obtained for the first 20 residues (Fig. 2A). The sequence, beginning with 3 serine residues, perfectly matched the predicted amino acid sequence of rat liver dienoyl-CoA isomerase (6) from residue 35 through residue 54. The missing residues 1 through 34 constitute a polypeptide that has the properties of a mitochondrial targeting sequence (Fig. 2B). This conclusion is supported by the following properties of this polypeptide: it is rich in positively charged and hydroxylated residues (3 arginine, 1 lysine, 4 serine, and 3 threonine residues), and it is devoid of acidic residues and does not have a large stretch of uncharged residues (Fig. 2B). Moreover, its N terminus forms a positively charged amphiphilic helix (Fig. 2B). The mitochondrial precur

sor protein seems to have only one cleavage site. Another potential cleavage site that is indicated by a hydrophilic residue and a serine at positions 27 and 30, respectively, is not susceptible to proteolysis, due to the absence of an arginine residue from position 25.

In an effort to characterize the peroxisomal dienoyl-CoA isomerase, the enzyme was isolated from purified rat liver peroxisomes. A better than 80-fold purification was achieved by chromatography on hydroxylapatite followed by chromatofocusing. The resultant preparation was composed of at least three proteins (Fig. 1A, lane 4). The major component with a molecular mass of 32 kDa was identified by immunoblotting as dienoyl-CoA isomerase (Fig. 1B, lane 4). N-terminal sequencing of the material corresponding to the 32-kDa band revealed the presence of several forms of dienoyl-CoA isomerase with different N termini. The N termini of the two most prominent polypeptides were located to positions 37 and 39 of the full-length protein (Fig. 2A). In an attempt to determine whether the peroxisomal dienoyl-CoA isomerase exists in vivo as a truncated protein, purified peroxisomes were incubated with boiling SDS incubation buffer and subjected to SDS-PAGE followed by immunoblotting with antibodies purified by affinity chromatography on a dienoyl-CoA isomerase-Sepharose column. As shown in Fig. 1C, only one band corresponding to a 32-kDa protein was observed. Thus, it seems that the native peroxisomal dienoyl-CoA isomerase is a truncated protein with a ragged N terminus.

**Mechanistic Study of Dienoyl-CoA Isomerase by Site-specific Mutagenesis**—The cDNA coding for dienoyl-CoA isomerase was cloned from a rat liver cDNA library by PCR. However, attempts to express the full-length protein in *E. coli* were unsuccessful. We therefore generated the cDNA for the mature rat heart isomerase from the full-length cDNA by PCR. This mature mitochondrial form of dienoyl-CoA isomerase was successfully expressed in *E. coli*. A 10-fold purification of dienoyl-CoA isomerase, beginning with a soluble extract of such cells, yielded the purified enzyme in 65% yield. This enzyme preparation exhibited an activity of 960 units/mg (Table I), which is significantly higher than the activity of the enzyme isolated from rat liver (3). The fact that the recombinant dienoyl-CoA isomerase also exhibited trienoyl-CoA isomerase activity, proved that both catalytic properties are associated with the same protein.

For the planned mechanistic study, mutant proteins were

![Fig. 1. SDS-PAGE and immunoblotting of rat dienoyl-CoA isomerases (DI), (A) after SDS-PAGE and staining with Coomassie Brilliant Blue: Molecular mass standards (lane 1), rat DI purified from liver (lanes 2 and 5), partially purified DI from rat heart (lane 3), and partially purified DI from rat liver peroxisomes (lane 4). B, immunoblot of the above DI's with antisemur to DI. C, immunoblot of partially purified DI from rat liver peroxisomes (lane 6) and of purified rat liver peroxisomes (lanes 7 and 8) using purified monospecific antibodies to DI.](image-url)
all these data indicates that the active site of dienoyl-CoA isomerase is identical with the active site of trienoyl-CoA isomerase and that the same acidic residues, Glu196 and Asp204, catalyze the proton transfers that result in the 3,5→2,4 and 3,5,7→2,4,6 isomerizations.

In an effort to further explore the mechanism of dienoyl-CoA/trienoyl-CoA isomerase, its activity with 2-trans,5-cis-octadienoyl-CoA as a substrate was evaluated. With the wild-type enzyme, a small but significant conversion of the 2,5 diene to the 2,4 isomer was observed (Table I). The rate of the 2,5→2,4 conversion was more than 10^4 times slower than the 3,5→2,4 isomerization. Surprisingly, mutants of Glu196 were more active than the wild-type enzyme in catalyzing this reaction (Table I). The E196Q mutant, which was 10 times as active as the wild-type isomerase, permitted a spectroscopic analysis of the 2,5→2,4 isomerization. The time-dependent spectral changes shown in Fig. 3A are indicative of a direct 2,5→2,4 isomerization rather than a sequential 2,5→3,5→2,4 conversion. These spectra do not provide evidence for the formation of a 3,5 intermediate with an absorbance maximum at 238 nm nor do they reveal a 3,5→2,4 isomerization as shown in Fig. 3A. Because the 2,5→2,4 conversion catalyzed by the E196Q mutant was 60 times faster than the 3,5→2,4 isomerization catalyzed by the same enzyme (Table I), the 2,5→2,4 isomerization seems to be a one-step conversion.

The Asp204 mutants also catalyzed a slow but detectable 2,5→2,4 isomerization. However, as shown in Fig. 4C, the formation of the 2,4 isomer proceeded with a lag. Spectral analyses of the reactions that occurred during (Fig. 4C, period A) and after the lag phase (Fig. 4C, period B) revealed an initial 2,5→3,5 isomerization indicated by an increase in the absorbance at 238 nm due to the formation of the 3,5 diene (Fig. 4A) followed by the formation of the 2,4 isomer detected at 300 nm (Fig. 4B). Overall, the product formation occurred by a 2,5→3,5→2,4 conversion that showed a pronounced lag in the formation of the 2,4 isomer, because the first reaction proceeded faster than the second reaction (Table I). Because the D204A mutant catalyzed the 2,5→3,5 conversion, it was expected to catalyze also the isomerization of 3-octenoyl-CoA to 2-octenoyl-CoA. This conversion was in fact observed and found to take place at a rate of 0.034 unit/mg as compared with 0.4 unit/mg for the 2,5→3,5 isomerization.

The question of whether the differences between the reaction rates observed with various mutants and substrates were due to changes in K_m values, V_max values, or both were addressed. The kinetic parameters listed in Table II clearly show that the K_m values varied little and that differences between V_max values were the major cause of rate differences observed at fixed substrate concentrations of 20 μM.

**DISCUSSION**

The main reason for the molecular characterization of the mature dienoyl-CoA isomerases was the need to produce a highly active recombinant form of this enzyme. Such enzyme had not been obtained when recombinant versions of the full-length protein and of an artificially truncated isomerase were generated (6). However, this study was also prompted by a desire to demonstrate unambiguously the dual location of this enzyme in mitochondria and peroxisomes. The presence of the same dienoyl-CoA isomerase in both organelles had been suspected when antibodies raised against the mitochondrial enzyme were found to cross-react with the peroxisomal isomerase (5). Cloning of dienoyl-CoA isomerase had revealed the presence of a peroxisomal targeting signal and an N terminus with properties of mitochondrial targeting sequence. Moreover, antibodies raised against a synthetic peptide corresponding to the C terminus of the enzyme recognized 32-kDa and 36-kDa pro-


Table I

| Mutant | 3,5→2,4 Specific | Relative | 3,5,7→2,4,6 Specific | Relative | 2,5→2,4 Specific | Relative | 2,5→3,5 Specific |
|--------|-----------------|----------|---------------------|----------|-----------------|----------|-----------------|
| WT     | 960             | 1        | 21                  |          | 0.037           | 1        | 0.037           |
| D176A  | 98              | 0.1      | 0.033               | 1        | 0.056           | 1.5      | 0.056           |
| E196D  | 32              | 3.3 × 10^{-2} | 0.12          | 5.7 × 10^{-3} | 0.11          | 3        | 0.11          |
| E196Q  | 0.0065          | 6.8 × 10^{-6} | 0.0002        | 9.6 × 10^{-6} | 0.41          | 11       | 0.41          |
| D204A  | 0.018           | 1.9 × 10^{-5} | 0.0002      | 9.6 × 10^{-6} | 0.0054        | 0.15     | 0.0054        |
| D204N  | 0.0038          | 4.0 × 10^{-6} | 0.0003      | 1.4 × 10^{-5} | 0.0007        | 0.019    | 0.0007        |

*a Specific activities in μmol/min/mg of protein were obtained with 20 μM substrate as described under "Experimental Procedures."

Figure 3: Spectrophotometric analysis of the isomerization of 3,5-octadienoyl-CoA and 2,5-octadienoyl-CoA catalyzed by dienoyl-CoA isomerase. A, spectral changes associated with the isomerization of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA (3,5→2,4) catalyzed by wild-type dienoyl-CoA isomerase. Spectrum 1 at time 0; spectra 2–4 were recorded 20 s, 1.5 min, and 10 min after the addition of enzyme. B, spectral changes associated with the isomerization of 2,5-octadienoyl-CoA to 2,4-octadienoyl-CoA (2,5→2,4) catalyzed by the E196Q mutant of dienoyl-CoA isomerase. Spectrum 1 at time 0; spectra 2–4 were recorded 20 s, 1.5 min, and 20 min after the addition of enzyme.

Figure 4: Spectrophotometric analysis of the isomerization of 2,5-octadienoyl-CoA to 2,4-octadienoyl-CoA catalyzed by the D204A mutant of dienoyl-CoA isomerase. Spectral changes observed during (A) the initial phase of the isomerization reaction (2,5→3,5); (B) the later phase of the isomerization reaction (3,5→2,4); and (C) absorbance change at 300 nm observed during the isomerization of 2,5-octadienoyl-CoA to 2,4-octadienoyl-CoA catalyzed by the D204A mutant as a function of time.

Table II

| Mutant | 3,5→2,4 | 3,5,7→2,4,6 | 2,5→2,4 |
|--------|---------|-------------|---------|
| WT     | 30      | 2450        | 13      |
| D176A  | 21      | 278         | 48      |
| E196D  | 21      | 51          | 1.2     |

*a K_m and V_max values are given in μM and μmol/min/mg of protein, respectively.

The successful expression of the mature cardiac dienoyl-CoA isomerase in E. coli achieved two goals. Foremost, a highly active form of this enzyme became available. In fact, the maximal specific activity of 2450 units/mg for the recombinant isomerase was 6 times higher than the V_max of the enzyme isolated from rat liver. Although this difference may be due in part to the use of HPLC-purified substrate in this study, it also reflects the preparation of a purer enzyme. In any case, the recombinant enzyme exhibited an activity well suited for the planned mechanistic study. The second achievement was the planned mechanistic study. The second achievement was the demonstration that both dienoyl-CoA isomerase and trienoyl-CoA isomerase are associated with the same protein. This result puts to rest any existing suspicion that the two activities may be expressions of distinct but similar proteins.

The successful creation and purification of several mutant forms of dienoyl-CoA isomerase permitted an analysis of its catalytic mechanism. Dramatic activity decreases were observed as the result of replacing Asp204 and Glu196 with neutral amino acids. This finding supports the proposed function of proteins in mitochondria and peroxisomes, respectively (6).

This study, besides revealing the N termini of the mature forms of dienoyl-CoA isomerase, confirms the dual localization of the enzyme to mitochondria and peroxisomes. However, in contrast to a previous report (6), the peroxisomal dienoyl-CoA isomerase was found to have a molecular mass of 32 kDa and hence to be a truncated form of the full-length protein. The removal of N-terminal sequences similar in size to the mitochondrial targeting sequence could be a consequence of the susceptibility of this region of the protein to proteolysis. This idea has not been tested nor have other explanations been ruled out. However, the detection of only one band corresponding to a 32-kDa protein when intact peroxisomes were solubilized with boiling SDS incubation buffer and then subjected to SDS-PAGE and immunoblotting, supports the conclusion that the truncated form(s) of the peroxisomal dienoyl-CoA isomerase is(are) present in vivo and are not artifacts of the isolation procedure. This study additionally demonstrates that the heart and liver enzymes are identical over a stretch of 20 amino acid residues. Hence, both enzymes are most likely products of the same gene.

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The successful creation and purification of several mutant forms of dienoyl-CoA isomerase permitted an analysis of its catalytic mechanism. Dramatic activity decreases were observed as the result of replacing Asp204 and Glu196 with neutral amino acids. This finding supports the proposed function of...
these residues in proton transfers from and to the substrate (7), because it agrees with the general prediction that the mutation of a residue that directly participates in a reaction as a general acid/base would be expected to cause a 10^5 or greater decrease in activity (17).

The use of 2,5-octadienoyl-CoA as a substrate analog revealed slow, but measurable 2,5→2,4 isomerizations. Because the different positional isomers of octadienoyl-CoA have distinct UV spectra, it was possible to analyze the mechanisms of these isomerizations. The spectral changes observed with mutant E196Q were suggestive of a direct 2,5→2,4 isomerization without the formation of an intermediate. The only alternative route, via a sequence of isomerizations with 3,5-octadienoyl-CoA as an intermediate, was ruled out because the 3,5→2,4 isomerization was much slower than the overall 2,5→2,4 isomerization. Hence the observed 2,5→2,4 isomerization must be the result of a 5→4 double-bond shift as shown in Fig. 5. Asp^204 is the obvious candidate to facilitate this monoene isomerization by catalyzing a 1,3-proton shift from carbon 4 to carbon 6. Such mechanism for single double-bond isomerizations has been proposed for cholesterol oxidase (18) and Δ^5,Δ^2-enoyl-CoA isomerase (19) based on observed intramolecular 1,3-hydrogen shifts. If mechanistically similar, the 1,3-proton shift catalyzed by Asp^204 may not be concerted, as shown in Fig. 5, but rather proceed in two steps by removal of a proton from carbon 4 and formation of a stabilized carbocation followed by addition of a proton to carbon 6. Noteworthy is the observation that the 2,5→2,4 isomerization catalyzed by mutant E196Q proceeded 11 times faster than the same reaction catalyzed by the wild-type isomerase. The lower rate detected with the wild-type enzyme may be due to a higher pK value of Asp^204 induced by Glu^196. Such electrostatic effect on Asp^204 would not be effective in the E196Q mutant.

The 2,5→2,4 isomerization catalyzed by mutant D204A was more complex than the conversion brought about by the E196Q mutant. The progress curve for the D204A-catalyzed 2,5→2,4 isomerization showed a lag that was shown to correspond to the conversion of 2,5-octadienoyl-CoA to its 3,5 isomer. The lag phase for the subsequent 3,5→2,4 isomerization, the 3,5 intermediate accumulated and initially was detectable. The formation of 3,5-octadienoyl-CoA was the result of a double-bond shift from carbon 2 to carbon 3. This double-bond isomerization must have been catalyzed by Glu^196, which is proposed to facilitate a 1,3-proton shift from carbon 4 to carbon 2 (Fig. 5). Again, the proton transfers may not be concerted as shown in Fig. 5 but may be sequential, resulting in the formation of a carbanion intermediate. An alternative route with a carbocationic intermediate represents an unlikely mechanism.

The analyses of the 2,5→2,4 isomerizations provide good evidence for Glu^196 being close to carbon 2 and Asp^204 close to carbon 6 as shown in Fig. 6. Both residues are necessary for the 3,5→2,4 isomerization that proceeds by a simultaneous shift of both double bonds (3). A similar mechanism is envisioned for the triene isomerization except that Asp^204 must be close to carbon 8 (Fig. 6). Such a dual role of Asp^204 suggests a certain flexibility of the residue and/or requires different positioning of the dienoyl-CoA and trienoyl-CoA substrates at the active site. Either way, the function of Asp^204 in the isomerization of the triene comes at a price that is reflected by the almost 50-fold lower activity of trienoyl-CoA isomerase as compared with dienoyl-CoA isomerase.

Altogether, this study demonstrates the need for two acidic residues to facilitate the proton transfers that result in positional isomerizations of dienes and trienes. In contrast, proton transfers that cause monoenes to shift by one carbon only require a single acidic residue as previously documented for other isomerases (18, 19).

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