Enzyme-Catalyzed Allylic Rearrangements

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Received May 17, 1990 (Revised Manuscript Received July 27, 1990)

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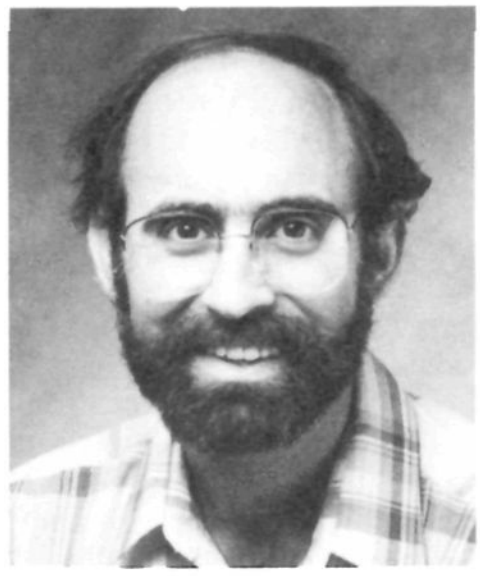
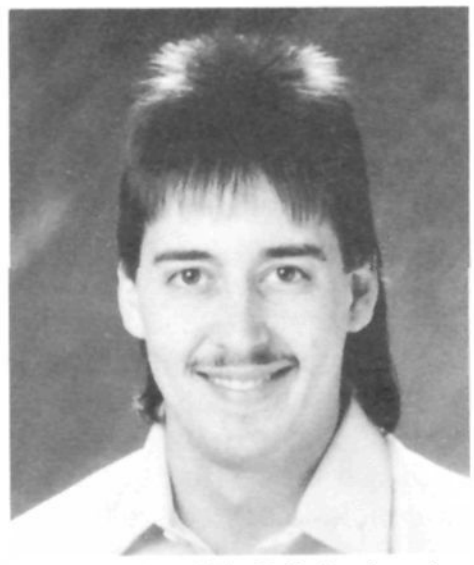


John M. Schwab majored in chemistry at the University of Michigan, from which he graduated in 1969 with a B.S. degree. While at Michigan he engaged in undergraduate research in Mark M. Green's laboratory, where his interest in stereochemistry and isotopic labeling was kindled. Dr. Schwab was a graduate student at Brandeis University in the Department of Chemistry (under the guidance of Ronald J. Parry), and he received his Ph.D. in 1976. He then spent 18 months in Konrad Bloch's laboratory at Harvard, as an NIH postdoctoral fellow. After 7 years at the Catholic University of America, Dr. Schwab moved to Purdue, where he currently holds the rank of Associate Professor of Medicinal Chemistry. His research interests include enzymatic reaction mechanisms, organic stereochemistry, and secondary metabolism.



Barry S. Henderson received his B.S. in chemistry in 1986 from the University of Tennessee (Chattanooga). He is currently a graduate student in the Purdue University Biochemistry (PUB) program, where he is working toward completion of his thesis research under John Schwab's direction. His project concerns characterization of the active site and the catalytic mechanism of *E. coli* β-hydroxydecanoyl thiol ester dehydrase.

I. Introduction

Allylic rearrangements are extremely common in biological settings. The chorismate mutase reaction, pyridoxal phosphate dependent transaminations, and even keto/enol tautomerizations such as those catalyzed by triose phosphate isomerase or phosphoglucose isomerase are allylic rearrangements. This review, however, deals only with the subset of enzyme-catalyzed allylic rearrangements that involve a net 1,3-hydrogen shift within a nonheteroatomic allylic system, as illustrated by the general equation below. While this equation

might imply a concerted, [1,3] sigmatropic hydrogen migration, conservation of orbital symmetry predicts that such a mechanism is only accessible photochemically,[1] and it apparently does not occur in nature.

Enzyme-catalyzed allylic rearrangements play central roles in important biochemical pathways, including the biosynthesis of terpenoids in general and of steroid hormones in particular, and in biodegradation. Mechanistically, these reactions are noteworthy due to their simplicity. They are single-substrate reactions, and in many cases, there are no cofactor requirements. For this reason, allylic rearrangements provide a convenient paradigm for studying the relationship between enzymes' structures and their catalytic mechanisms.

There have been no comprehensive reviews of enzymatic allylic rearrangements, although a brief review on the topic has appeared,[2] and the literature on some of the enzymes described herein has been reviewed separately. Most notably, reviews dealing with Δ^5-3-ketosteroid isomerase, β-hydroxydecanoyl thiol ester dehydrase, and isopentenyl pyrophosphate isomerase are available, as mentioned in the appropriate sections.

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This article deals with the literature up to the spring of 1990.

In describing various examples of enzyme inactivation, kinetic constants are sometimes cited. Lucid explanations of these terms can be found on pages 12–16 of the first volume of Silverman's monograph on mechanism-based enzyme inactivation.[3]

In brief, our use of kinetic constants for describing irreversible enzyme inactivation is described by eq 1.

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{inact}} E^* \qquad (1)$$

Additionally, K_I is the concentration of an irreversible enzyme inactivator that produces half of the maximal rate of inactivation, and K_i is the concentration of a reversible enzyme inhibitor that leads to half of the maximal rate of substrate turnover.

II. Relatively Acidic Substrates

The substrates for enzymatic allylic rearrangements can be considered in two general groups: (1) those from which resonance-stabilized carbanions could be formed (whether or not these carbanions are actual enzymatic reaction intermediates) and (2) those with isolated allylic systems. (An alternative, more inclusive system of classification has also been described.[4])

A. Δ^5-3-Ketosteroid Isomerase

Δ^5-3-Ketosteroid isomerase (KSI) is associated with both biodegradation (in bacteria) and the biosynthesis of steroid hormones (in mammals). In each setting, the isomerase shifts a double bond from the 5(6)-position of a 3-oxosteroid to the 4(5)-position, as illustrated below. The equilibrium favors the thermodynamically more stable conjugated enone.

At the time of the most recent comprehensive review article on KSI,[5] the vast majority of mechanistic research had been carried out on the soluble, biodegradative KSI from *Pseudomonas testosteroni*. While the proportion of data on mammalian KSIs (which are generally membrane-bound) has increased in the intervening 14 years, papers concerning KSI from *P. testosteroni* still dominate the literature. Important insights concerning the structure and the mechanism of action of the bacterial isomerase have resulted from chemical modification, X-ray crystallography, magnetic resonance, and site-directed mutagenesis.

In light of earlier reviews,[5-9] no attempt will be made to discuss in detail the literature from years prior to 1976, although key findings will be summarized as appropriate.

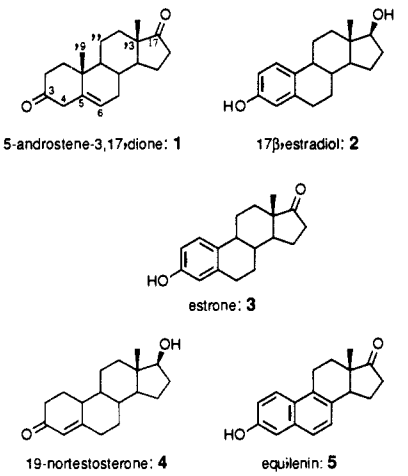
1. Pseudomonas testosteroni KSI

An inducible enzyme, KSI is available in fairly substantial quantities from wild-type *P. testosteroni*.[10] A straightforward purification scheme[10,11] (subsequently improved by the addition of an affinity chromatography step[12]) affords homogeneous enzyme that is amenable to crystallization.[13-16]

In its native state, KSI is a homodimeric enzyme[17-19] comprising 125 amino acid residues,[20] with a protomer molecular mass of 13 399 (as deduced from the nucleotide sequence of the gene).[21-23] KSI is unusually rich in clusters of hydrophobic amino acid residues,[20] a fact that is consistent with both the nonpolar nature of its substrates and its tendency to undergo concentration-dependent aggregation.[18,19]

a. Substrate Binding. *P. testosteroni* KSI accepts a wide variety of steroid substrates and inhibitors. Some special classes of enzyme-inactivating substrate analogues will be discussed in the following sections.

In evaluating structural features critical to substrate recognition, Weintraub et al. tested a large number of compounds, including both steroids and synthetic steroid analogues, as inhibitors of the KSI-catalyzed isomerization of 5-androstene-3,17-dione (1).[24] In the A ring, a carbonyl or hydroxyl group at C-3 appears necessary (although other electronegative substituents were not tested), and relatively small substituents at positions 1 and 2 are acceptable. C-11 cannot be substituted. In the D ring, bulky substituents in the 17β-position do not seem to affect binding, but 17α-substituents are acceptable only if ring A is aromatic, and not in Δ^4-3-oxosteroids. Methyl or ethyl groups at C-13 of Δ^4-3-oxosteroids enhance binding to KSI; this effect, however, is substantially reduced in the relatively more planar $\Delta^{4,9}$- or $\Delta^{4,9,11}$-17α-substituted derivatives. Surprisingly, (−)-17β-estradiol (2) and its enantiomer are almost equally effective as enzyme inhibitors. Anomalous results were also observed for the binding of 17β-estradiol and estrone (3).



On the basis of these data, a crude topological model for the enzyme's active site was proposed. A critical assumption was that the A rings of Δ^4-3-oxo- and phenolic steroids (e.g., testosterone and estradiol) should be superimposable, since positions 3–6 of substrate molecules must be fixed relative to the enzyme's catalytic acid–base groups. When the A rings are optimally superimposed, it is found that the C and D rings are displaced from one another by 3–4 Å. It should be noted, however, that the A-ring binding orientation is only critical for substrates, and not necessarily for inhibitors. Furthermore, in light of the subsequent finding that KSI binds certain substrate analogues in a "backward" mode (i.e., with A- and D-ring positions interchanged; see below),[25,26] the observations of Weintraub et al.[24] probably need to be reevaluated.

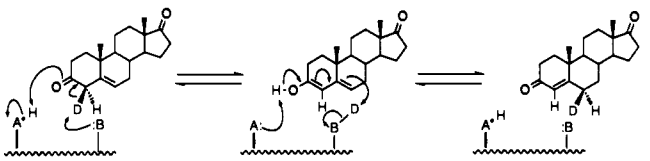
Hopefully, with further refinement of the present 6 Å crystal structure of KSI,[27] the actual nature of enzyme-substrate and enzyme-inactivator interactions will become clear.

Three research groups have determined the number of active sites per KSI dimer,[28-30] with conflicting results. Vincent et al. conducted fluorescence titration studies with 19-nortestosterone (4) and equilenin (5), as well as equilibrium dialysis experiments with radiolabeled 19-nortestosterone and 17β-estradiol (2) (all three of which are competitive inhibitors).[28] They concluded that KSI exhibits "half-of-the-sites" reactivity and proposed a "flip-flop" mechanism for the isomerization process. On the other hand, photoinactivation experiments by Benisek and co-workers (see below) resulted in the modification of approximately one active site per protomer.[31,32] Furthermore, both titration of the UV chromophore of 19-nortestosterone with KSI as well as titration of the fluorescence chromophore of the enzyme with 19-nortestosterone have implicated two steroid binding sites per dimer (after the molecular mass of the enzyme and the molar extinction coefficient had been adjusted to their correct values).[33] Ogez and Benisek repeated the equilibrium dialysis experiments,[30] using three competitive inhibitors (one of which had been employed by Vincent et al.[28]). Scatchard plots of the data clearly showed the formation of inhibitor/enzyme–dimer complexes with 2:1 stoichiometries, in contrast to the earlier finding. The latter results were corroborated by Penning et al.,[29] who (1) repeated and extended the UV and fluorescence titration experiments, (2) quantified the stoichiometry of mechanism-based inactivation of KSI by a tritium-labeled acetylenic secosteroid (see below), and (3) determined the amount of tritiated progesterone that becomes bound to the enzymatically active, crystalline KSI (the protein concentration of which is accurately known).[29,34] All of the results obtained by Penning et al. support the existence of one independent steroid binding site per KSI protomer. The reasons for the discrepancy between the results of Vincent et al., on the one hand, and those of Benisek and Penning and their respective co-workers, on the other, remain unclear.

b. Early Mechanistic Studies. P. testosteroni KSI is one of the most active enzymes known, with a turnover number for 5-androstene-3,17-dione (1) of 8.4×10^6 min^{-1} per enzyme dimer.[35] It also has a broad substrate specificity, acting on a variety of $\Delta^{5(6)}$- and $\Delta^{5(10)}$-3-oxosteroids. Kinetic parameters for a number of these substrates have been reported.[36] The equilibrium constant for the isomerization, which can be calculated from substrate and product pK_a values, strongly favors the conjugated enone. For the equilibration of 5-androstene-3,17-dione (1) with 4-androstene-3,17-dione (cf. **6** ⇄ **7**), $K_{eq} = 2400$.[37]

Malhotra and Ringold investigated the mechanisms of both the acid-catalyzed nonenzymatic isomerization, as well as the KSI-catalyzed isomerization of β,γ-unsaturated 3-oxosteroids.[36] On the basis of substrate and solvent isotope effects it was suggested that the dienol derivative of the substrate is the key intermediate in the non-enzyme-catalyzed reaction and that removal of a proton from C-4 of the Δ^5-3-oxosteroid is the rate-determining step. By using a series of Δ^5-3-oxosteroids stereoselectively mono- and dideuterated at C-4 and by

SCHEME 1



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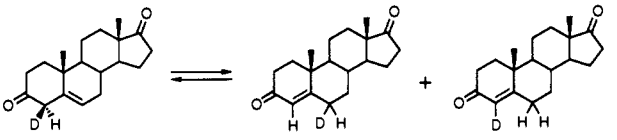
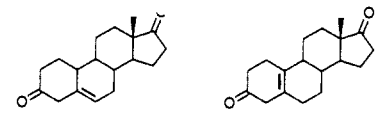


6 7 8

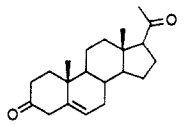
analyzing the configurations of the deuterium atoms at C-6 of the Δ^4-3-oxosteroid products, the enzyme-catalyzed isomerization was determined to be stereospecific, involving the axial 4β- and 6β-hydrogens. Deuterium crossover experiments showed that hydrogen transfer is intramolecular, although a small degree of exchange with the medium has been observed.[33,38,39] These facts, along with a substantial 4β-deuterium isotope effect on V_{max}[38] and a pH rate profile that is flat between pH 6 and pH 9,[33] implicated two ionizable functional groups in the catalytic mechanism. It was suggested that either tyrosine or lysine, and either aspartate or glutamate might function as the acidic (A–H) and basic (B:) groups, respectively (see Scheme 1).[33,38] As discussed later in this review, these have proven to be excellent predictions.

More recently, some of Malhotra and Ringold's findings have been reinvestigated by Marquet and her co-workers.[40,41] It was the latter group's original intent to assess the extent of exchange versus intramolecular hydrogen transfer for a variety of KSI substrates, which might in turn implicate differences in enzyme-substrate interactions. When they repeated the KSI-mediated isomerization of 5-[4β-$^2H_1$]androstene-3,17-dione (**6**), mass spectral analysis showed the formation of three product isotopomers (Scheme 2): product with deuterium at C-6 (**7**; ca. 50%), unlabeled product (ca. 25%; reflecting exchange of deuterium into the aqueous medium), and, surprisingly, product with deuterium only in the vinylic position at C-4 (**8**; ca. 25%). When unlabeled 5-androstene-3,17-dione was incubated with KSI in D_2O, deuterium was again found at both positions 4 and 6 in the product. Qualitatively similar results were obtained with two other KSI substrates, 5-estrene-3,17-dione (**9**) and 5(10)-estrene-3,17-dione (**10**). Rearrangement of 5-[4α-$^2H_1$]androstene-3,17-dione only gave product deuterated at C-4; none of the deuterium was transferred to C-6. In contrast to the results obtained with **6**, **9**, and **10**, 5-pregnene-3,20-dione (**11**) suffered exchange of *only* the 4β- and 6β-protons, with no exchange of the 4α-proton.

To explain their results,[40,41] Marquet et al. proposed a variation on the Malhotra and Ringold mechanism. The key feature of their mechanism is a second active site base, situated proximal to the α-face of the substrate. Abstraction of the 4α-hydrogen by this base, and addition of a solvent-derived proton to the β-face of the resulting enolate (or enol) would give C-4-epimerized substrate. Allylic rearrangement of the latter by the usual mechanism would result in retention of the ori-



5-estrene-3,17-dione: 9 5(10)-estrene-3,17-dione: 10



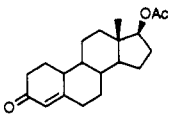
5-pregnene-3,20-dione: 11

ginal 4β-hydrogen. This mechanism may be plausible; however, for removal of the 4α-hydrogen to be stereoelectronically favorable, it would have to shift into an axial position, which would require a substantial change in the overall conformation of the substrate A ring.[42] Inspection of Dreiding models indicates that the carbonyl oxygen would have to move by nearly 1.5 Å. Whether the acidic functional group (A–H, above) would still be suitably disposed to protonate the carbonyl oxygen is unclear. The accessibility of water to the hydrophobic active site environment is also unproven. In fact, a very recent report by Eames et al.[43] indicates that KSI-bound steroid is largely inaccessible to the solvent. Pollack et al. have also commented on the acidities of the C-4 hydrogens, with particular reference to the dihedral angles formed by the π-orbital of the double bond with the bond to the α- or β-hydrogen.[37] They, too, have noted that the 4α-hydrogen should be removed less easily than the 4β-hydrogen. Although the putative second basic group has not been identified, inactivation studies by Robinson and Talalay and their co-workers (section II.A.1.c.ii) have resulted in the covalent modification of an asparagine residue that otherwise has not been directly implicated in the enzyme's mechanism of action.[44-46] Other potential bases may also be present.[47]

Alternatively, "upside-down" binding of substrates (section II.A.1.c.iii) may be responsible for Marquet's results. If this were the case, then the fact that compound 11 does not undergo exchange of the 4α-proton may reflect adverse steric interactions between KSI and the C-17 acetyl side chain when the steroid is bound in an upside-down orientation. Once again, further refinement of the X-ray crystal structure[27] may provide answers to these questions.

c. Chemical Modification. Over the years, many chemical modification experiments have been carried out with the aim of identifying amino acid residues in the vicinity of KSI's active site. Since the publication of the 1976 review on KSI,[5] three different approaches have been particularly successful, affording significant new information.

i. Photoaffinity Labels. Benisek and his co-workers reported in the mid-1970s that KSI is irreversibly inactivated when irradiated at wavelengths over 290 nm in the presence of a Δ^4-3-oxosteroid such as 3-oxo-4-estren-17β-yl acetate (19-nortestosterone acetate; **12**).[31,48,49] The inactivation process is oxygen independent and appears to result in the modification of a single aspartic acid residue. Histidine can also be modified if the photoirradiation is conducted in the presence of O_2; however, the first-order rate constants for modification of aspartic acid and for photoinactivation of KSI are equal and also considerably greater than the first-order rate constant for destruction of histidine. Photoinactivation by **12** is slowed in the presence of the competitive inhibitor 3β-hydroxy-5-androstene-17β-carboxylic acid (**13**), demonstrating that the process is active site directed.



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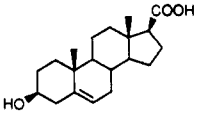


13

Improvement of the conditions used for photoinactivation, as well as the use of a single isozyme of KSI (rather than the mixture of all three that had been used in previous experiments[31,48]) permitted refinement of the prior results.[32] Amino acid analysis and Edman degradation of chymotryptic peptides derived from the photoinactivated enzyme revealed that aspartic acid-38 had been changed to alanine-38. (Interestingly, previous investigations had misidentified residue 38 as asparagine.[20]) The fact that this single, specific modification of KSI led to inactivation of the enzyme suggested that Asp-38 is the active site base or is critical to substrate binding. As discussed below, this conclusion has been supported by several other lines of evidence.

In addition to the D38A "mutant", a minor amount of a photoinactivation product that was characterized by a covalent linkage between KSI and the steroid was obtained.[48,50] The adduct could be separated from the photodecarboxylated enzyme by the use of an immobilized photosensitizer, Δ^6-testosterone–agarose, the photoactive component of which is 4,6-androstadien-3-one.[51-53] Following photoirradiation, any species not covalently linked (including unreacted KSI and photodecarboxylated KSI) were washed away. Chymotrypsin digestion of the covalently linked isomerase followed by gentle hydrolysis of the steroid–agarose bond led to the identification of a modified 25-residue peptide. Edman sequencing of this peptide implicated Asp-38 as the modified amino acid and therefore as the site of covalent attachment of the isomerase to the affinity reagent.[53,54]

The work of Hearne and Benisek[53,54] is significant for several reasons. First, they developed an efficient technique for separation of various classes of enzyme photoinactivation products and for identification of specific sites of reaction. Second, the use of a supported photoaffinity reagent provided important information regarding the location of the catalytic apparatus within KSI's binding "pit" (section II.A.1.d.i). Specifically, since the D ring of the photolabile steroid derivative is attached to the solid support via a bulky, hydrophilic linker, it must be the A ring, and not the D ring, of the steroid that penetrates the enzyme's substrate binding pit most deeply. Since the photoreactive group is the unsaturated ketone moiety in the inactivator's A ring, Asp-38 must be toward the bottom of the pit.

ii. Acetylenic and Allenic Secosteroids. KSI undergoes irreversible inactivation[44,45] by the acetylenic 5,10-secosteroids **14** and **15** (Scheme 3).[56] Inactivation is a pseudo-first-order process, with K_I values of 56 μM and 32 μM, and k_{inact} values of 1.98×10^{-3} and 4.10×10^{-3} s^{-1} for **14** and **15**, respectively.[3,44] The competitive inhibitor 19-nortestosterone (**4**) protects KSI against

SCHEME 3

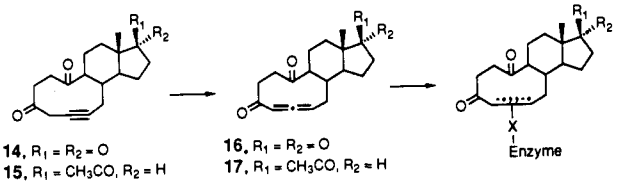


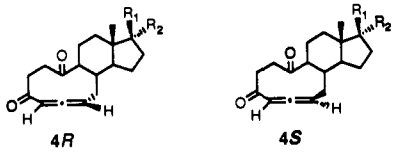
TABLE I. Secosteroid Inactivators of *P. testosteroni* KSI

compd	functional gp	half-life of KSI,[a] s
14	acetylene	564
4*R*-16	allene	540
4*S*-16	allene	660
15	acetylene	152
4*R*-17	allene	168
4*S*-17	allene	333

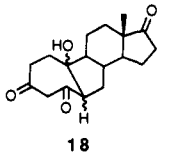
[a] Data taken from ref 58.

inactivation, and one molecule of inactivator binds to each enzyme subunit.[57]

In a manner analogous to the inactivation of *Escherichia coli* β-hydroxydecanoyl thiol ester dehydrase by 3-decynoyl-NAC (section II.B), there is strong evidence that 14 and 15 act as mechanism-based inactivators of KSI, via the corresponding allenic ketones.[58] This hypothesis was evaluated by using the purified, synthetic allenes, made by treatment of each acetylenic ketone with triethylamine. Pairs of allenic secosteroid diastereomers, 4*R*- and 4*S*-16, and 4*R*- and 4*S*-17, were obtained from 14 and 15, respectively. A number of lines of evidence, including protection of KSI by 4 against allene-mediated inactivation, indicated that the inactivation process is active site-directed.[57] The half-lives for the pseudo-first-order inactivation of KSI by the various secosteroids are provided in Table I.

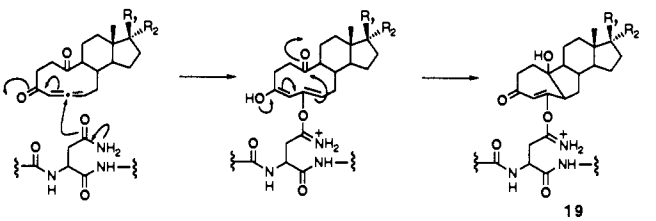


In each series, the 4*R* epimer of the allene inactivates KSI significantly faster than does the 4*S* epimer, and the rates of inactivation by acetylene and 4*R* allene are essentially the same. That the allenes are generated enzymatically was shown by HPLC analysis of mixtures in which KSI was incubated with the acetylenes. In fact, both acetylenes were found to be converted by the enzyme into *mixtures* of 4*R* and 4*S* allene epimers, with the 4*R* predominating in each case. Interestingly, both of the 4*S* allenes were unstable in phosphate buffer, with half-lives on the order of 40 min. Compound 18, the product resulting from hydration and cyclization of 4*S*-16, had no effect on KSI.



The data imply a common rate-determining step for the inactivation of KSI by the acetylenes and the corresponding 4*R* allenes and demonstrate that the 4*R* allenes are kinetically competent to serve as intermediates in the inactivation of KSI by the acetylenic ketones. The 4*S* allenes themselves may or may not inactivate KSI. The equilibrium for the propargylic rearrangement strongly favors the allene; nevertheless, KSI rapidly interconverts the diastereomeric allenic ketones.[59] In light of the faster rate of inactivation of the enzyme by the 4*R* allenes, it seems likely that inactivation by each 4*S* allene requires prior enzyme-catalyzed conversion of it to the 4*R* isomer, via the acetylene.

SCHEME 4



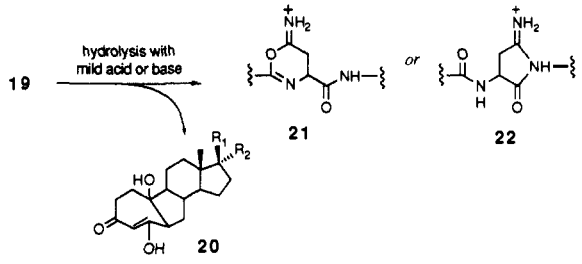
Talalay and his colleagues have conducted extensive and elegant studies of this inactivation process.[45,46,57] The KSI-inactivator linkage was found to be sensitive to both acid and base, and reduction of the adduct with $NaBT_4$ resulted in the incorporation of tritium into only the steroid moiety. When KSI was inactivated with [7-^{3}H]14 and the inactivated enzyme hydrolyzed under mildly acidic conditions, the radioactive product had a chromatographic R_f value identical with that of synthetic 18. A number of types of linkage were ruled out by these observations, which are, however, consistent with an enol ester bond.[57]

KSI that had been inactivated with [7-^{3}H]14 was subjected to proteolysis with proteinase K.[45] Careful fractionation by a combination of chromatographic techniques ultimately led to the identification of a radiolabeled, modified tetrapeptide. On the basis of amino acid composition and N-terminal analysis data, plus the known amino acid sequence of the enzyme, it was concluded that the modified tetrapeptide was derived from residues 55–58 of KSI, with the sequence Y-A-N-S.[45] This peptide includes three potential nucleophiles that upon reaction with 14 would give an enol ester linkage: the hydroxyl groups of tyrosine and serine, and the carboxamide group of asparagine. Thorough analyses were carried out, primarily by two-dimensional high-voltage electrophoresis, on both the modified tetrapeptide as well as its acid hydrolysis product (i.e., with the steroid moiety removed). Largely on the basis of the fact that acid hydrolysis confers enhanced basicity on the remaining peptide moiety, it was concluded that asparagine-57's carboxamide group is the nucleophile. Thus, an enol *imidate* ester linkage between the steroid derivative and asparagine-57, presumably formed as shown in Scheme 4, was implicated.

Upon mild hydrolysis, steroid fragment 20 (cf. structure 18) is released from 19, and the protein is converted to an enzymatically inactive form. On the basis of its ion exchange behavior and electrophoretic mobility, the hydrolysis product of the modified tetrapeptide could be represented by either 21 or 22 (Scheme 5).[45]

In an attempt to differentiate between structures 21 and 22 for the steroid-free modified peptide, fast-atom-bombardment mass spectrometry studies were carried out.[46] Curiously, the major $(M + H)^+$ peak for the modified peptide was found not at m/z 472 (which

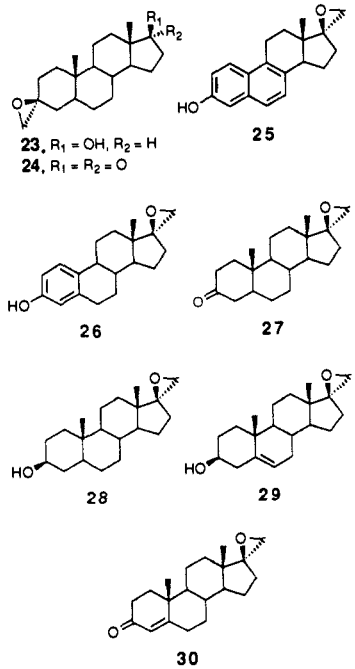
SCHEME 5



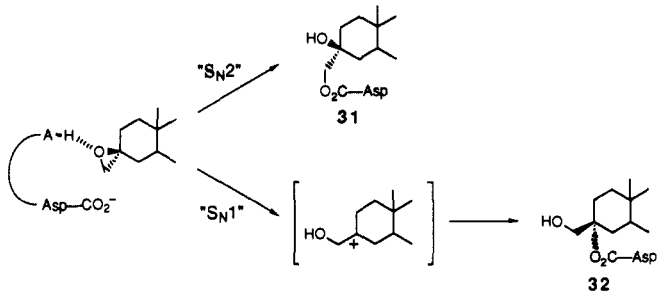
would have been expected for either **21** or **22**), but at m/z 455, which corresponds to Y-A-D-S. This tetrapeptide could have been obtained by selective hydrolysis of either **21** or **22**. Indeed, an additional $(M + H)^+$ peak was observed at m/z 253, indicative of an additional hydrolysis product, the dipeptide Y-A. From the observation of $(M + H)^+$ peaks at both m/z 472 and 253, as well as the absence of a peak at m/z 472, it would appear that the modified peptide had undergone hydrolytic decomposition prior to mass spectral analysis. While this was an unfortunate development, the inconclusive nature of the mass spectrometry studies represents only a minor blemish on a beautifully reasoned set of analytical experiments. There seems to be no doubt regarding the central conclusion that the acetylenic secosteroid **14** reacts with Asn-57 of KSI so as to produce a modified protein that is best represented by structure **19**.

iii. Oxiranes. Using oxiranyl steroid substrate analogues as affinity labels for KSI, Pollack and his associates have also identified Asp-38 at the active site, in accord with the results obtained by Benisek et al., as described in section II.A.1.c.i. In addition, Pollack has demonstrated the existence of at least two distinct modes of substrate binding by KSI.

Pollack found that KSI is irreversibly inactivated by (3*S*)-spiro[5α-androstan-3,2′-oxirane]-17β-ol (**23**),[60] (3*S*)-spiro[5α-androstan-3,2′-oxirane]-17-one (**24**),[61] and by several 17β-oxiranyl steroids, including (17*S*)-spiro-[estra-1,3,5(10),6,8-pentaen-17,2′-oxiran]-3β-ol (**25**),[25] (17*S*)-spiro[estra-1,3,5(10)-trien-17,2′-oxiran]-3β-ol



SCHEME 6



(**26**),[62] (17*S*)-spiro[5α-androstan-17,2′-oxiran]-3-one (**27**),[62] (17*S*)-spiro[5α-androstan-17,2′-oxiran]-3β-ol (**28**),[25] (17*S*)-spiro[androst-5-en-17,2′-oxiran]-3β-ol (**29**),[25] and (17*S*)-spiro[androst-4-en-17,2′-oxiran]-3-one (**30**).[25]

Compound **23** exhibits saturation kinetics, with K_I = 17 μM and the first-order rate constant for inactivation $k_{inact} = 1.13 \times 10^{-3}$ s^{-1}. 19-Nortestosterone (**4**) suppresses the inactivation of KSI by **23**, demonstrating that the inactivation process takes place at the enzyme's active site,[60] and one molecule of **23** was found to bind to each KSI protomer.[63] The C-3 epimer of **23** (with the oxirane oxygen in the α-configuration), although an effective reversible inhibitor of KSI (K_i = 21 μM), fails to irreversibly inactivate the enzyme.[60] The dependence of the mode of inhibition upon the configuration at C-3 highlights the specificity of the interaction between KSI and these inhibitors and supports the contention that the irreversible inactivation of KSI by **23** is an active site directed event, related to substrate binding. Indeed, the pH dependence for the inactivation of KSI by **24** is the same as for KSI-catalyzed isomerization of substrate **10**,[61] suggesting mechanistic similarity between normal catalysis and inactivation of KSI by the 3β-oxiranyl steroids.

The nature of the linkage between **23** and KSI was probed by the isolation and chemical characterization of two steroid-containing modified peptides, derived from residues 14–45 of the enzyme.[63] In each peptide, the linkage was found to be base labile but relatively acid stable. Hydrolysis of the two modified peptides afforded a single peptide product but two distinct steroid moieties. These facts, as well as the relative electrophoretic mobilities of the modified peptides and the corresponding peptide from the native enzyme, suggested that the inactivator becomes bound to the enzyme by an ester linkage to an enzyme carboxyl group. By a clever application of oxygen-18 labeling, partial structures of the enzyme-bound inactivator were identified as **31** and **32** (Scheme 6).[63] These products had apparently resulted from S_N2- and S_N1-like attack, respectively, by the enzyme carboxyl group (later identified as the β-carboxyl of Asp-38[64]) on an activated form of the oxirane. From the stereochemical configurations of **31** and **32**, it is clear that in the course of enzyme inactivation Asp-38 is proximal to the α-face of the steroid.

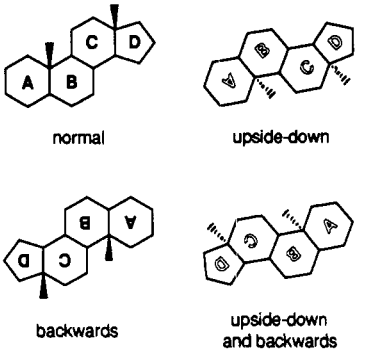
Characterization of the location of Asp-38 relative to the oxirane inactivators led to some conclusions regarding the role of this residue in KSI catalysis. That Asp-38 might be the base that shifts protons between positions 4β and 6β[38] of the substrates was deemed unlikely, since Asp-38 attacks the α-face of **23**. Asp-38

also did not appear to be the general acid that protonates the C-3 carbonyl oxygen, since pH versus V_{max} data implicated a general acid with a pK_a of 9.3.[65] (In subsequent investigations, however,[61] Pollack et al. found that KSI rapidly denatures at pH values over 9, calling into question the reported pK_a of 9.3.)

Two alternative suggestions were made for the role of Asp-38.[63] One role would be to provide electrostatic stabilization for a substrate oxocarbonium ion reaction intermediate. Such stabilization would facilitate the protonation of the weakly basic carbonyl group of the substrate, thereby promoting deprotonation at C-4. A second suggestion was that Asp-38 might stabilize the enzyme's acidic functional group (A–H) by hydrogen bonding. When substrate is bound, the hydrogen bond would be broken, thus destabilizing A–H, and facilitating proton transfer to the carbonyl oxygen.

The assumption that Asp-38 is out of position to serve as the catalytic base may be, however, in conflict with results gained from crystallographic and NMR studies of KSI's interactions with a spin-labeled substrate analogue.[47] These studies indicated that Asp-38 is actually situated over the β-face (*not* the α-face) of the substrate. As discussed below, mutagenesis studies[35] have suggested that Asp-38 functions as the general base, rather than acting to stabilize the substrate oxocarbonium ion intermediate, or serving as the general acid.

Interestingly, the enzyme is irreversibly inactivated by 17-oxiranyl steroids **25–30** in a manner that parallels enzyme inactivation by the 3-oxiranyl steroid **23**. Compounds **25** and **30** also modify Asp-38, leading to ester-linked adducts between enzyme and inactivator, with structures analogous to **31** and **32**.[26,66] The similarities in the inactivation chemistry, as well as the fact that neither 3α- nor 17α-oxiranyl steroids afford covalent adducts, suggest that the oxirane moieties of all of the inactivators bind to KSI in a similar fashion. This curious result requires that KSI be able to bind inhibitors (and possibly substrates as well) in a "backward" mode (i.e., rotated by 180° about an axis perpendicular to the plane of the steroid ring system),[25,26,62,66] so that the positions of the A and D rings would be interchanged. In fact, superimposition of the C-3 oxygen



of **23** with the C-17 oxygen of **28** and of the C-17 oxygen of **23** with the C-3 oxygen of **28** can be accommodated by a slight sideways displacement of the steroid ring systems relative to one another.[67] In this arrangement, the C-10 angular methyl group of each compound occupies nearly the same space occupied by the C-13 angular methyl group of the other compound.

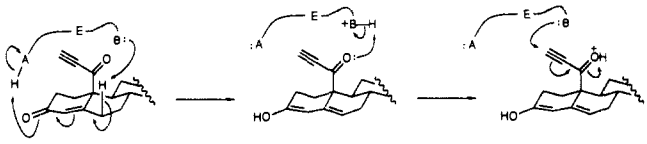
The notion of backward binding of certain substrates to KSI was supported by studies of KSI inactivation by the naphthalenic 17β-oxiranyl steroid **25**. Fluorescence[25] and UV spectroscopy[62] measurements implicated two enzyme/inactivator complexes: one with **25** bound in the normal orientation, and one with **25** bound backward. Normal binding (which is reversible) leads to fluorescence at 420 nm and absorbance at 250 nm, and backward binding leads to enzyme inactivation. Since inactivation is irreversible, all of the enzyme eventually ends up with backward-bound inactivator. Significantly, while **25** binds more tightly to KSI than does 17β-oxirane **29**, the rate of enzyme inactivation is slow relative to the rate of inactivation by **29**. In effect, normal-mode binding of **25** affords protection to KSI against the irreversible inactivation that accompanies backward binding of this compound. Furthermore, analysis of k_{inact}/K_I values for a series of 3β- and 17β-oxiranes indicates that the activation energies for the enzyme inactivation processes are comparable.[25,62]

Evidence of "wrong-way" binding has been reported for several other enzymes of steroid metabolism, including 3β(17β)-hydroxysteroid dehydrogenase,[68,69] 3α-(20β)-hydroxysteroid dehydrogenase,[70,71] and hydroxysteroid sulfotransferase.[72] In fact, it has been suggested[70,71] that the latter dehydrogenase could bind its substrates "upside-down and backward".

Although in the case of 3α(20β)-hydroxysteroid dehydrogenase the available evidence does not allow discrimination between the "upside-down and backward" and "backward" substrate binding modes, the upside-down and backward binding of substrates by KSI might allow rationalization of some heretofor contradictory observations. Crystallographic and NMR studies have implicated Asp-38 as the base that shuttles protons between substrate 4β- and 6β-positions. It is conceivable that Asp-38 is alkylated by 3β-oxirane **23** when the oxiranyl steroid is upside-down and by **25–30** when they are upside-down and backward relative to the productive mode of substrate binding. In addition, upside-down binding could explain the exchange of the 4α-hydrogen observed by Viger et al.[40,41,43] On the other hand, upside-down binding would place the angular methyl groups on the "wrong" side of the substrate relative to the enzyme, possibly leading to adverse steric interactions. Furthermore, if Asp-38 is situated proximal to the substrate's β-face in the normal binding mode, then one might possibly expect 3α- and 17α-oxiranes to irreversibly inactivate KSI when bound in the normal mode.[60]

iv. Other Approaches. Additional evidence that Asp-38 plays a role in KSI catalysis comes from amidation studies conducted by Benisek et al., who studied the effect on the enzyme of ammonium ion or various amines, in the presence of a water-soluble carbodiimide coupling reagent.[73] This procedure was expected to lead to the amidation of one or more of the enzyme's 15 carboxyl groups. When cystamine was coupled with KSI in this fashion, the kinetic course of the derivatization reaction was best explained by the rapid amidation of a single carboxyl group (k = 0.407 min^{-1}) along with a much slower amidation of KSI's fourteen other carboxyl groups (k = 0.0057 min^{-1}). In the presence of 19-nortestosterone acetate (**12**; 410 μM), only the hyperreactive carboxyl group was protected (k = 0.037 min^{-1}). Proteolysis of amidated KSI led to the isolation of a modified peptide whose amino acid composition

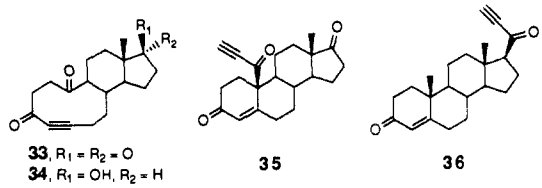
SCHEME 7



corresponded to residues 31–48 of the native enzyme, and Edman degradation of this peptide suggested that Asp-38 had been the site of reaction.

The finding that Asp-38 is hyperreactive toward amidation with cystamine, that this reaction is suppressed by competitive inhibitor **12**, and that the amidated enzyme is catalytically inactive, clearly implicates Asp-38 as an active site residue. The loss of catalytic activity could be ascribed to a failure of the modified enzyme to bind substrate, due merely to the bulk of the cystamine blocking group; however, enzyme modified in a similar manner but with ammonium ion (rather than cystamine) is also inactive.[73] This suggests that the polarity or the charge of Asp-38 is critical to KSI's function.

Penning et al. have investigated the inactivation of KSI by various affinity reagents.[74,75] Since acetylenic secosteroids **14** and **15** clearly are bound at KSI's active site, Penning et al. synthesized and studied the conjugated secosteroid ynones **33** and **34**, along with oxopropynyl steroids **35** and **36**, as prospective enzyme inactivators.[74] All of these compounds are potential Michael acceptors. In fact, all four compounds act as active site directed, irreversible inactivators of KSI.



33, $R_1 = R_2 = O$
34, $R_1 = OH$, $R_2 = H$

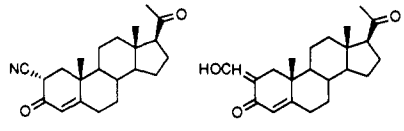
35 **36**

Of the four compounds investigated, **36** was found to bind most tightly (K_I = 14.5 μM); however, k_{inact} was quite low (0.13×10^{-3} s^{-1}; cf. $k_{inact} = 2.3 \times 10^{-3}$ s^{-1} for the β,γ-acetylenic analogue of **33**).[74] Penning et al. did not attempt to rationalize these findings. It is tempting to speculate, though, that tight binding of **36** reflects extremely favorable interactions when it is bound in the normal mode; inactivation, however, might require that **36** be bound in a backward mode. Thus, **36** itself could protect KSI against inactivation, in much the same manner as proposed by Pollack for compound **25**.

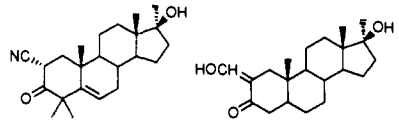
Compound **35**, on the other hand, binds reasonably well to KSI (K_I = 66.0 μM), and k_{inact} is quite high (12.5×10^{-3} s^{-1}). Thus it is a very potent inactivator of the enzyme. On the basis of crystallographic studies,[76] Penning et al. suggested that the enzyme-base that shuttles the proton between the 4β- and 6β-positions is likely to be located quite close to the C-terminus of the Michael acceptor system. A modified version of Penning's mechanism is the notion that **35** is actually a mechanism-based inactivator (Scheme 7). Thus, protonation of the C-3 carbonyl, followed by abstraction of the 6β-proton from **35** by the enzyme base gives a dienol. If the proton were then delivered to the carbonyl oxygen at C-19, rather than to the 4β- or 6β-position, the tendency of the oxopropynyl system to undergo Michael attack by either the enzyme base itself or some other nucleophile would be greatly increased. The steric bulk of the oxopropynyl group may promote enzyme inactivation as well, since unproductive upside-down binding may lead to even more unfavorable interactions than are encountered in normal binding. This hypothesis could be tested by comparing the rates of inactivation of KSI by **35** and [6,6-$^2H_2$]**35**.

Of Penning's secosteroids, **33** has approximately the same effectiveness as Robinson's compound **14**, as judged by a comparison of the k_{inact}/K_I values for the two compounds. Compound **34** is not a very good inactivator, however, since it is bound relatively weakly and since the inactivation constant k_{inact} is quite low. The basis of these observations is not entirely clear, although it certainly has something to do with molecular shape and/or the polarity of the C-17 hydroxyl group. In any case, the poor binding appears to be consistent with the trends observed by Weintraub et al.[24]

Penning has also synthesized compounds **37** and **38** and reported that they are active site directed, irreversible inactivators of KSI.[75] Interestingly, Neville and Engel had reported earlier that a closely related pair of compounds, **39** and **40**, are potent *competitive* inhibitors of *P. testosteroni* KSI, and that upon prolonged dialysis (six changes of buffer, over a total of 72 h), the inhibited enzyme regains full activity.[77] Goldman



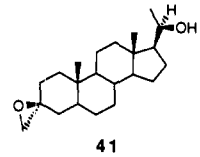
37 **38**



39 **40**

conducted a similar study using the same compounds, with essentially the same results, although it is not clear whether inhibition could be reversed fully under his less extensive dialysis conditions.[78] Penning's dialyses[75] were conducted under conditions similar to those used by Goldman.[78]

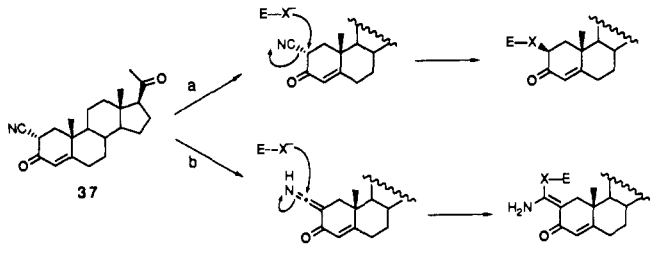
Penning found[75] that **37** and **38** were both less reactive as inactivators of KSI than was 3β-oxiranyl steroid **41**. Of these three compounds, only **41** is comparable in reactivity to Pollack's oxiranes (e.g., **23**[60] or **28**[25]), Robinson's acetylenic secosteroids (e.g., **14** or **15**[74]), or the oxopropynyl steroids of Penning et al. (e.g., **33** and **35**[74]).



41

Penning considered two mechanisms by which α-cyanoketone **37** might inactivate KSI (Scheme 8). While aguments were presented in support of mechanism a, the evidence is inconclusive, and the modified form of the enzyme was not characterized. 2-Mercaptoethanol was found to protect KSI against inactivation by **37**. It was suggested that protection re-

SCHEME 8



37

sults from scavenging of the cyanoketone via S_N2 displacement of cyanide. However, the products formed in a control reaction between 2-mercaptoethanol and **37** were not characterized, and so the proposed mechanism of protection must still be considered speculative. Furthermore, if mechanism a were correct, then it would be very surprising that 2α-bromo-3,4-dihydrotestosterone, 2α-bromotestosterone, and 2α,4α-dibromo-17β-acetoxy-5α-androstan-3-one are only *competitive* inhibitors of *P. testosteroni* KSI[79] and that they would not alkylate the enzyme by an analogous direct displacement mechanism.

Although **38** exists largely in its enolized form (as shown) at pH 7.0 and might therefore be an effective Michael acceptor, even 50 mM 2-mercaptoethanol fails to protect KSI from inactivation by **38**.[75] This is understandable since Michael addition would lead to an aldehyde tetrahedral addition product, which would be formed reversibly. It was suggested that **38**, a 1,3-dicarbonyl compound, might modify an arginine residue.[75] Indeed, arginine residues are subject to modification by malondialdehyde, in a reaction carried out in 10 N HCl and accompanied by extensive peptide bond cleavage.[80] Other dicarbonyl reagents that modify arginine under milder conditions are 1,2-dicarbonyl rather than 1,3-dicarbonyl compounds.[80] This intriguing suggestion appears not to have been investigated further.

d. Structure Determination. The overall picture of the enzyme's structure and mechanism has been substantially clarified by biophysical investigations utilizing techniques that include X-ray crystallography and NMR spectroscopy. Such studies, in combination with the kinetic characterization of site-specific mutants of KSI (section II.A.1.e), are helping investigators to interpret the results obtained by other methods.

i. X-ray Crystallography. Two morphologically distinct crystal forms of the *P. testosteroni* KSI have been characterized. Monoclinic crystals were obtained at pH 7.0 in the presence of ammonium or lithium sulfate as the precipitant.[13] They belong to space group $P2_1$, with unit cell dimensions a = 140.4 Å, b = 85.0 Å, c = 94.5 Å, and β = 130.1°. The asymmetric unit contains 12 protomers, with two asymmetric units per unit cell. These crystals, which tend to grow in polycrystalline clusters, diffract to better than 2.8 Å.[15]

Hexagonal crystals of *P. testosteroni* KSI are obtained under similar conditions, albeit at lower pH (5.5).[16] This crystal form belongs to space group $P6_322$, with unit cell dimensions $a = b$ = 65.38 Å, c = 504 Å, and four protomers (two dimers) per unit cell. While the crystals diffract to better than 2.5 Å, a complete set of diffraction photographs was obtained at 2.7 Å.[16]

The crystal structure for the hexagonal form has been described at a resolution of 6 Å.[27] Phase information was obtained by multiple isomorphous replacement, by using data collected from crystals of native enzyme that had been soaked in either $PtCl_4$, UO_2, or 4-(acetoxymercuri)-17β-estradiol (Hg-estradiol). Significantly, the hexagonal crystals of the native enzyme retain their enzymatic activity.[34]

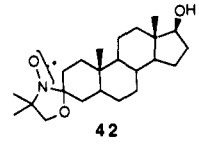
The most interesting gross structural feature of the enzyme dimer is the extensive contact surface between the subunits, most notably the insertion of an arm of each protomer approximately 10–12 Å into a deep pit of the other protomer. This pit, which is where Hg-estradiol binds, appears to be the normal substrate binding site, since Hg-estradiol is a powerful competitive inhibitor of KSI (K_i = 3 μM).[27]

As reported in 1987, substantial progress has been made toward a partially refined 2.5 Å X-ray structure for KSI.[47] Details have yet to be published, however.

ii. NMR Spectroscopy. Although the high-resolution crystal-structure analysis is still incomplete, critical structural and mechanistic insights have been gained by other approaches, providing a rather detailed understanding of the catalytic mechanism of KSI. The key experiments have featured NMR spectroscopy, as well as kinetic analyses of KSI point mutants.

In an early set of experiments, Benisek and Ogez studied the isomerase using one-dimensional ^{1}H NMR spectroscopy at 360 MHz.[81] Their attention was focused primarily on the C-2 protons of histidine residues and the aromatic protons of tyrosine and phenylalanine residues, since (1) these NMR signals are well-separated from those of other amino acids and are therefore relatively easy to assign and (2) prior investigations had suggested that histidine[5,6,65,82] and/or tyrosine[5,33,82] might play an important role in catalysis. Upon binding of the competitive inhibitor 17β-estradiol (**2**) the *only* significant effect on the enzyme's spectrum was a substantial (0.2–0.4 ppm) upfield shift of 4.8–7.8 aromatic protons. This suggested that a histidine is not involved in catalysis. The upfield shift of the aromatic protons is consistent with the recent finding that tyrosine residues 14, 55, and 88 are all relatively close to the A-ring of the substrate when the latter is bound in the normal, productive mode.[47] Indeed, Tyr-14 (now known to be the catalytic general acid,[35] as described below) should certainly be close enough to the aromatic portion of 17β-estradiol for its protons to be affected by the aromatic ring current of the steroid.

An especially informative series of studies conducted by Kuliopulos et al. probed the position of the spin-labeled substrate analogue doxyl-DHT (**42**) within the substrate binding site of *P. testosteroni* KSI.[47] (Although the sample of **42** that was used was apparently a mixture of C-3 epimers, the basic conclusions resulting from the experiments described below are apparently valid.) After ascertaining that **42** binds stoichiomet-



42

rically at KSI's active site, the longitudinal relaxation rates ($1/T_1$) of water protons were measured, and it was concluded that the doxyl moiety is both highly immobilized and accessible to the bulk medium. By analysis of the paramagnetic effects of the nitroxyl group on the T_1 values of a number of protein proton resonances,

distances between the spin label and these protons were defined. This allowed the nitroxyl moiety of doxyl-DHT to be docked by computer graphics methods within a partially refined 2.5 Å X-ray structure of the native isomerase. The remainder of the steroid nucleus of 42 was positioned in the highly hydrophobic binding "pit" by minimizing the van der Waals interactions between the atoms of the steroid and those of the isomerase, while maintaining the position of the nitroxyl group. A very close correspondence was found between the site occupied by 42 and that occupied by Hg-estradiol in the 6 Å X-ray structure.[27]

If the substrate were bound in the same fashion as is 42, there would not be an effective proton donor in the vicinity of the C-3 carbonyl oxygen. This suggests that 42 is bound by KSI in a backward mode. If so, then both Tyr-14 and Tyr-55 would be close to the C-3 carbonyl group during the catalytic event. Tyr-55 was tentatively identified as the general acid since it appeared to be slightly closer to the substrate's carbonyl oxygen. In addition, both Asp-38 and Asn-57 are near C-4 and C-6 of the substrate. On the basis of the enzyme's titration behavior, Asp-38 was judged most likely to serve as the general base. The proximity of Asn-57 to this region of the substrate is entirely consistent with its alkylation by acetylenic secosteroids 14 and 15 (section II.A.1.c.ii). Kuliopoulos et al. suggested[47] that if the C-3 oxygen were to undergo complete or partial protonation in an initial step of the reaction mechanism, then the positively charged intermediate could be stabilized by its proximity (5 Å) to the negative end of the dipole associated with the enzyme's sole α-helical segment. Although it was suggested[47] that the carboxylate groups of Asp-32 and/or Glu-37 might provide additional charge stabilization, further refinement of the X-ray structure has shown that these groups are oriented toward the periphery, rather than toward the active site of the enzyme.[83]

In fact, Tyr-14 interacts strongly with substrate carbonyl groups. By selective deuteration of a KSI mutant with only a single tyrosine, at position 14, Kuliopulos et al. have now assigned critical NMR resonances and determined that Tyr-14's ^{1}H signals are shifted downfield upon binding of 19-nortestosterone.[84] (This effect is reminiscent of the 17β-estradiol-induced shifts observed by Benisek and Ogez.[81]) It was hypothesized that the downfield shift owes to the deshielding effect of the C-3 carbonyl group. Binding of the inhibitor also perturbs the proton resonances of eight phenylalanines, which suggested a steroid-induced conformational change. The true significance of these findings could be clarified by having results from the complementary NMR study involving a mutant with only Tyr-55.

e. Cloning and Mutagenesis. There has been rapid progress in the application of molecular biology to the study of *P. testosteroni* KSI, which has been cloned, overexpressed, and sequenced by two groups.

Kuliopulos et al.[21] created a λgtWES library from a partial *Eco*RI digest of *P. testosteroni* genomic DNA. When plated on *E. coli*, 20 clones (of 20 000 that were examined) hybridized to a synthetic oligodeoxynucleotide probe, and of these, six colonies were found to contain an insert of 5400 base pairs (bp). A 1370-bp restriction fragment was subcloned into pUC 19, giving a recombinant plasmid, pAK 1370. The thiogalactoside-induced expression system, *E. coli*/pAK 1370, afforded KSI to the extent of 10% of the total cellular protein. Fifty percent of the total KSI activity could be extracted with buffer, giving enzyme with a purity of 40%. Physical and kinetic characterization of the recombinant enzyme showed it to be indistinguishable from the native isomerase.[21]

A nearly identical strategy was used by Choi and Benisek, who estimated that their *E. coli* expression system afforded approximately 100 times the amount of KSI obtained from fully induced *P. testosteroni*.[23]

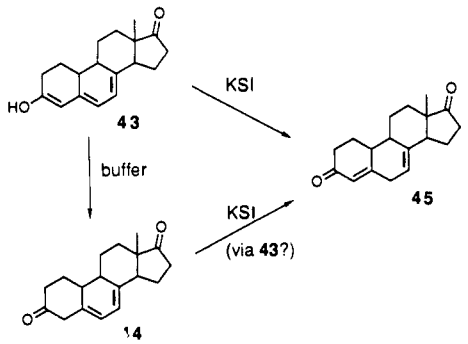
The most significant observation from the deduced amino acid sequence obtained by both groups was that residue 38 is indeed aspartic acid, rather than asparagine as had been reported on the basis of peptide sequencing.[20] In addition, the nucleotide sequence indicated that residues 22, 24, and 33 are also aspartic acid (rather than asparagine[20]), and that residue 77 is glutamic acid (rather than glutamine[20]). Thus, on the basis of the nucleotide sequence, the molecular mass of the KSI protomer is 13 399.[21,23]

In an attempt to assess the roles of specific amino acid residues in catalysis by KSI, several site-specific mutant forms of the enzyme have been prepared and analyzed.[35] In particular, Asp-38 was changed to asparagine (D38N), Tyr-14 to phenylalanine (Y14F), and Tyr-55 to phenylalanine (Y55F). These substitutions were selected on the basis of a variety of experiments that had implicated Asp-38 as the enzyme's general base[26,32,47,64,66,73] and either Tyr-14 or Tyr-55 as the general acid.[47,81]

The kinetic properties of the purified mutant enzymes were enlightening.[35] At pH 7.5, with 1 as the assay substrate, the Y14F and D38N mutants had k_{cat} values that were $10^{4.7}$- and $10^{5.6}$-fold less than that of the native isomerase. On the other hand, the k_{cat} of the Y55F mutant was diminished by only a factor of 4. All three mutants had K_m values that were about 3-fold *less* than that of the wild-type enzyme, indicating that mutagenesis had effected no major changes in the substrate binding site, and also that the residual activity shown by the mutants could not be attributed to contamination by small amounts of wild-type isomerase. These results clearly implicate Tyr-14 as the active site general acid and Asp-38 as the general base.

Ultraviolet spectroscopy experiments also produced interesting results.[35] When the Y55F and D38N mutants, as well as the recombinant wild-type enzyme, were each incubated with 19-nortestosterone, red-shifted absorption maxima attributed to the carbonyl-protonated form of the inhibitor were produced. The Y14F mutant, on the other hand, failed to induce such a spectral perturbation. When 17β-estradiol was incubated with the wild-type enzyme, the Y55F mutant, or the D38N mutant, spectral changes attributed to the phenolate anion were induced, while with the Y14F mutant there was no such effect. The surprising implication is that Tyr-14 can act as a proton acceptor *and* as a proton donor. Furthermore, if the D38N mutant retains the ability to protonate 19-nortestosterone, then substrate deprotonation would not precede protonation of the C-3 oxygen, and therefore a carbanion could not be an enzymatic reaction intermediate. Hence, if enolization of the substrate were not a concerted process,

SCHEME 9



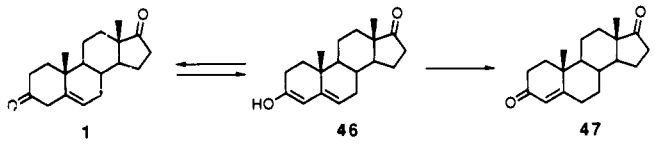
protonation of the carbonyl group would have to precede deprotonation at C-4, affording an oxonium ion intermediate. Such an intermediate might be stabilized by the negative end of the α-helix dipole.

f. Recent Studies and Views on the Mechanism of Action. Evidence in favor of the dienol reaction intermediate originally proposed by Wang et al.[33] and supported by the experiments of Malhotra and Ringold[38] has recently been presented by Bantia and Pollack,[85] who have studied both the enzymatic and nonenzymatic tautomerization of synthetic trienol **43** (Scheme 9).

When incubated with KSI in pH 4.5 acetate buffer at 10 °C, 5,7-estradiene-3,17-dione (**44**) was converted to 4,7-estradiene-3,17-dione (**45**), with $k_{cat}/K_m = 2.5 \times 10^9$ M^{-1} min^{-1}. A synthetic sample of trienol **43**, a plausible intermediate in the enzymatic reaction, was then incubated with KSI under the same conditions (since **43** is reasonably stable at pH 4.5). It, too, underwent pseudo-first-order conversion to **45**, with $k_{cat}/K_m = 7.2 \times 10^9$ M^{-1} min^{-1}. That the tautomerization was enzyme-catalyzed was supported by two lines of evidence: (1) it was inhibited by 19-nortestosterone (**4**), and (2) in the absence of enzyme, the trienol is slowly rearranged to compound **44**, rather than to **45**. Importantly, the rate data demonstrate that trienol **43** is kinetically competent to serve as an intermediate in the KSI-catalyzed rearrangement of **44** to **45**.

While **43** is a logical intermediate in the overall reaction, the significance of the particular rate constants that were obtained is not clear. First, in its resting state the enzyme has both a general acid (A–H; for protonation of, or hydrogen bonding to, the substrate carbonyl) and a general base (:B; for removal of the 4β- or 6β-proton). If the purpose of A–H is to protonate the substrate, then when trienol **43** is formed during turnover of **44** the general acid will be *deprotonated*, and the general base with be *protonated*. If A–H merely hydrogen bonds to the substrate, then under normal turnover conditions this acidic group as well as the basic group will be protonated when the trienolate (rather than the trienol) is formed. In either case, in Pollack's experiments, it would appear that the enzyme was not in the appropriate protonation state for turnover of **43**. (Turnover of **43** requires that A be deprotonated and B be protonated.) Furthermore, if A–H doesn't give up its proton during normal catalysis but instead stabilizes the reaction intermediate through hydrogen bonding, then trienolate anion, **43**$^-$, rather than trienol **43** itself, is the actual intermediate. It is, of course, impossible to say how fast **43** (or **43**$^-$) would be turned over by the appropriate form of the isomerase. Second, Pollack et al. have reported critical pK_a values of 4.6–4.8 for free KSI and 4.7–5.5 for ES and EI complexes.[61] It is likely that the particular group with this pK_a is the general base, now known to be Asp-38. Thus, at pH 4.5 *both* the general acid (Tyr-14) and the general base (Asp-38) will be largely protonated, and so the enzyme will again not be in a catalytically competent form for turnover of **43**. (In this case it *would* be in the proper form for turnover of **43**$^-$, but the concentration of **43**$^-$ should be infinitesimal at pH 4.5.) Despite these complications, enzymatic conversion of **43** to **45** was several times as fast as the overall conversion of **44** to **45**, and so **43** is certainly kinetically competent to serve as a reaction intermediate.

SCHEME 10



As implied in the preceding paragraph, there is some question as to whether a dienol or a dienolate is more likely to be the normal reaction intermediate in the enzymatic isomerization of an enone substrate.[86] While most mechanisms proposed for KSI have assumed a neutral enolic reaction intermediate (produced via transfer of a proton from A–H to the substrate carbonyl),[5,8,86] a case has also been made for an enolate intermediate.[43,86] The C-4 protons of steroidal β,γ-enones such as 5-androstene-3,17-dione (**1**) are unusually acidic (pK_a = 12.72[87]). Thus, it has been argued that proton transfer to the substrate carbonyl may not be necessary to facilitate deprotonation at C-4. Furthermore, the dienol is sufficiently acidic (p$K_a \approx 10.0$[88]) that the corresponding dienolate may derive little additional stabilization from proton transfer. Instead, hydrogen bonding between the enzyme and the C-3 oxygen of the substrate or reaction intermediate has been proposed. Supporting the notion of an anionic reaction intermediate is the fact that several investigators have observed chromophores that are consistent with the formation of phenolate ions upon binding of A-ring phenolic substrate analogues to KSI.[33,35,43,62] These chromophores do not result from deprotonation of an enzyme tyrosine residue, since binding of 17β-dihydroequilenin (a naphthalenic steroid) and the phenolic steroid 17β-estradiol afford different enolates.[33] If there is a neutral dienol reaction intermediate, however, then the enzyme-mediated deprotonation of the phenolic substrate analogues and stabilization of the derived phenolate ions might not bear a direct relationship to the normal catalytic mechanism.

Eames et al. have very recently reported on the KSI-catalyzed partitioning of the suspected isomerization reaction intermediate, dienol **46** (Scheme 10).[89] This compound was made by treating **1** with dilute sodium hydroxide and quenching with mild acid. When the quench buffer was pH 4.7 acetate containing KSI, the turnover of **46** could be followed by the use of stopped-flow spectrophotometry. The results were consistent with an initial partitioning of **46** between **47** and **1**, along with the slower KSI-catalyzed conversion of **1** to **47**. Kinetics analysis led to the conclusion that 68 ± 3% of enzyme-bound dienol **46** is converted to unbound **1**, while 32 ± 3% goes to free **47**. Similar

results were obtained from an experiment in which 46 was produced enzymatically from the dienol phosphate and 1 was trapped by reduction with NADH in the presence of 3α-hydroxysteroid dehydrogenase. These findings are noteworthy in light of the equilibrium constant for the equilibration of 1 with 47, which favors the conjugated enone by a factor of 2400.[37]

The studies of the partitioning of the dienol intermediate are especially significant since for the first time, the partial reactions catalyzed by KSI have been observed separately. The overall relevance of the experimentally derived partition ratio is not clear, however, because of the same factors that complicate interpretation of the experiments involving trienol 43. That is, at pH 5.2 (the final pH of the reaction mixtures studied by Eames et al.[89]) the resting enzyme will be largely in the wrong protonation state.

The KSI double mutant Y14F/D38N has recently been constructed and found to bind substrate analogues tightly, but to have greatly diminished catalytic activity.[90] Under normal assay conditions, the ratio ($\leq 10^{-9.8}$) of the double mutant's k_{cat} to that of the wild-type enzyme is comparable to the product ($10^{-10.3}$) of the fractional k_{cat} values for the single mutants Y14F ($10^{-4.7}$) and D38N ($10^{-5.6}$).[35] This is consistent with the involvement of both Tyr-14 and Asp-38 in a single chemical step, presumably enolization of the substrate.[90]

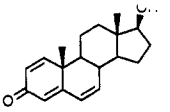
The concertedness of the enolization step was evaluated by analyzing solvent and substrate isotope effects.[91,92] With 4β-^{2}H substrate, or in a ^2H_2O buffer, both the wild-type enzyme and the Y55F mutant experience substantial k_{cat} isotope effects. For both enzymes, the double isotope effects are increased slightly over the corresponding single isotope effects. This is consistent with a concerted mechanism for enolization of the substrate. With the Y14F mutant (i.e., lacking the general acid), the double isotope effects are substantially less than the corresponding solvent and substrate deuterium isotope effects. This argues against a concerted enolization mechanism for this mutant, and in favor of an enolate ion reaction intermediate. Analysis of V/K isotope effects for substrate deuterated in the 4α-position, the 6-position, or in both the 4α- and 4β-positions suggested that substrate enolization by the wild-type enzyme is both concerted and rate-limiting.[93]

2. *Pseudomonas putida* KSI

Δ^5-3-Ketosteroid isomerase activity has also been detected in *Pseudomonas putida* Biotype B.[94] Gel exclusion chromatography and sedimentation equilibrium experiments gave a native molecular weight in the vicinity of 27 000, and SDS gel electrophoresis was consistent with a subunit molecular weight of between 14 000 and 15 500. These values, as well as the isoelectric point of 4.8, are very similar to those of the *P. testosteroni* enzyme.

Kinetic analysis of the *P. putida* isomerase revealed many similarities but some marked contrasts between the two enzymes.[95] The pH dependencies of V_{max} and K_m proved to be quite different. The V_{max} of the *P. putida* isomerase (for 1) was quite insensitive toward pH, whereas pH versus V_{max} for the *P. testosteroni* enzyme showed inflection points at about 5 and 9.[65] In addition, the K_m of the *P. putida* enzyme is constant up to pH 8, above which it rises dramatically. In contrast, a plot of pH versus pK_a for the *P. testosteroni* isomerase is sigmoidal, with an inflection point at pH 5.1.[65]

The *P. putida* isomerase undergoes active site directed photoinactivation in the presence of 1,4,6-androstatrien-3-one-17β-ol (48).[96] The steroid does not become covalently linked to the enzyme; rather, analysis of the protein indicated a direct relationship between enzyme inactivation and the loss of a single sulfhydryl group. This is in marked contrast to photoinactivation of the *P. testosteroni* enzyme, which results in the modification of Asp-38.[31,32]



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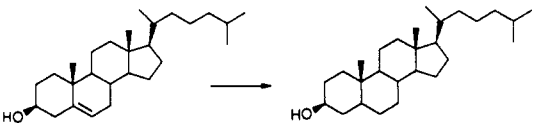
Subsequently, the entire *P. putida* enzyme was sequenced,[97] and it was found to be a homodimer, with subunits of 14 536 Da. The only protein with which *P. putida* KSI showed significant homology was *P. testosteroni* KSI. Even so, there were only 44 identical matches, giving a relatively low value of 34% sequence homology. Significantly, residues 31–39 (including Asp-38) of *P. testosteroni* KSI are identical with residues 33–41 of the *P. putida* enzyme, and Tyr-14 of *P. testosteroni* KSI is conserved also.

3. Miscellaneous Bacterial Steroid Isomerases

There have been a few reports of KSI in bacterial genera other than *Pseudomonas*.

Using a mixed culture obtained from rat fecal material, Björkhem and Gustafsson found that [4β-^{3}H,4-^{14}C]cholesterol (49) is converted to coprostanol (50) with retention of 60% of the tritium.[98] Chemical degradation was carried out to localize the tritium label at C-6 of coprostanol. The migration of tritium from the 4β-position to C-6 provided indirect evidence for a steroid isomerase. As a follow-up, Parmentier and Eyssen repeated the experiment of Björkhem and Gustafsson with a pure culture of a *Eubacterium* species that had been isolated from rat fecal contents and obtained essentially the same results as with the mixed culture.[99]



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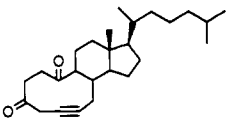
Cholesterol oxidase, which converts cholesterol into 4-cholesten-3-one, has been reported in a number of microorganisms. Smith and Brooks have determined that in the reaction catalyzed by *Nocardia erythropolis* cholesterol oxidase, the 4β-hydrogen is transferred to position 6β.[100] Once again, this implies the intermediacy of 5-cholesten-3-one, which could be generated via KSI activity. That 5-cholesten-3-one is an intermediate was confirmed by isotope dilution analysis.[100] Finally, Smith and Brooks incubated cholesterol oxidase with acetylenic secosteroid 51, an analogue of secosteroids 14 and 15, which are potent inactivators of *P. testosteroni* KSI.[100] Compound 51 destroys both the isomerase and the oxidase activities in a time-dependent manner. The fact that both activities are affected implies a single

active site for this bifunctional enzyme.



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4. Mammalian Steroid Isomerases

In mammals, KSI activity is essential to the biosynthesis of androgens, progestagens, estrogens, and corticosteroids. Although the presence of KSI in mammalian tissues has long been accepted and KSI activity could be demonstrated in crude tissue preparations, structural and mechanistic insights have evolved slowly. This owes mostly to the fact that mammalian KSI is membrane-associated.

KSI is usually accompanied by 3β-hydroxysteroid dehydrogenase, and there is evidence that in most mammalian tissues the isomerase and dehydrogenase activities belong to a single bifunctional protein. The extent of any structural similarity among the KSIs obtained from different mammals is unclear, since the enzymes are difficult to purify, and the physical characterization data are still incomplete. The best characterized dehydrogenase/isomerase enzymes are those from rat and human tissues.

a. Purification. Ishii-Ohba and co-workers have purified the dehydrogenase/isomerase from both rat adrenals[101] and rat testes.[102,103]

The adrenal enzyme[101] was solubilized by sodium cholate and purified by ion exchange, hydroxyapatite, gel filtration, and affinity chromatography, with elution buffers containing 20% glycerol as well as low concentrations of the nonionic detergent Emulgin 913. The purified protein was obtained in 4% yield (based upon isomerase activity), representing a 15.3-fold purification from the starting microsomal preparation. This protein ran as a single band upon SDS polyacrylamide gel electrophoresis, with a mobility that was consistent with a protomer M_r of 46.5 kDa. Gel filtration suggested a native M_r of 91 kDa. The ratio of isomerase to dehydrogenase activity varied substantially from step to step during the course of the purification, possibly reflecting the removal of one or more inhibitory species, or the separation of several hydrogenases and/or isomerases. In fact, evidence has been presented by several groups for the presence, in certain mammalian tissues, of multiple isomerases with somewhat overlapping substrate specificities.

The testicular dehydrogenase/isomerase has been purified by the same scheme used for the adrenal enzyme. In earlier work[102] the gel filtration and affinity chromatography steps were omitted. This procedure afforded partially purified enzyme, with a ratio of isomerase to dehydrogenase activity of approximately 10. Two major and several minor bands were evident upon SDS gel electrophoresis, which indicated a subunit M_r of 46.5 kDa (the same value obtained for the adrenal enzyme) for the testicular dehydrogenase/isomerase. Subsequently, the testicular enzyme was purified by the full scheme used for the adrenal enzyme.[103]

Dehydrogenase/isomerase has been purified 500-fold to apparent homogeneity from human placenta,[104] by a shortened version of the procedure used for purification of the enzyme from rat tissue.[101,102] The molecular mass of the native placental enzyme is 76 kDa (by gel filtration), and the subunit M_r (by SDS polyacrylamide gel electrophoresis) is 19000. A tetrameric quaternary structure is therefore indicated.

Talalay's group has characterized a soluble,[105] reduced glutathione (GSH) dependent KSI activity in mammalian liver tissue with activity toward both C_{19} and C_{21} Δ^5-3-ketosteroids.[106] Physical and immunological characterization studies have shown that the activity resides in several GSH *S*-transferases.[107] These are proteins that are normally involved in the elimination of electrophilic xenobiotics through covalent conjugation with the sulfhydryl group of GSH. In the isomerization, GSH is not consumed but is used catalytically, presumably as an acidic or basic catalyst. Since a substantial nonenzymatic component to steroid isomerization has been found with boiled placental microsomes,[108] various peptides,[106] liver enzyme preparations in the absence of GSH,[106] and even phosphate buffer,[109] it seems unlikely that the GSH-dependent isomerase activity from liver has any physiological significance.

Although the Δ^5-3-ketosteroid isomerase activity of bovine adrenocortal microsomes has been studied by a number of groups, there is still relatively little that is known about the enzyme(s) responsible for it.

In contrast to the steroid isomerases from most other mammalian tissues, the bovine adrenal enzyme appears not to be part of a bifunctional isomerase/3β-hydroxysteroid dehydrogenase.[110,111] This has been shown most conclusively by Gallay et al., who chromatographically separated C_{19} and C_{21} dehydrogenase activities from C_{19} and C_{21} isomerase activities.[110] In addition, Hiwatashi and co-workers have purified a dehydrogenase from bovine adrenal tissue and found it to be devoid of isomerase activity.[112] Efforts to solubilize the isomerase have been partially successful and have utilized both detergents[110,113-115] as well as Ca^{2+}.[116] Several groups have reported that there may be more than one Δ^5-3-ketosteroid isomerase present in the bovine adrenal cortex, with differential selectivities toward C_{19} and C_{21} substrates.[110,111,113,115] Attempts to purify and physically characterize the enzyme(s) beyond this point have apparently not been successful.

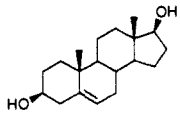
Ford and Engel have described the purification of the dehydrogenase/isomerase from sheep adrenocortical microsomes,[117] by detergent solubilization, followed by ion-exchange chromatography. A subunit M_r of 40000 was indicated on the basis of SDS polyacrylamide gel electrophoresis; however, the number of subunits present in the native protein could not be determined. The dehydrogenase and isomerase activities copurified.

b. Mechanism. The adrenal and testicular dehydrogenase/isomerase proteins from rat behaved identically in virtually all ways, with the exception of the dehydrogenases' K_m values for NAD^+, which differed by a factor of two. NAD^+ and NADH (to a somewhat lesser extent) are positive effectors of isomerase activity. Not surprisingly, the nucleotide-like affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) completely inactivates the dehydrogenase activity. However, when the dehydrogenase is inactivated by FSBA, the isomerase retains 10% of its original activity, even in the absence of NAD^+. Although this phenomenon is not completely understood, it suggests that FSBA binds at the NAD^+ binding site, leading to

partial allosteric activation of the isomerase.[103]

As is the case with the rat dehydrogenase/isomerase, the placental isomerase is activated by NAD^+,[104,118,119] and the isomerase is considerably more active (based on V_{max} values) than is the dehydrogenase. Thus, the dehydrogenation step is rate-limiting in the presence of saturating levels of substrate. The purified placental enzyme exhibited comparable activity toward C_{19} and C_{21} substrates 1 and 11, respectively. Crossover inhibition studies indicated that isomerization of 1 and 11 take place at the same active site.[104] Analogous results were obtained for the dehydrogenase activity. Blomquist et al. have reported that the isomerase is not inhibited by either substrates or competitive inhibitors of the dehydrogenase, thus leading to the conclusion that the isomerase and dehydrogenase activities do not share a common active site.[120] Consistent with this conclusion, Edwards et al. have observed selective inhibition of the isomerase activity.[121] Both of these studies utilized crude placental enzyme preparations, and so further experiments involving highly purified dehydrogenase/isomerase are necessary.

Akhtar et al. have evaluated the stereochemical course of the isomerization catalyzed by the placental dehydrogenase/isomerase.[122] They incubated 5-androstene-3β,17β-diol (**52**), bearing tritium in the 4α-, 4β-, or 6-position, with a crude microsomal enzyme preparation and carried out chemical and microbiological derivatizations of the isomerized products. As



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with the *P. testosteroni* enzyme, it is the 4β-hydrogen that is removed, and the new hydrogen at C-6 is in the β-configuration. In contrast to what was found with the bacterial enzyme,[33,39] however, there was no evidence for intramolecular proton transfer. It was concluded that either the placental enzyme utilizes two acid/base groups (one to remove the C-4 proton, and one to protonate C-6) or that there is rapid exchange of the protonated base with the aqueous medium. The fact that the isomerization is a suprafacial process suggests, however, that only a single acid/base is required.[123,124]

In contrast to Akhtar's observation, Talalay and coworkers found that the isomerization of unlabeled 1 catalyzed by the partially purified bovine adrenal isomerase proceeds without significant exchange with a deuterated medium.[109] The same reaction was reinvestigated by Viger et al., using [4β-^{2}H]1, and significant exchange *was* observed.[125] Since they also replicated Talalay's finding,[109] they concluded that the microsomes are slow to exchange. A small but significant amount of the label from the stereospecifically labled substrate was transferred to C-6, and a much larger portion remained at C-4 of the rearranged product (cf. 6 → 7 + 8). It was concluded that the isomerization requires a single active site acid/base, which as its conjugate acid is susceptible to exchange with the medium. As with the *P. testosteroni* enzyme,[40,41] a competing epimerization at C-4 of 1, catalyzed by a second acid/base group, was proposed.[125]

Several groups have studied environmental effects on the activity of the bovine adrenocortical isomerase. Incubation of a microsomal isomerase preparation with phospholipase A leads to a loss of enzyme activity that is proportional to the extent of lipid hydrolysis.[126] The lost activity is completely restored by reconstitution with an aqueous dispersion of native microsomal lipids. Wehrle and Pollack also observed the importance of the lipid environment of the bovine isomerase.[114] They found that detergent-solubilized isomerase possesses only 1–2% of the activity of the original microsomal enzyme. Reconstitution into asolectin vesicles restores full activity. This is in contrast to the finding of Geynet et al., who had been unable to restore activity to the phospholipase A treated microsomes by addition of asolectin.[126] In native microsomes or reconstituted vesicles, both K_m and V_{max} rose dramatically at pH values over 8.5. Detergent-solubilized isomerase did not show this pH effect. Wehrle and Pollack's findings are in conflict with an earlier report by Neville and Engel,[113] who had found that microsomes that had been rehomogenized with sodium deoxycholate retained 80% of their original isomerase activity toward both C_{19} and C_{21} substrates.

Neville and Engel also observed that isomerization of 1 by the microsomal enzyme was stimulated by NAD^+ at concentrations as low as 10^{-8} M; however, the deoxycholate-treated enzyme preparation was unaffected by up to 50 μM NAD^+.[113] Similar observations concerning isomerase activation by NAD^+ and by NADH were made by Brandt and Levy,[127] in their studies of partially purified dehydrogenase/isomerase preparations. (For these studies, there was no special effort made to separate dehydrogenase and isomerase activities from one another.[128]) NAD^+ is a positive effector of isomerase activity in solubilized as well as microsomal forms.[127] Since the bovine adrenal isomerase has been shown by other workers not to be part of a single bifunctional dehydrogenase/isomerase protein, this effect of NAD^+ on isomerase activity may be explained by assuming that the dehydrogenase and the isomerase are not only metabolically coupled but also physically contiguous in their microsomal environment. In this way, activation of the dehydrogenase may lead to a conformational change that also effects activation of the isomerase.

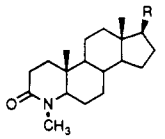
Overall, it appears that an intact phospholipid bilayer environment is essential for proper functioning of the isomerase. Still, the interpretation of experiments by different research groups is complicated by the possible existence of multiple isomerases and/or dehydrogenases in any given mammalian tissue.

In addition to NAD^+, serum albumin (1–2 μM)[129] and divalent cations (1 mM)[130] have been identified as activators of the membrane-imbedded bovine adrenocortical isomerase. The observed stimulation has in each case been ascribed[114] to effects on membrane structure rather than to direct interactions with the isomerase. Serum albumin is known to be a scavenger of free fatty acids, which could otherwise inhibit the isomerase by exerting a detergent effect.[131]

A number of compounds have been evaluated as inhibitors of the mammalian isomerases. Penning found that acetylenic secosteroids 14 and 15 inactivate the bovine adrenal isomerase at rates that are very similar to the rates at which the same compounds inactivate the *P. testosteroni* enzyme. Compound 15 inactivates

both enzymes faster than does 14, by a factor of 2.[132] While it was assumed that 14 and 15 are mechanism-based inactivators of the bovine isomerase, this was not proven to be the case. The two secosteroids destroyed the ability of the enzyme to isomerize either C_{19} or C_{21} Δ^5-3-ketosteroids, although neither compound destroyed the two isomerase activities at the same rate. This suggests that the enzyme may have two active sites or that there may be two or more isomerases with differing substrate specificities. Penning and Covey determined that ynones **33–36** are also inactivators of the bovine isomerase.[133] Once again, the rates of inactivation of the C_{19}- and C_{21}-isomerase activities were nonidentical. Isomerase inactivation by **36** suggests the possibility of backward binding of substrates and inhibitors to the bovine adrenal enzyme.

On the basis of preliminary results reported by Cooke and Robaire,[134] Brandt and Levy investigated the effect of several 3-oxo-4-aza steroids (**53–56**, among others) on a bovine adrenal Δ^5-3-ketosteroid isomerase/3β-hydroxysteroid dehydrogenase preparation.[127] Each of

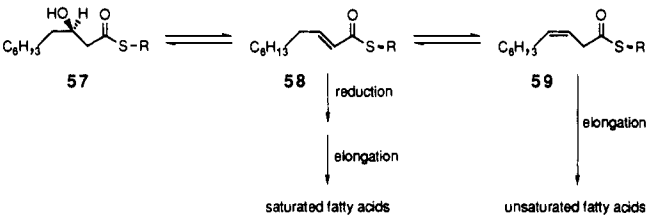


53 R = –CH(CH$_3$)CH$_2$OH
54 R = –C(O)N[CH(CH$_3$)$_2$]$_2$
55 R = –C(O)N(C$_2$H$_5$)$_2$
56 R = –CH(CH$_3$)COOH

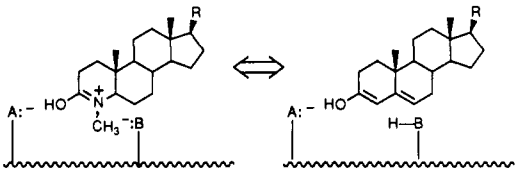
these compounds was found to inhibit the dehydrogenase rather effectively (up to 97%, in the presence of 1 μM **53**); however, the ratio of Δ^4- to Δ^5-3-ketosteroid products was unaffected. This suggested that either the dehydrogenase was inhibited preferentially, or that both enzyme activities were inhibited more or less equally. Indeed, both **53** and **54** at submicromolar concentrations were also competitive inhibitors of isomerase-catalyzed conversion of Δ^5-pregnenedione to progesterone. In the presence of the positive effector NAD^+, inhibition of the isomerase was much weaker than was inhibition of the dehydrogenase. In fact, NADH proved to be 5–6 times better than NAD^+ as an effector of isomerase inhibition by compounds **53–56**.

Inhibition of the steroid isomerase by **53–56** was rationalized by suggesting that these compounds may mimic the dienol reaction intermediate, as portrayed below. This is consistent with the fact that in amides electron density tends to be polarized toward the carbonyl oxygen and away from the nitrogen atom. Thus, the amide should be favorably disposed toward accepting a proton from an active site acid (the conjugate base of which is represented by A:$^-$ in the cartoon below) corresponding to Tyr-14 in the *P. testosteroni* enzyme. Indeed, normal catalysis by the bovine adrenal steroid isomerase, as well as inhibition of this enzyme by **54**, is dependent upon an enzyme functional group with $pK_a \approx 7.8$ being in its protonated state.[127] Another appealing feature of the suggested mechanism of isomerase inhibition presented below is the fact that protonation of the amide carbonyl is *not* accompanied by deprotonation elsewhere by the active site base (B:$^-$, below). Thus, the inhibitor/isomerase complex may be electrostatically stabilized, through ion pairing between the active site base (Asp-38 in the *P. testosteroni* enzyme) and the positively charged amide nitrogen. This, of course, would not be the case for 3-aza-3-oxo steroids that lack the *N*-methyl group. Two such compounds

SCHEME 11



(not shown here) were found to be poor inhibitors of the steroid dehydrogenase. No results were reported for purified isomerase;[127] however, Levy has noted that since the dehydrogenase assays utilized a coupled dehydrogenase/isomerase system, any significant inhibition of the isomerase by either of these compounds probably would have been noticed.[128]



B. β-Hydroxydecanoyl Thiol Ester Dehydrase

While unsaturated fatty acids are ubiquitous, there are significant differences between those found in eukaryotes and those that occur in prokaryotes. Most eukaryotic unsaturated fatty acids have a fixed number of carbons between the double bond and the carboxyl group, while those made by prokaryotes tend to have the double bond in a constant position relative to the terminal methyl group.[135] These structural trends reflect biosynthetic distinctions. Eukaryotes make unsaturated fatty acids by an oxygen-dependent desaturation of full length fatty acids,[136] a process that is unavailable to anaerobic organisms. Prokaryotes biosynthesize their unsaturated fatty acids through a process in which the double bond is introduced by dehydration rather than dehydrogenation.[137,138] The discovery of this pathway, by Bloch, grew out of observations on the structures of unsaturated fatty acids and their distribution in nature.[139,140]

The key enzyme in the biosynthesis of unsaturated fatty acids in most (if not all) prokaryotes is β-hydroxydecanoyl thiol ester dehydrase.[141] Dehydrase interconverts thiol esters of (*R*)-3-hydroxydecanoic acid (**57**) with those of (*E*)-2-decenoic acid (**58**) and (*Z*)-3-decenoic acid (**59**). In vivo, **58** (R = acyl carrier protein; ACP) is reduced to decanoyl-ACP, which is elongated via the usual process to full-length saturated fatty acids. Compound **59** is homologated without prior reduction, leading to the common monounsaturated fatty acids (Scheme 11).

1. Purification and General Properties

The original procedures for the purification of dehydrase, developed by Bloch and his coworkers, utilized *E. coli* strain B as the source of enzyme.[142-145] In recent years, the yield of enzyme has been improved substantially by the use of a cloned, overproducing bacterial strain, *E. coli* DM51A, along with minor variations on the earlier purification scheme.[146,147]

The most convenient assay for the enzyme utilizes the *S*-(*N*-acetylcysteamine) (NAC) thiol ester of **59** as the assay substrate. The formation of **58**, which absorbs

CHART I. Amino Acid Sequence of *E. coli* β-Hydroxydecanoyl Thiol Ester Dehydrase and Nucleotide Sequence of the *fabA* Gene

```
...CC ATG GCC ATT ACG TTG GCT GAA CTG GTT TAT TCC GAA CTG ATC GGA CTT GTT CAG CGT ACA CGT

                                                                [met] val asp lys arg glu ser
GTT AGC TAT CCT GCG TGC TTC AAT AAA ATA AGG CTT ACA GAG AAC ATG GTA GAT AAA CGC GAA TCC

                10                                  20
tyr thr lys glu asp leu leu ala ser gly arg gly glu leu phe gly ala lys gly pro gln leu
TAT ACA AAA GAA GAC CTT CTT GCC TCT GGA CGC GGT GAA CTG TTT GGC GCT AAA GGC CCG CAA TTG

    30                                  40                                  50
pro ala pro asn met leu met met asp arg val val lys met thr glu thr gly gly asn phe asp
CCA GCA CCG AAC ATG CTG ATG ATG GAC CGT GTG GTC AAA ATG ACC GAA ACG GGT GGT ACC TTC GAC

                        60                                  70
lys gly tyr val glu ala glu leu asp ile asn pro asp leu trp phe phe gly cys his phe ile
AAA GGC TAT GTT GAA GCA GAA CTG GAT ATC AAT CCG GAT CTG TGG TTC TTC GGA TGC CAC TTT ATT

                    80                                  90
gly asp pro val met pro gly cys leu gly leu asp ala met trp gln leu val gly phe tyr leu
GGC GAT CCG GTT ATG CCG GGA TGC CTG GGC CTG GAC GCA ATG TGG CAG CTG GTA GGC TTC TAC CTC

                100                                 110
gly trp leu gly gly glu gly lys gly arg ala leu gly val gly glu val lys phe thr gly gln
GGC TGG CTG GGC GGC GAA GGT AAA GGC CGC GCG CTG GGC GTT GGC GGA GTG AAA TTC ACT GGT CAG

        120                                 130
val leu pro thr ala lys lys val thr tyr arg ile his phe lys arg ile val asn arg arg leu
GTA CTG CCG ACA GCG AAA AAA GTG ACC TAC CGT ATT CAC TTT AAA CGC ATT GTT AAC CGT CGT CTG

    140                                 150                                 160
ile met gly leu ala asp gly glu val leu val asp gly arg leu ile tyr thr ala ser asp leu
ATT ATG GGC CTG GCG GAT GGC GAA GTG CTG GTT GAT GGT CGT CTG ATC TAT ACC GCC AGC GAC CTG

                    170
lys val gly leu phe gln asp thr leu phe
AAA GTC GGT CTG TTC CAG GAT ACT CTG TTC TGA TTA TAA TCA GAC AGA GTA TCA AAA GGC GAA ACC

TCC GCA ATG CGG AGG TTT CTT TTT AAA GAG ACA GAA TCA GGC CAT TAC TAC CCT GTC CGC CAT GG
```

at 263 nm, is monitored spectrophotometrically.[145] Substrate NAC thiol esters have been used for nearly all studies of dehydrase-catalyzed reactions since they are considerably easier to synthesize[147,148] and to rigorously purify than are the natural ACP thiol esters.[149]

Kass[144,145] and Helmkamp and Bloch[150] reported specific activities of 3350 and 6000 units mg^{-1}, respectively, for highly purified dehydrase. (One unit corresponds to the formation of 1.0 nmol of **58** formed per minute under a standard set of spectrophotometric assay conditions.[145]) By careful spectrophotometric monitoring of chromatographic column effluents, so as to exclude contaminating proteins, Li obtained dehydrase with a specific activity of 8700 units mg^{-1}.[47]

Very recently, Henderson and Schwab have verified[153] the importance of dehydrase substrate purity and have developed procedures for obtaining highly purified **59**. By the use of HPLC-purified assay substrate **59** that had been synthesized by an optimized semihydrogenation procedure, highly purified dehydrase has given specific activities as high as 25 000 units mg^{-1}.[153]

2. Protein Structure

Dehydrase is a homodimeric protein[150] with subunits of 18 794 Da. This number is deduced on the basis of the nucleotide sequence[154] of the *fabA* gene, which codes for dehydrase[155–157] (Chart I).

There are two histidines per subunit. Various chemical modifications have pointed toward a key role for histidine in catalysis.[150] Photooxidation of the enzyme in the presence of methylene blue, as well as incubation with any of several α-bromocarbonyl compounds, leads to loss of enzyme activity concomitant with the modification of one histidine per subunit. The most conclusive observation is that incubation of dehydrase with the mechanism-based inactivator 3-decynoyl-NAC (section II.B.3.a) leads to the modification of a single histidine residue,[150] which by peptide mapping of covalently inactivated enzyme has been identified as His-70.[154]

Dehydrase is also inactivated by treatment with the tyrosine-selective reagent tetranitromethane.[150] Protection against tyrosine modification is afforded by 3-decynoyl-NAC, suggesting that there is an active site tyrosine residue. On the other hand, *N*-acetylimidazole, which is also a tyrosine modification reagent, has no effect on dehydrase's activity.[150] Thus, it is not clear if tyrosine has a direct role in catalysis.

There are two cysteines per subunit. Helmkamp and Bloch had reported that dehydrase fails to give a positive test for free sulfhydryl groups with either 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) or *p*-hydroxymercuribenzoate and that the enzyme can be carboxymethylated with iodoacetate only following treatment with dithiothreitol.[150] On this basis, they concluded that dehydrase has two disulfide bonds per dimer. Electrophoretic evidence suggested that these are intrasubunit disulfide bonds. On the other hand, in preliminary experiments Henderson and Schwab have found that dehydrase is sensitive to inactivation by *S*-methyl methanethiosulfonate (MMTS) and by methylmercuric chloride.[153] The oxidation states of the cysteine residues and the possible role(s) of cysteine sulfhydryls in catalysis will ultimately be revealed by X-ray crystallographic studies and/or by site-directed mutagenesis experiments.

The prospects for determining the three-dimensional structure of dehydrase are excellent now that the en-

zyme has been crystallized.[158] Dehydrase crystallizes in two forms, monoclinic and orthorhombic, and both crystal forms diffract to at least 1.6 Å. Efforts are currently under way in Smith's and Schwab's labs to produce crystals from dehydrase that has been inactivated with 3-decynoyl-NAC, as well as to produce crystals that are doped with heavy atoms, so that phase information can be obtained.

3. Mechanism

Consistent with the limited range of unsaturated fatty acids found in *E. coli*, dehydrase has a rather narrow substrate specificity. The initial rates of dehydration of C_8 and C_{12} 3-(hydroxyacyl)-NAC derivatives are no more than 5% that of the C_{10} compound, and C_8 and C_{12} β,γ-enoyl-NAC derivatives are not isomerized at an appreciable rate.[159] While the natural ACP thiol ester of 3-hydroxydecanoic acid is dehydrated fastest, the coenzyme A, NAC, and pantetheine thiol esters are also acceptable substrates. The initial rate of ACP thiol ester turnover is only 6 times that of the NAC thiol ester, and 10 times that of the pantetheine thiol ester. Coenzyme A[159] and 4-phosphopantetheine[141] thiol esters are dehydrated at about the same rate as the pantetheine thiol ester. Curiously, the *N*-acetyl-β-alanylcysteamine thiol ester of 3-hydroxydecanoic acid is a much poorer substrate than either the NAC or pantetheine thiol ester.[141] The relatively broad thiol ester selectivity of dehydrase is in contrast to those of the fatty acid elongating enzymes, whose requirements for ACP thiol esters are nearly absolute.[160] (*E*)-3-Decenoyl-NAC[159] and (*Z*)-2-decenoyl-NAC[141] are not isomerization substrates; thus, the double bond configurations of dehydrase's substrates are critical. Only the *R* enantiomer of 3-hydroxydecanoyl thiol ester is a substrate for enzymatic dehydration.[143,159] The K_m values for (*R*)-**57** and for **59** are 170 and 50 μM, respectively.[144]

The ratio of the three substrates at equilibrium depends upon the state of purity of dehydrase, since *E. coli* possesses enzymes for which **57**–**59** are substrates. With highly purified enzyme, the ratio is approximately 73:25:2, **57**/**58**/**59**.[159] It is curious that the concentration of **59** should be so low, since this compound is the precursor of all unsaturated fatty acids in *E. coli* and there is another dehydratase (a part of the fatty acid synthase) that can produce **58** from **57**.[161] In vivo, however, dehydrase is metabolically coupled[159] to β-ketoacyl-ACP synthase I,[162] which apparently has a greater affinity for **59** than for **58**. The level of expression of β-ketoacyl-ACP synthase I controls the proportion of saturated to unsaturated fatty acids.[162]

The sequence of interconversion of the three dehydrase substrates was proven by isotopic labeling to be as shown above.[163] By using doubly labeled substrates, it was shown that the dehydration of **57** to the nonconjugated enoyl thiol ester **59** involves the loss of one hydrogen atom from C-2. A similar result was obtained for the reverse of this reaction. Thus, **58** was implicated as an intermediate in the dehydrase-catalyzed dehydration of **57** to **59**. That it is an enzyme-bound intermediate was demonstrated by the fact that the reaction is not inhibited by added *N*-acetylcysteamine, which adds to free **58** in a Michael fashion. The dehydration of [2,2-$^2H_2$]**57** to **58** is accompanied by a kinetic isotope effect of 2.25;[163] thus, C_α–H bond breaking is the rate-determining step in the dehydration.

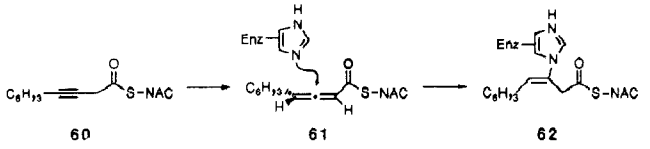
The fact that a C-2 hydrogen atom is lost to the solvent in the interconversion of **57** and **59** indicates that the allylic rearrangement cannot be accompanied by 1,3-hydrogen transfer.[163] This is consistent with the two-base mechanism proposed by Helmkamp and Bloch (section II.B.3.b).[150]

a. Mechanism-Based Inactivation. Dehydrase rapidly undergoes irreversible[164] inactivation by the acetylenic substrate analogue 3-decynoyl-NAC (**60**). The inactivation of dehydrase by **60**[159,165] is the first recognized example of mechanism-based enzyme inactivation.[3] The discovery[151,152] of **60** as a mechanism-based inactivator of dehydrase is a marvelous example of the importance of both serendipity and curiosity to the advancement of science. As already mentioned, (*Z*)-3-decenoyl-NAC (**59**) is the preferred substrate for the assay of dehydrase activity. In Bloch's laboratory, **59** was synthesized by the semihydrogenation of 3-decynoic acid, followed by thiol esterification of the olefinic acid. One particular sample of **59** was found to give anomalously low values for dehydrase activity. Further inspection showed the presence of a 5% impurity that was irreversibly inactivating the enzyme. A thorough analysis indicated that the impurity was **60**. Thiol esterification of the mixture of the olefinic acid plus the residual acetylenic acid (from the incomplete reduction of 3-decynoic acid) led to a mixture of substrate plus inactivator.

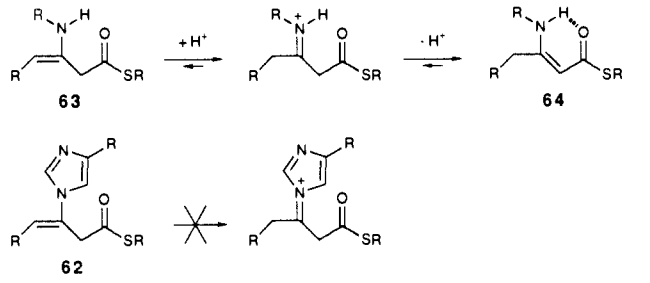
The mechanism by which **60** inactivates dehydrase has been thoroughly studied by Bloch et al. and subsequently by Schwab et al. At low concentrations of **60** (less than 1 μM), the inactivation of dehydrase is time-dependent.[164] Analysis of a Lineweaver–Burk plot of the inactivation data afforded an apparent K_I of 130 nM.[164] The apparent K_I values for a homologous series of 3-ynoyl-NAC derivatives parallel the $1/v_0$ values for an analogous series of 3-enoyl-NAC substrates.[164] This suggests that the inactivation is an active site directed phenomenon. That the inactivation of dehydrase by **60** is irreversible was demonstrated by a variety of methods, including dialysis, gel filtration, and electrophoresis.[164] Amino acid analysis showed that a single histidine residue is modified.[150] Competition experiments strongly implied that the same histidine residue is modified by **60** as by bromoacetate. In addition, photooxidized dehydrase loses the ability to bind **60**.

A critical clue to the mechanism of inactivation of dehydrase by **60** came from the observation of a kinetic deuterium isotope effect (k_H/k_D = 2.6) associated with C_α–H bond breaking.[166] Subsequently, 2,3-decadienoyl-NAC (**61**) was found to be an even more potent inactivator of dehydrase.[166] Inactivation of dehydrase by [2,4-$^2H_2$]**61** takes place without a primary kinetic deuterium isotope effect. Although dehydrase is not inactivated by 3-decynoic acid, it is inactivated by 2,3-decadienoic acid (albeit at higher concentrations).[166,167] This suggests that the role of the thiol ester moiety is to facilitate α-deprotonation, leading either to propargylic rearrangement of **60**, or to allylic rearrangement or dehydration of the normal substrates. It was suggested that **60** is isomerized by dehydrase to allenic thiol ester **61**, which is the actual enzyme inactivator (Scheme 12). The isomerization of **60** to **61** is directly analogous

SCHEME 12



SCHEME 13

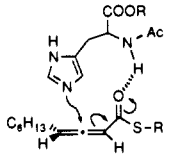


to the "normal" dehydrase-catalyzed isomerization of **58** to **59**.

The fate of the allenic thiol ester **61** was originally addressed by Morisaki through model chemistry[167] and by Stein, through chemical degradation of dehydrase that had been inactivated with **60**.[168]

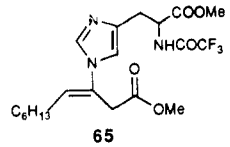
Morisaki characterized the adducts formed between *S*-ethyl 2,3-decadienethioate and each of several histidine derivatives.[167] Both *N*-acetylhistidine and *N*-acetylhistidine methyl ester react with the allenic thiol ester to give primarily vinylimidazole thiol ester adducts that have the double bond in the β,γ-position (as in structure **62**). The product of the reaction of histidine methyl ester with the allenic thiol ester is an α,β-enoate adduct, however. It was subsequently shown by Ho and Schwab (by high-field NMR analysis) that this adduct had resulted from attack of the α-amino group (rather than the imidazole moiety) on the allenic linkage (cf. **63**).[169] The reason for this difference in reactivity probably stems from the fact that **63** is an enamine, and its amino group is able to participate in the isomerization of the double bond (Scheme 13). Adduct **62**, however, is a vinylimidazole, and the lone pair on the lower nitrogen atom is part of the heterocycle's aromatic sextet. Although the nonconjugated enoyl thiol ester is the kinetic product,[147,170] the conjugated compound (e.g., **64**) is more stable and accumulates under the equilibrating reaction conditions.

Interestingly, 4-methylimidazole fails to react with *S*-ethyl 2,3-decadienethioate.[167] This implies a direct role for the non-imidazole portions of the histidine derivatives in their alkylation by the allenic thiol ester. A plausible mechanism is illustrated below.

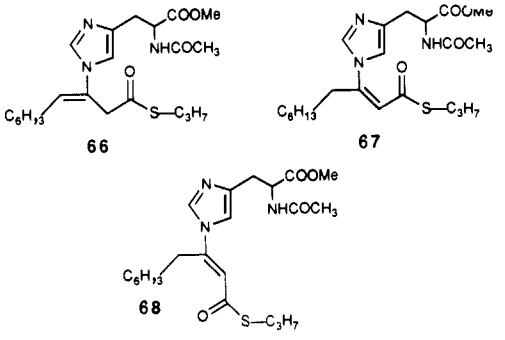


The ultimate fate of **60** in the course of enzyme inactivation has been addressed both by chemical degradation[168] and ^{13}C NMR analysis of inactivated dehydrase.[147]

Stein[168] inactivated dehydrase with *S*-ethyl [1-^{14}C]3-decynethioate and subjected the modified protein to acid hydrolysis. Two radioactive species were identified in the hydrolysate by ion exchange chromatography. (Similar observations were made previously by Helmkamp[171] and subsequently by Schwab et al.[147]) The major species was derivatized with methanolic HCl followed by trifluoroacetic anhydride. The radioactive derivative had a gas chromatographic retention time identical with that of **65**, made from one of Morisaki's model adducts.[167]



Schwab et al. extended Stein's work, by the application of ^{13}C NMR spectroscopy to the problem.[147] 3-[2-^{13}C]Decynoyl-NAC (**60**) was incubated with homogeneous dehydrase. ^{13}C NMR analysis showed the rapid appearance of a new peak at 45.0 ppm (line width = 36 Hz), and the slower appearance of a second signal, at 110.2 ppm (28 Hz). After two weeks at 4 °C the 110.2 ppm signal had increased at the expense of the 45.0 ppm signal. The signals were assigned by reference to the ^{13}C NMR spectra of model adducts **66**–**68**. Compound **66** (the *E* diastereomer, as shown) has a C-2 chemical shift of 45.1 ppm. Compounds **67** and **68** have C-2 chemical shifts of 116.4 and 114.0 ppm, respectively. Thus, **66** is an excellent model for the initial adduct formed between dehydrase and **60**, and **67** and **68** may be reasonably good models for the species responsible for the 110.2 ppm signal.



C-2 of the *Z* diastereomer of **66**, obtained by HPLC purification of the crude adduct, resonates at 51.3 ppm, which is significantly different from the chemical shift of the initial enzyme/inactivator adduct. This suggests that the inactivator moiety of the adduct has the double bond configuration shown in **66**.

The stoichiometry of the inactivation of dehydrase by **60** was also determined by the use of ^{13}C NMR spectroscopy.[172] A sample of dehydrase whose concentration had been rigorously calibrated by gravimetric analysis was titrated with 3-[2-^{13}C]decynoyl-NAC. After the addition of 2.0 subunit equivalents of the inactivator, *only* the 45.0 and 110.2 ppm signals were observed (in addition to the background spectrum of the protein). After the addition of a third subunit equivalent, two additional signals appeared. The major signal, at 27.5 ppm (line width = 3 Hz), corresponds to 3-[2-^{13}C]decynoic acid, resulting from nonspecific, protein-catalyzed hydrolysis of the thiol ester function. A minor signal, at 45.4 ppm (line width = 13 Hz), was assigned to an adduct analogous to the active site adduct (cf. **62** or **66**), but involving His-129, rather than His-70. Presumably **60** is isomerized by His-129 to **61**,

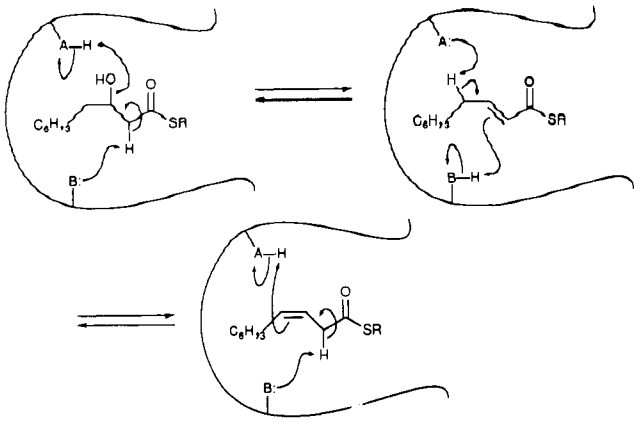


Figure 1. Hypothetical two-base mechanism of action for *E. coli* β-hydroxydecanoyl thiol ester dehydrase.

which alkylates the imidazole ring. In light of the intermediate line width of this signal, it was concluded that His-129 is on the periphery of the protein.

The discovery of *two* active-site enzyme/inactivator species by ^{13}C NMR spectroscopy provides an explanation for the observation made by both Stein and Helmkamp of two peaks of radioactivity in the hydrolysate of dehydrase inactivated with 3-[1-^{14}C]**60**. Although Stein correctly identified one of the adducts as a 3-decenoyl thiol ester species (cf. **62**), the structure of the other adduct and the process by which it arises were not addressed. Furthermore, the use of ^{13}C NMR spectroscopy allowed the configuration of the double bond of the inactivator moiety to be determined. Finally, the titration experiment provided a definitive answer to the nagging question of whether dehydrase has two independent active sites or a single functional active site per enzyme dimer.[150,164,166,168,171,173]

The fact that **60** is a potent inactivator of an enzyme that is unique to prokaryotes suggested that this compound might be an antibacterial agent in vivo. In fact, **60** inhibits the growth of *E. coli*,[174] while it has no effect on yeast or mammalian cells. Growth of *E. coli* can be sustained by the addition of oleic acid. Unfortunately, **60** proved not to be a useful antibiotic,[151] apparently due to enzyme-catalyzed decomposition. A mammalian enzyme has been found to isomerize **60** to the allenic thiol ester (**61**)[175-177] which is a substrate for crotonase-catalyzed hydration, to 3-(oxodecanoyl)-NAC.[178] Alternatively, the thiol ester function could simply undergo enzyme-catalyzed hydrolysis. As mentioned, dehydrase is unaffected by 3-decynoic acid.

b. Reaction Stereochemistry. On the basis of their chemical modification studies, Helmkamp and Bloch proposed that both histidine and tyrosine play direct roles in the supply and removal of protons at C-2, C-3, and C-4 of the substrate.[150,171] Such a "two-base" mechanism is illustrated in Figure 1.

An alternative mechanism, utilizing a single active site acid/base group for the supply and removal of protons at C-2, C-3, and C-4 of the substrate, can also be envisioned (Figure 2).

Rose and Hanson have suggested that the stereochemical course of an enzyme-catalyzed allylic rearrangement is suggestive of the number of active site acid/base groups.[123,124] Specifically, a suprafacial steric course implies a one-base mechanism, while an antarafacial steric course implies a two-base mechanism.

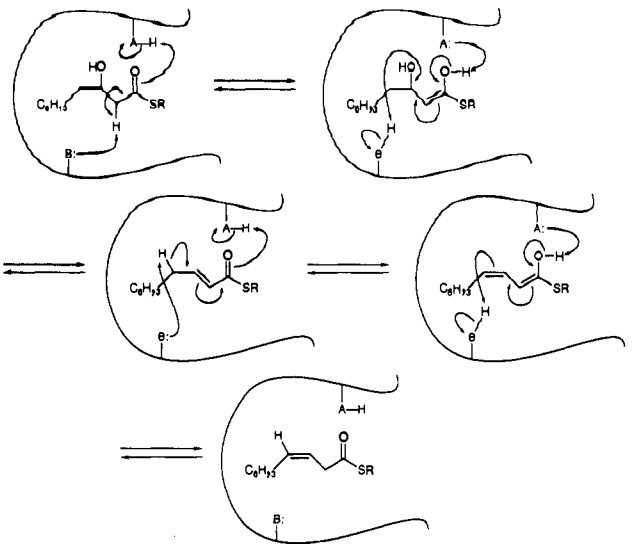


Figure 2. Hypothetical one-base mechanism of action for *E. coli* β-hydroxydecanoyl thiol ester dehydrase.

Using deuterium-labeled substrate samples and ^{2}H NMR spectroscopy for product analysis, Schwab and Klassen determined that the dehydrase-mediated allylic rearrangement has a suprafacial steric course,[179-181] indicative of a one-base mechanism. The *pro*-4*R* proton of **58**[179] and the *pro*-2*S* proton of **59**[180] are involved, as illustrated below. The extensive chemical modification,

58 **59**

amino acid sequence, and peptide mapping studies that have been described implicate His-70 as the active site acid/base. The fact that Rando and Bloch had observed loss of a C-2 proton in the course of the allylic rearrangement[163] (an observation confirmed during our stereochemical studies[181]) indicates that the conjugate acid of His-70 readily exchanges with the solvent.

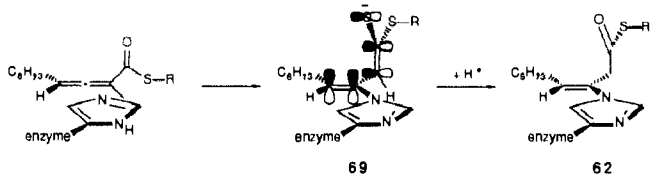
Schwab, Klassen, and Habib determined that in the dehydrase-catalyzed dehydration reaction, the *pro*-2*S* hydrogen of **57** is removed.[182,183] Since the C-3 hydroxyl group of **57** is in the *R* configuration, the dehydration must a syn elimination.

57 **58**

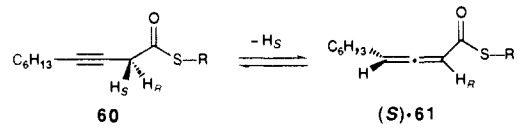
In both the dehydration and the allylic rearrangement, all of the groups that are involved (i.e., the hydroxyl group and the protons at C-2 and C-4) are on the same "face" of the substrate. This is again consistent with a one-base mechanism.

The mechanism-based inactivation of dehydrase by 3-decynoyl-NAC (**60**) is also understood in terms of a one-base mechanism. As mentioned, **60** is rearranged to **61**, which is the compound that alkylates His-70. Only (+)-**61** is highly inhibitory toward dehydrase.[176,177] (Dehydrase is also inactivated by (+)-2,3-decadienoic acid but not the (−)-enantiomer.[166,167]) If the mechanism by which **60** is converted to **61** is directly related to the enzyme's normal mechanism of action, then the stereochemical course of the propargylic rearrangement is predictable. In this case, the *pro*-2*S* proton of **60** should

SCHEME 14



be removed, and C-4 should be protonated from in front of the plane of the page, as shown below, giving (*S*)-**61**. As shown by X-ray crystallographic analysis of a derivative of (+)-2,3-decadienoic acid, this is the case.[184,185]



The stereochemical course of the alkylation of His-70 is also understandable in terms of a one-base mechanism (Scheme 14). For proper orbital overlap, a nucleophile must approach C-3 of **61** within the plane defined by carbons 2–5 and the C-4 hydrogen. If His-70 is the base that is responsible for substrate protonation and deprotonation, it should attack (*S*)-**61** from above the plane of the page. This leads, after protonation of the resulting enolate (**69**) at C-2, to adduct **62** with the *E* configuration about the C-3(4) double bond. As shown by ^{13}C NMR spectroscopy (see above),[147] this is the case.

The fact that **62** (rather than an isomer with a C-2(3) double bond (cf. **67** and **68**)) is the kinetically favored product is also readily understandable (Scheme 14). In intermediate **69**, the π-system of the enolate moiety is *orthogonal* to the C-3(4) π-system. In order for this enzyme-bound "dienolate" to be protonated at C-4, it would have to twist by 90° about the C-2(3) bond. For steric reasons alone, this may be unfavorable. Thus, it is logical that the adduct that is formed first is **62**.

The mechanism by which adduct **62** is isomerized to the species with the C-2 chemical shift of 110.2 ppm (cf. **67** or **68**) must be unrelated to dehydrase's normal mechanism of action. This is because in adduct **62**, His-70 (dehydrase's single active site base) is alkylated and can no longer remove a proton from C-2. It is for this reason that the rate of isomerization of **62** is slow. It is also not surprising that **62** does isomerize, since the conjugated product is more stable. It is possible that water (which must have access to the active site of dehydrase) is the catalyst in the rearrangement of **62**.

c. Additional Mechanistic Questions. New Experimental Approaches. Questions remain as to the nature of groups other than His-70 that participate in catalysis by dehydrase. Of particular interest is the mechanism of substrate deprotonation and whether the reaction intermediates are enolates or enols. Helmkamp and Bloch proposed that tyrosine may be involved in catalysis.[150,171] As with Δ^5-3-ketosteroid isomerase, it is conceivable that one of dehydrase's tyrosines (of five per subunit) participates in the concerted enolization of the substrates, or that it at least stabilizes enolate intermediates through hydrogen bonding. On the other hand (as conceded by Helmkamp and Bloch[150]), the evidence for an essential tyrosine is not overwhelming, and so this auxiliary role may be fulfilled by some other amino acid.

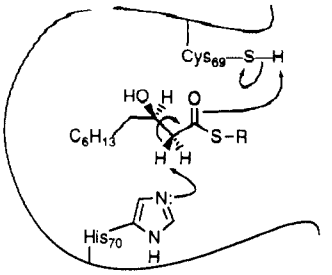


Figure 3. Proposed mechanism of substrate deprotonation by *E. coli* β-hydroxydecanoyl thiol ester dehydrase.

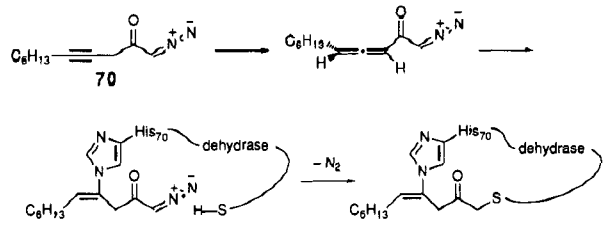
In a concerted enolization, the substrate in effect acts as a conduit for the deprotonation of an enzyme acid by an enzyme base. Histidine is a weak base (imidazolium $pK_a = 6.0$[186]), and tyrosine is a weak acid (phenol $pK_a = 10.1$[186]); therefore the equilibrium for histidine-mediated deprotonation of tyrosine strongly favors the neutral species. Dehydrase has a cysteine residue in position 69, directly adjacent to active site His-70.[154] While Helmkamp and Bloch had concluded that dehydrase's cysteines form intrasubunit disulfide bonds,[150,171] preliminary data from Schwab's lab suggest that there may be an active site sulfhydryl group.[153] Hydrogen bonds involving sulfhydryl groups are generally weaker than those involving hydroxyl groups;[187,188] nevertheless, the ability of cysteine to form useful hydrogen bonds in the context of enzyme-substrate interactions is proven.[189,190] At least in the free amino acid, cysteine's sulfhydryl group is relatively acidic ($pK_a = 8.35$[186]), and so from this perspective cysteine would be a better general acid than would be tyrosine. A working hypothesis for dehydrase-catalyzed substrate enolization is shown in Figure 3.

In an attempt to determine the nature of the general acid, Henderson et al. have begun to characterize the chemistry of the potential crosslinking reagent 1-diazo-4-undecyn-2-one (**70**).[191] Compound **70** irreversibly inactivates dehydrase in a time- and concentration-dependent manner. Substrate **59** protects the enzyme against inactivation. The inactivation is stoichiometric: one molecule of **70** becomes bound to each dehydrase subunit. That **70** is a mechanism-based inactivator rather than an affinity label is indicated by the kinetic deuterium isotope effect of 5.2 for breaking the C(3)–H bond. Also, the saturated analogue, 1-diazo-2-undecanone, does not inactivate dehydrase. Finally, FT-IR experiments have shown that the diazo ketone moiety is destroyed during the inactivation of dehydrase by **70** when all incubations and manipulations are carried out in the dark. (The diazo ketone group was originally chosen because photoirradiation can lead to a ketene, via Wolff rearrangement.) This behavior is reminiscent of the inactivation of various enzymes with essential sulfhydryl groups (e.g., papain) by α-diazo ketone substrate analogues[192-194] and suggests that dehydrase may also have an essential sulfhydryl. A working hypothesis for the mechanism of dehydrase inactivation by **70** is shown in Scheme 15. Experiments are in progress to shed light on the actual mechanism and the nature of the modifications to the protein.

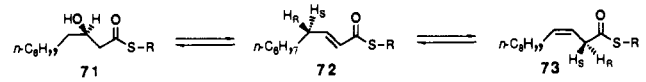
C. β-Hydroxydodecanoyl Thiol Ester Dehydrase

In *Brevibacterium ammoniagenes*, unsaturated fatty acids are made by a series of reactions that closely

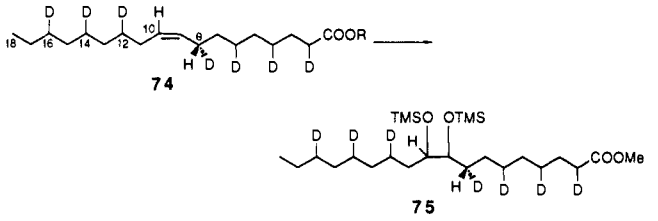
SCHEME 15



SCHEME 16



SCHEME 17



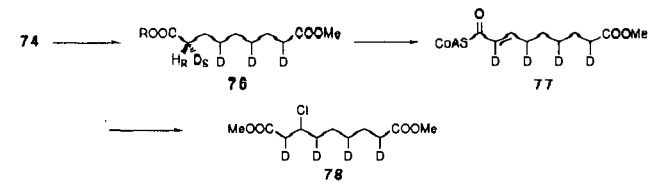
parallels the pathway used in *E. coli*. The critical enzyme in the *B. ammoniagenes* pathway is β-hydroxydodecanoyl thiol ester dehydrase. This enzyme, which is functionally analogous to β-hydroxydecanoyl thiol ester dehydrase of *E. coli*, interconverts the 12-carbon thiol esters **71**, **72**, and **73** (Scheme 16). In contrast to the *E. coli* enzyme, however, the *B. ammoniagenes* dehydrase is part of a fatty acid synthase (FAS) multi-enzyme complex with a molecular mass estimated to be 1.2×10^6.[195-197]

Kawaguchi and his co-workers have investigated the stereochemical course of the allylic rearrangement catalyzed by the *B. ammoniagenes* β-hydroxydodecanoyl thiol ester dehydrase.[198] Since the dehydrase cannot be separated from the other FAS enzymes, the reaction had to be studied indirectly. [2,2-$^2H_2$]Malonyl-CoA was converted to oleic acid by using the purified FAS, and the positions and configurations of the deuterium atoms in the labeled oleic acid were analyzed as follows.

The methyl ester (**74**, R = Me) of the labeled oleic acid was oxidized (OsO_4) to the corresponding diol, which was converted to bis(trimethylsilyl) ether **75** (Scheme 17). Analysis of **75** by mass spectrometry showed that the C-10 to C-18 segment of the labeled oleic acid contained no more than three deuterium atoms. Since [2,2-$^2H_2$]malonyl-CoA labels the even-numbered positions of fatty acids, it was concluded that the three deuterium atoms in the C-10 to C-18 segment were located at positions 12, 14, and 16 and that there could be *no* deuterium at position 10. Previous experiments had been interpreted as showing that deuterium from [2,2-$^2H_2$]malonyl-CoA is incorporated by the *B. ammoniagenes* FAS into the *pro-S* positions at even-numbered carbons,[199] and so it was concluded that the *pro*-4*S* hydrogen of enzyme-bound intermediate **72** is lost in the rearrangement.

The configuration at C-8 of **74** was investigated (Scheme 18) by converting a portion of it to monomethyl nonanedioate (**76**),[200] and thence to **77**, with acyl-CoA synthetase and acyl-CoA oxidase.[198] (The

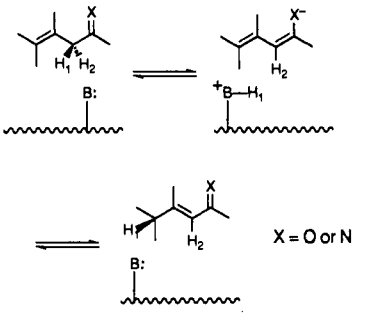
SCHEME 18



oxidase had previously been shown to effect anti elimination of the *pro*-2*R* and *pro*-3*R* protons of stearoyl- and nonanoyl-CoA.[201]) Compound **77** was methanolized and converted to chlorinated diester **78**, by treatment with HCl.

The intensities of the $M^+ - (CH_3OH + Cl)$ mass spectral ions derived from the isotopomers of **78** were compared to those calculated for two possible cases: (i) in which deuterium occupied the *pro*-2*S* position of **76** (R = H) (corresponding to the *pro*-2*R* position of **73**) and had not been abstracted by acyl-CoA oxidase; and (ii) in which deuterium occupied the *pro*-2*R* position of **76** (R = H) (corresponding to the *pro*-2*S* position of **73**) and had been abstracted by acyl-CoA oxidase. The experimental results fell *between* these two calculated extremes. Nevertheless the authors concluded that deuterium was in the *pro*-2*S* position of **76** (R = H), and therefore inferred that a proton is added to the *si* face (the top face, as shown in Scheme 16) of the double bond at C-2 of **72** during the allylic isomerization. (C-2 of **72** synthesized from [2,2-$^2H_2$]malonyl-CoA is deuterated.) This suggests[123,124] a two-base, antarafacial rearrangement.

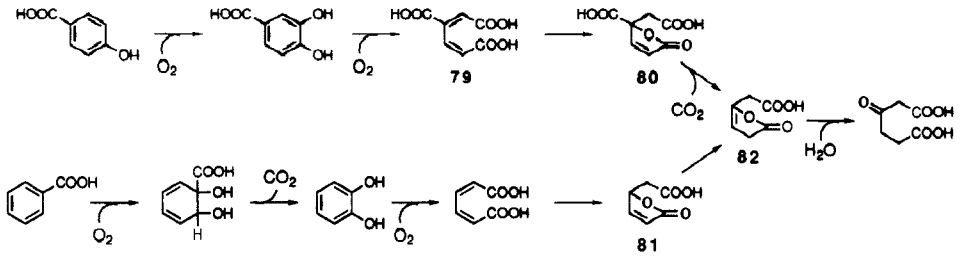
As noted previously,[181] there are a number of allylic rearrangements in which resonance-stabilized carbanion (or the corresponding enol or enol-like) intermediates can be formed from the substrates or products. These allylic rearrangements tend to have suprafacial steric courses, thus implicating one-base mechanisms. Presumably, then, evolution has selected for enzymes with the minimal number of acid/base groups needed to effect the "easy" allylic rearrangements.



On the basis of the experiments of the Japanese group, the *B. ammoniagenes* β-hydroxydodecanoyl thiol ester dehydrase appeared to be the *sole* exception to this general rule. Certain factors, however, made it attractive to reinvestigate the steric course of this reaction. These are the complex and indirect nature of the analytical scheme,[198] questionable assumptions concerning the stereo- and regiospecificity of acyl-CoA oxidase with monomethyl nonanedioate, and the fact that the experimental mass spectral ion intensities fell *between* the values calculated by assuming *si*- or *re*-face protonation at C-2 of intermediate **72**.

Very recently, the steric course of this allylic rearrangement has been redetermined,[202] by the application

SCHEME 19



of degradative chemistry and spectroscopic techniques that had been developed in the laboratory of Vederas.[203-207] In brief, *B. ammoniagenes* was grown on [$^2H_3$,2-^{13}C]acetate, and labeled oleic acid was isolated. The absence of deuterium at C-10 of the labeled oleic acid was confirmed by one-dimensional ^{13}C NMR spectroscopy. A portion of the oleic acid was degraded to octanoic acid (corresponding to C-11 through C-18 of oleic acid) and to monomethyl nonanedioate (corresponding to C-1 through C-9 of oleic acid, with the esterified carboxyl of the derivative matching C-1 of oleic acid). The degradation products were converted to their methyl mandelate ester derivatives, which were analyzed by deuterium-decoupled ^{1}H–^{13}C correlation spectroscopy. Analysis in this fashion confirmed that deuterium at C-8 of the labeled oleic acid occupies the *pro-S* position. However, *in contrast* to the claim of Saito et al.,[199] the 2D NMR experiments clearly showed that the configuration at C-12 of oleic acid biosynthesized from deuterated acetate is *R*. Thus it is the *pro-4R* proton of the enzyme-bound 12-carbon biosynthetic intermediate that is removed in the course of the allylic rearrangement. The overall stereochemical course of the allylic rearrangement catalyzed by the *B. ammoniagenes* dehydrase is therefore *suprafacial.*

D. Muconolactone Isomerase

Muconolactone isomerase (MLI) converts (+)-muconolactone (**81**) to β-ketoadipate enol-lactone (**82**), an integral step in the bacterial pathway for degradation of catechol.[208] The catechol pathway is shown in Scheme 19, along with the closely related protocatechuate pathway. Both of these pathways feed into the β-ketoadipate pathway, providing a general, convergent, substrate-inducible[209] route used by a variety of aerobic bacteria for the degradation of aromatic substances.

1. Purification and Properties

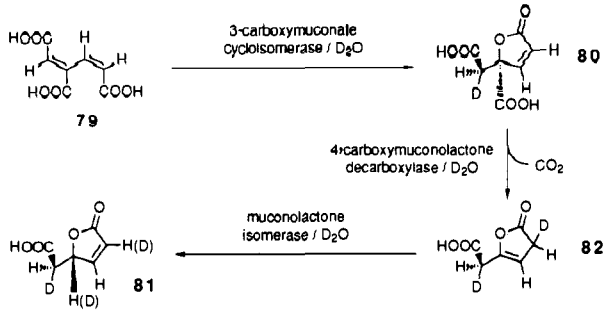
The purification of MLI originally reported by Ornston[210] gave a crystalline enzyme with a specific activity of 695 U/mg and an overall recovery of 19% of the starting activity. (One unit of MLI activity corresponds to the isomerization of 1.0 μmol of **81**/min.) MLI activity was found to be maximal at pH 8.5 in 33 mM Tris-Cl, and the enzyme's molecular mass was estimated at 93 kDa by gel filtration.[211] Subsequent purification of MLI by an improved procedure gave homogeneous enzyme of specific activity 856 U/mg in 74% recovery.[212] This enzyme preparation was shown to be immunologically pure, unlike that obtained previously.[210] The subunit M_r of 12000 suggests that the native enzyme comprises eight identical subunits.[212] Subsequently, sedimentation equilibrium ultracentrifugation gave a relative molecular mass for the native protein of 111000, which is consistent with a decameric structure.[213] Cross linking with dimethyl suberimidate led to the characterization of two species: a major one with M_r 62000–65000, and a minor one with M_r 124000. These results suggested that MLI could be a decamer or a dodecamer, comprising stacked disks of pentamers or hexamers.[213] Indeed, low-resolution crystallographic studies subsequently showed that MLI is a homodecamer with a five-fold axis of symmetry.[214]

The amino acid sequence of the *P. putida* MLI has very recently been deduced on the basis of the cDNA sequence. Each subunit comprises 96 residues and has a molecular mass of 11068.[215] Partially on the basis of this information, the crystal structure has now been refined to 3.3 Å resolution.[216] Secondary and tertiary structural motifs within the subunits were characterized, as were the intersubunit interactions. The decamer is nearly spherical and has a large, solvent-accessible, hydrophilic cavity in the center. It was hypothesized that the active site occupies a hydrophobic region at the interface between subunits and that portions of neighboring subunits are involved. An attempt was also made to identify amino acid residues that might be involved in catalysis. Nevertheless, meaningful suggestions along these lines and the assignment of roles in catalysis to specific amino acids will have to await further refinement of the structure and, ideally, obtention of a structure of a complex between MLI and a substrate or substrate analogue.

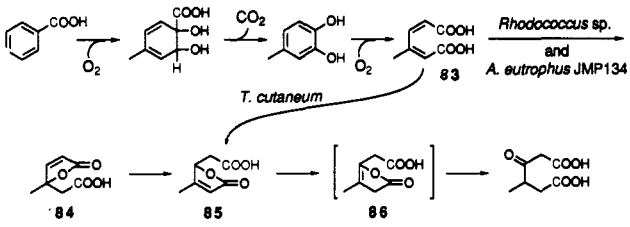
MLI and 4-carboxymuconolactone decarboxylase occupy parallel positions in the catechol and protocatechuate pathways, respectively. The substrates for the two enzymes (**81** and **80**, respectively) are similar, and the same product (**82**) is obtained from the two reactions. Both enzymes catalyze double bond rearrangements, and both reactions involve protonation at C-2 of the lactone ring. Since they catalyze similar reactions, the structural relationship between the two enzymes was of considerable interest. This has been investigated from several perspectives. Using enzyme purified by the original procedure, Ornston was able to show that one unit of MLI caused the disappearance of no more than 1.0 nmol of **80** per minute.[210] This demonstrated both the relative purity of the enzyme preparation as well as its specificity for its own substrate and reaction mechanism.

Amino acid sequencing of enzymes of the β-ketoadipate pathway from both *P. putida* and *Acinetobacter calcoaceticus* revealed interesting structural similarities.[217] The N-terminal regions of the MLIs and the decarboxylases show substantial sequence identity. In addition, there is significant sequence identity between residues 1–12 of the decarboxylases and residues 24–35 of the isomerases.[218] Analogous sequence homologies have been reported for *Pseudomonas* MLI and *Acinetobacter* enol-lactone hydrolase, although the sequences of these two enzymes from a single organism, either

SCHEME 20



SCHEME 21



Acinetobacter or *Pseudomonas*, are quite different.[213,219,220] These findings indicate complex evolutionary relationships between the proteins and suggest the existence of a common ancestral gene. A review on this subject has been published.[221]

2. Mechanism

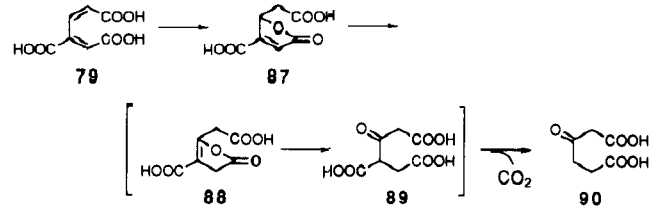
The stereochemical course of the reaction catalyzed by MLI has been investigated by a very clever ^1H NMR based "ricochet" analysis (Scheme 20).[222] The experiment utilizes three coupled enzymatic reactions and allows determination of the stereochemical courses of two of the reactions. A prerequisite was prior knowledge of the absolute configurations of enzymatically-produced (+)-81 (S) and 80 (*R*).[223]

In this experiment, **79** was converted to (4*R*,5*R*)-[5-$^2H_1$]**80** by 3-carboxymuconate cycloisomerase from *P. putida*, in D_2O. Enzymic decarboxylation gave dideuterated β-ketoadipate enol-lactone (**82**) retaining the *R* configuration at C-5, and presumably chirally deuterated at C-2 as well. The enol-lactone was then "ricocheted" by *P. putida* MLI to deuterated (4*S*,5*R*)-**81**. This experimental design exploits the favorable equilibrium for the MLI reaction[208] when run in the absence of β-ketoadipate enol-lactone hydrolase (to which it is physiologically coupled).

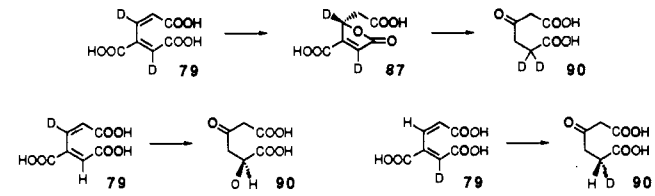
The ^1H NMR spectrum of the deuterated **81** included a signal for a proton at C-4 (δ_H 5.32 ppm). Since the enzymatic reactions had been performed in D_2O, this proton could not have come from the solvent; therefore it must have arisn by direct transfer from C-2 of **82**. This implies a suprafacial proton transfer by a single, univalent base. Since (+)-**81** has the 4*S* configuration, the transfer of the proton rather than the deuteron from C-2 of **82** implies that the latter has the *R* configuration at C-2, and therefore indicates that the decarboxylation must also be a syn process. Identical results were obtained for the analogous process in *A. calcoaceticus*.[222]

A functionally similar lactone isomerase apparently participates in the degradation of 4-methylcatechol by the eukaryotic microorganism *Trichosporon cutaneum* (Scheme 21).[224] In this organism, dioxygenase-mediated ring cleavage of 4-methylcatechol provides 3-methyl-*cis*,*cis*-muconate (**83**), which is cyclized to 3-

SCHEME 22



SCHEME 23



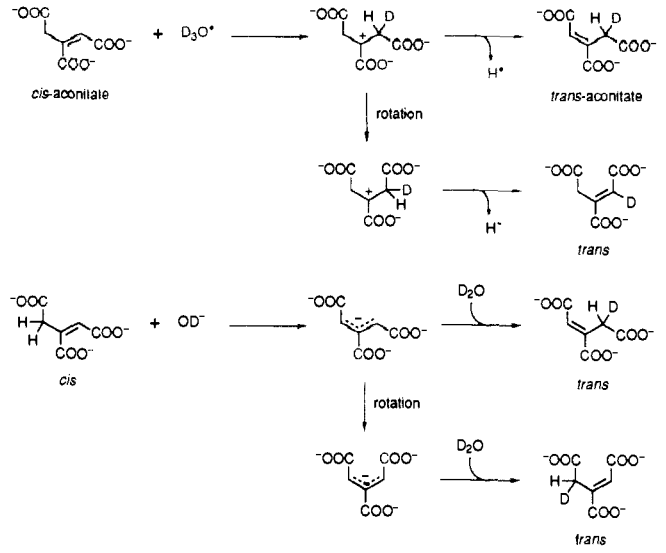
methylmuconolactone (**85**). Compound **85** is converted to (−)-3-oxo-4-methyladipate, which undergoes further degradation, ultimately leading to pyruvate and acetyl-CoA. The metabolism of **85** presumably involves an allylic rearrangement to enol-lactone **86**, which is hydrolyzed to the keto diacid. The intermediacy of **86** in this process has not been proven, however.

A curious variation on this pathway occurs in the metabolism of 4-methylbenzoic acid by various species of *Rhodococcus*[225] and in the degradation of (4-methylphenoxy)acetate (via 4-methylcatechol) by *Alcaligenes eutrophus* JMP134 (Scheme 21).[226-228] In both pathways, **83** is produced, by reactions that parallel those of the catechol pathway (Scheme 19). Compound **83** undergoes cyclization to 4-methylmuconolactone (**84**). The latter is isomerized to **85**, which is degraded to 3-oxo-4-methyladipate. While the rearrangement of **85** to **86** is implied by each overall pathway, the latter compound has not been identified as a discreet intermediate.[225,226]

Another interesting variation on the above pathway is seen in the metabolism of protocatechuate by fungi.[229,230] *p*-Hydroxybenzoate or protocatechuate is converted to 3-carboxy-*cis*,*cis*-muconate by the same series of reactions utilized by bacteria (Scheme 19). However, the triacid is cyclized to 3- rather than 4-carboxymuconolactone (Scheme 22). Then, by a pathway that remained obscure for over 20 years[229,231] and which has still not been characterized very well at the enzyme level, the lactone ring is opened, carbon dioxide is lost, and 3-oxoadipate (**90**) is produced. This is subsequently degraded to succinate and acetate.

In the pathway shown in Scheme 22, **88** and **89** are mechanistically plausible metabolic intermediates whose existence had been purely speculative until put to the test by deuterium labeling experiments (Scheme 23).[231] Incubation of [2,4-$^2H_2$]**79** with a cell-free preparation from a strain of *Neurospora crassa* led to the isolation of *did*euterio-**90**. This clearly implicates a migration of deuterium from C-5 of the substrate, presumably via a 1,3 allylic rearrangement involving lactone intermediates **87** and **88**, as portrayed above. By employing two singly deuterated samples of **79**, two samples of [5-$^2H_1$]**90** were obtained. Their absolute configurations were established by correlation with (*R*)- and (*S*)-[$^2H_1$]succinic acid. Since **87** produced by *N. crassa* has the *S* configuration,[232] the presumed allylic rearrangement of the lactone intermediate must be a suprafacial process. Since little or no exchange of label

SCHEME 24



was observed, the rearrangement must utilize a single, univalent base.

E. Aconitate Isomerase

Aconitate isomerase,[233,234] which interconverts *cis*- and *trans*-aconitate, presented a particularly intriguing subject for study because distinctly different, chemically plausible, and biochemically precedentated mechanisms could be envisioned (Scheme 24). These include (a) cis–trans isomerization via torsion about the C-2/C-3 bond (perhaps involving nucleophilic addition followed by elimination (cf. maleate isomerase[235])) and (b) 1,3-allylic rearrangement. Either mechanism could involve a carbanion or a carbocation intermediate and would be consistent with extensive exchange of substrate protons with the aqueous reaction medium.

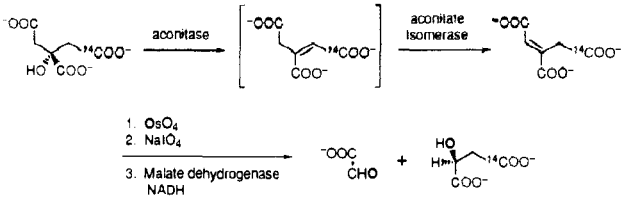
Aconitate isomerase was originally obtained from a pseudomonad grown on *trans*-aconitate as its sole carbon source.[233] The enzyme is assayed by monitoring the concentration of citrate, formed by the action of aconitase on *cis*-aconitate.

Aconitate isomerase was subsequently purified by 50- to 100-fold, such that it was estimated to comprise somewhere between 3 and 40% of the total protein preparation.[234] By preincubation with EDTA, the isomerase was shown not to have a requirement for exogenous metals (although it is conceivable that it possesses tightly bound metal ion). The native enzyme has a relative molecular mass of 78 000 ± 10 000. By thorough analysis of a great deal of kinetic data, it was deduced that the isomerase is susceptible to dissociation and that its two subunits either are nonidentical or form active dimers through an unsymmetrical association.[234]

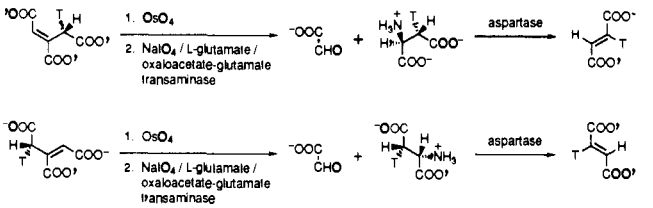
In preliminary studies, with no preincubation of the enzyme, an initial burst of activity, followed by a slower steady-state rate, was observed. Testing of various supplements to the assay cocktail revealed that in the presence of 20% glycerol, the rate of decay of the activity burst is greatly diminished. Extensive kinetic studies of the glycerol effect were carried out, and it was concluded that glycerol activates the isomerase through stabilization of its undissociated form.[234]

Klinman and Rose also conducted isotopic-labeling experiments to distinguish between the various mechanisms portrayed in Scheme 24.[236] Unlabeled *cis*-aconitate was incubated with aconitate isomerase in a D_2O buffer, and ^1H NMR analysis of the resulting *trans*-aconitate indicated that one atom of deuterium had been incorporated in an *allylic* position. Thus, the second mechanism in Scheme 24 (involving a carbocation intermediate or addition/elimination, with rotation about the C-2/C-3 bond) was eliminated. To determine if there were double bond rearrangement (consistent with the first or third mechanism), [1-^{14}C]citrate was synthesized, and this was converted to *trans*-aconitate, via *cis*-aconitate, by the combined action of aconitase and aconitate isomerase (Scheme 25). By degradation of the *trans*-aconitate to glyoxylate and [^{14}C]malate, it was deduced that the isomerization had involved migration of the double bond. This is consistent only with the first and third mechanisms in Scheme 24, each involving an allylic rearrangement.

SCHEME 25



SCHEME 26



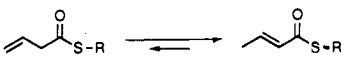
The stereochemical course of the allylic rearrangement was then evaluated.[236] *cis*- and *trans*-aconitate were equilibrated in tritiated water, and the site of tritium labeling at the allylic methylene carbon of each aconitate sample was ascertained by the analysis portrayed in Scheme 26. Aconitate was oxidized to hydroxycitrate, as before. This was converted to glyoxylate plus tritiated L-aspartate, which was incubated with aspartase (known to produce fumarate by removal of the substrate *pro*-3*R* hydrogen). Since very little of the tritium from both the aspartase-equilibrated samples was found to be volatile, it was concluded that both aconitate samples had the *S* configuration at carbon-4. Thus, it could be concluded that the aconitate isomerase reaction is a suprafacial 1,3-allylic rearrangement. A single catalytic base could be inferred both from the steric course of the reaction.[123,124] and from the fact that there is a small but finite amount of proton transfer between the rearrangement termini. The simplest explanation for any degree of proton transfer is that C–H bond breaking precedes C–H bond making, thus implying a carbanion reaction intermediate. A carbocation intermediate is also conceivable (albeit unlikely), in which the proton transfer would have to be intermolecular rather than intramolecular. Also, a thorough analysis of tritium isotope effect data implicates a univalent enzyme base, and appears to rule out lysine as the active site base.

Finally, calculations of the rate constant for the nonenzymatic, base-catalyzed isomerization of *cis*-aconitate and of the turnover number of aconitate isom-

erase were carried out.[236] The enzymatic rate acceleration was calculated to be in the neighborhood of (1–12) × 10^8. It was noted that the enzyme's role may be largely to neutralize the negative charge on the three carboxyl groups (all of which are ionized at high pH) and therefore to facilitate formation of a substrate carbanion. The enforcement of conformational rigidity in the substrate is also clearly critical, since the carbanion intermediate cannot be formed without proper orbital overlap.

F. Vinylacetyl-CoA Isomerase

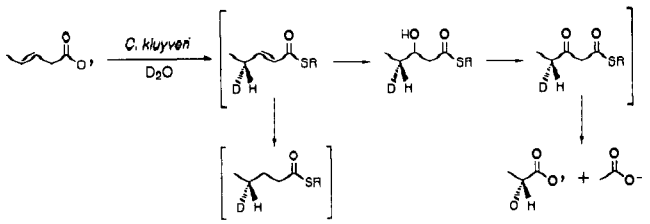
Vinylacetyl-CoA isomerase interconverts thiol esters of short-chain Δ^2- and Δ^3-carboxylic acids. It is found in mammals[237,238] and in microorganisms such as various species of *Clostridium*.[238,239] Purification of the bacterial enzyme has proven difficult due to its instability.[238] The greatest success has been reported by Schleicher and Simon,[240] who used a modification of an earlier procedure[238] for the purification of the isomerase from *Clostridium kluyveri*. Enzyme thus obtained had been purified by 32-fold; however, only 8.5% of the activity present in the crude cell extract was recovered.



The relative molecular mass of the bacterial enzyme has been estimated at 170 000 by gel filtration.[240] This figure may be an underestimate, however, since it was noted that the isomerase emerges in the void volume of the Sephadex G-200 column. Inhibition of the isomerase by *p*-chloromercuribenzoate[238] is consistent with an essential thiol group, leading Bartsch and Barker to suggest a possible "X-group" mechanism of action. Such a mechanism would involve nucleophilic attack of the enzyme sulfhydryl group at C-3 of the substrate 3-butenoyl thiol ester, with protonation at C-4. This would be followed by deprotonation at C-2 and expulsion of the sulfhydryl group. A simple prototropic shift mechanism was also considered.[238] Subsequently, it was found that the isomerase is rapidly and completely inactivated by 3-butynoyl-NAC.[240] This is reminiscent of the inactivation of *E. coli* β-hydroxydecanoyl thiol ester dehydrase by 3-decynoyl-NAC[166] and of *P. testosteroni* Δ^5-3-ketosteroid isomerase by acetylenic secosteroid substrate analogues.[55] As already noted, both of these enzymes catalyze allylic rearrangements via enol or enolate intermediates rather than by addition/elimination mechanisms.

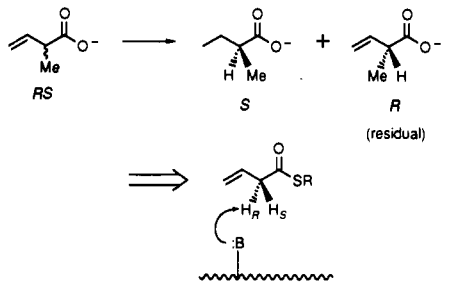
The enol/enolate mechanism is supported by isotope exchange experiments.[240] When 3-butenoyl-NAC was incubated with the isomerase in D_2O, 0.56 D was incorporated at C-4. In TOH, only 0.07 T was incorporated. That this low figure could be attributed to a solvent tritium isotope effect was confirmed by running the same experiment in a mixture of TOD and D_2O. In this experiment, 0.27 T was incorporated at C-4, along with 0.60 D. In the complementary experiment, enzymic isomerization of 3-[2,3-^{3}H]butenoyl-NAC in H_2O was accompanied by the transfer of 0.46–0.48 T to C-4. (The missing tritium was found in the aqueous medium.) Substantial transfer of tritium to C-4 is most easily explained by an mechanism involving an enol or enolate intermediate by using a single active site base. The enzyme base is unlikely to be a lysine amino group, since statistically no more than 0.33 T should have been transferred in the experiments involving C-2 labeled substrate. Clearly, when the enzyme base is in its conjugate acid form, it is accessible to the medium, since intramolecular transfer of label is not quantitative, and because label is incorporated from D_2O, TOH, or TOD. Finally, one incubation involving the tritiated 3-butenoyl-NAC was carried to completion, and the other was terminated after 19% of the substrate had been isomerized. Essentially identical amounts of tritium were found at C-4, indicating that C–H bond breaking is not rate-determining.

SCHEME 27



Although *C. kluyveri* vinylacetyl-CoA isomerase has a relatively narrow substrate specificity, the precise range of substrates that can be accommodated is not clear. Bartsch and Barker incubated 3-butenoyl-NAC, 3-methyl-3-butenoyl-NAC, 3-pentenoyl-NAC, and 3-hexenoyl-NAC with their 4- to 8-fold-purified enzyme. They found little if any isomerization of the latter three compounds.[238] In addition to the NAC thiol ester, pantetheine and coenzyme A thiol esters of 3-butenoic acid were found to be acceptable substrates.[238] On the other hand, Simon and co-workers' studies of the steric course of the isomerization were predicted upon the assumption that the *C. kluyveri* isomerase would process thiol esters of 2-methyl-3-butenoic acid and 3-pentenoic acid.[241]

Simon and co-workers[240,241] used washed, whole *C. kluyveri* cells to show that (*R*,*S*)-2-methyl-3-butenoic acid is only partially converted to 2-methylbutyrate (via isomerization to 2-methyl-2-butenoate, followed by reduction of the double bond of the latter).[241] The residual 2-methyl-3-butenoic acid was found to be entirely the *R*-(−) isomer, suggesting that only the *S*-(+) enantiomer had reacted. This implies that it is the *pro*-2*R* hydrogen atom of 3-butenoyl-CoA that is removed en route to 2-butenoyl-CoA.



A complementary experiment was conducted, involving fermentation of 3-pentenoate by washed cells in D_2O (Scheme 27). One equivalent of 2-pentenoate was reduced to pentanoate, while one equivalent was degraded to propionate plus acetate. The propionate was isolated, and its absolute configuration was shown to be *R*.[242,243] Consequently it was inferred that hydrogen had entered from the *re* side at C-3 of 3-pentenoate. Taken along with the results of the previous experiment, the vinylacetyl-CoA isomerase reaction is therefore a suprafacial proton transfer process.

TABLE II. Substrates for Mammalian Vinylacetyl-CoA Isomerase

compound	rel act. (V_{max})[a,b]	ref
3-butenoyl-CoA	100	237
3-methyl-3-butenoyl-CoA	100	237
(*Z*)-3-hexenoyl-CoA	48	245
(*E*)-3-hexenoyl-CoA	10	237, 245
(*Z*)-3-octenoyl-CoA	8.1	245
(*E*)-3-octenoyl-CoA	0.48	245
(*Z*)-3-decenoyl-CoA	7.6	245
(*E*)-3-decenoyl-CoA	0.48	245
(*Z*)-3-dodecenoyl-CoA	7.6	245

[a] Although slightly different assay conditions were used by Rilling and Coon[237] and Struijk and Beerthuis,[245] (*E*)-3-hexenoyl-CoA serves as a benchmark, since its rate of reaction was evaluated by both groups. [b] Both (*E*)- and (*Z*)-3-alkenoyl-CoA substrates are converted to (*E*)-2-alkenoyl-CoA products.[245]

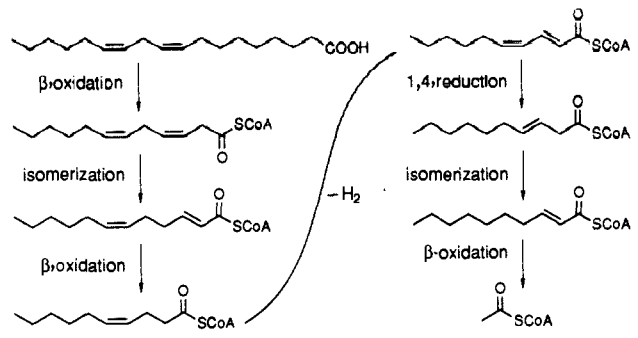
The fact that Simon and his associates used whole cells for their stereochemical studies may be cause for caution in the interpretation of the experimental results. This is because it had been reported that 3-pentenoyl-NAC is *not* a substrate for the partially purified isomerase.[238] That 3-pentenoic acid was successfully utilized in Simon's experiments suggests either that 3-pentenoyl-CoA *is* a substrate although 3-pentenoyl-NAC is not, or that there are multiple routes for degradation of 3-pentenoyl-CoA. On the other hand, it is reassuring that Simon's results point to a suprafacial mechanism, which conforms to the general trend for isomerases that act on relatively acidic substrates.

Vinylacetyl-CoA isomerase activity has also been found in a variety of mammalian tissues. An enzyme with this activity has been partially purified by an estimated 80-fold from ox liver mitochondria.[237] It was suggested[237] that the ox liver isomerase's physiological function may be to participate in the degradative metabolism of unsaturated fatty acids. As mentioned in section II.G, (*E*)-3-decenoyl-CoA is an intermediate in the enzymatic degradation of linoleic acid, and it is proposed that this compound is isomerized to (*E*)-2-decenoyl-CoA, which enters into the β-oxidation pathway.[244] In this context, the results of substrate specificity studies are rather perplexing, since (Table II) (*E*)-3-decenoyl-CoA is turned over by the ox liver enzyme at only a small fraction of the rate of turnover of 3-butenoyl-CoA or 3-methyl-3-butenoyl-CoA. (The K_m values for all of the substrates listed below are not strikingly different from one another.[245])

The data in Table II are even more perplexing in light of the uncertain metabolic role of 3-methyl-3-butenoyl-CoA. An interesting alternative explanation is that the isomerase activity of the ox liver enzyme is an artifact. In the course of leucine catabolism, 3-methyl-2-butenoyl-CoA is carboxylated to 3-methylglutaconyl-CoA. It is conceivable that the carboxylase catalyzes the isomerization by adventitiously removing an α-proton of 3-methyl-3-butenoyl-CoA (rather than a γ-proton of 3-methyl-2-butenoyl-CoA, as would be the enzyme's normal function). Indeed, it is noted that "attempts to free the liver carboxylase of the isomerase have not yet proved successful".[237] Still, it could be merely coincidental that 3-butenoyl-CoA and 3-methyl-3-butenoyl-CoA are turned over so much faster than are the longer, straight-chain enoyl-CoAs.

In contrast to the *C. kluyveri* isomerase, the ox liver enzyme is quite stable either frozen or when stored at 5 °C, and it is not air-sensitive. In addition, no tritium is incorporated into 3-methyl-3-butenoyl-CoA when the isomerization is run in TOH. This implies a direct transfer of a proton from C-2 to C-4, by a single, univalent base. As mentioned, 3-butenoyl-CoA and 3-methyl-3-butenoyl-CoA are optimal substrates for the isomerase, and 3-hexenoyl-CoA is also turned over, but only 10% as fast. The pantetheine thiol ester of 3-butenoic acid is isomerized, but at just 0.1% the rate of isomerization of the coenzyme A thiol ester. The liver isomerase is sensitive to inhibition by *p*-chloromercuribenzoate and by *N*-ethylmaleimide. Inhibition by the former can be reversed by low concentrations of glutathione. The enzyme is maximally active at pH 8–9.[237]

SCHEME 28



G. 2,3-Enoyl-CoA Isomerase

For the most part, there is relatively little known about the 2,3-enoyl-CoA isomerases that participate in the degradative metabolism of unsaturated fatty acids. Though there have been several reports on 2,3-enoyl-CoA isomerases from both eukaryotes and prokaryotes, the structural and mechanistic relationships between the isomerases from these different sources remain unclear.

The current view[246] of β-oxidation of unsaturated fatty acids in mammals is based largely upon studies by Kunau,[247] Stoffel,[248] and Schulz.[244] The major pathway is shown in Scheme 28. Linoleic acid is degraded to (*Z*,*Z*)-3,6-dodecenoyl-CoA, which is rearranged to the *E*,*Z*-2,6 isomer. The latter compound is converted by standard β-oxidation to (*Z*)-4-decenoyl-CoA, which is oxidized by acyl-CoA dehydrogenase II to (*E*,*Z*)-2,4-decadienoyl-CoA. This compound undergoes 1,4-reduction to (*E*)-3-decenoyl-CoA, which is rearranged to (*E*)-2-decenoyl-CoA, a substrate for classical β-oxidation. As illustrated in Scheme 28, 2,3-enoyl-CoA isomerase is required for the degradation of fatty acids with double bonds at both odd- and even-numbered positions. Two allylic rearrangements are required to completely degrade linoleic acid. Since there is no evidence for more than one mitochondrial 2,3-enoyl-CoA isomerase, a single isomerase is assumed to catalyze both reactions.

2,3-Enoyl-CoA isomerase has been purified to homogeneity from rat liver mitochondria.[249] This enzyme comprises protomers with M_r 30 000, which, despite a strong tendency to dimerize, retain full enzymatic activity in the presence of sodium dodecylsulfate.[249] It loses little activity upon extended storage and has a broad pH optimum between pH 7 and 9.

In terms of substrate specificity and kinetic characteristics, the rat liver mitochondrial 2,3-enoyl-CoA isomerase is similar to the ox liver vinylacetyl-CoA

isomerase described above. The rat enzyme exhibits little chain-length selectivity for (*E*)-3-enoyl-CoA substrates between C_6 and C_{16}. While it isomerizes both (*E*)- and (*Z*)-3-enoyl substrates, the K_m values for the *E* isomers are about 10 times higher than those for the *Z* isomers, and the rates of isomerization for the *Z* isomers are greater than those for the *E* isomers, again by a factor of about 10. All tested substrates lead to (*E*)-2-enoyl-CoA products.[250] This includes the linoleic acid degradation product (*Z*,*Z*)-3,6-dodecenoyl-CoA, which is rearranged by the partially purified rat liver isomerase to (*E*,*Z*)-2,6-dodecenoyl-CoA.[248]

In mammals, (*E*,*Z*)-2,4-decadienoyl-CoA is reduced by 2,4-dienoyl-CoA reductase to 3-decenoyl-CoA. Strong evidence has been obtained by Dommes and Kunau that it is the *E* isomer that is formed (as shown in Scheme 28),[246,251] and which is therefore the substrate for the second of the two allylic rearrangements catalyzed by 2,3-enoyl-CoA isomerase. On the other hand, Mizugaki et al. have presented evidence[252] that is in conflict with that of Dommes and Kunau. According to Mizugaki, (*E*,*E*)-2,4-decadienoyl-CoA is reduced to (*E*)-3-decenoyl-CoA, but (*E*,*Z*)-2,4-decadienoyl-CoA is reduced to roughly equal parts of (*E*)- and (*Z*)-3-decenoyl-CoA. Unfortunately, there was no discussion of the reductase's apparent lack of stereoselectivity. If Mizugaki's data are correct, then both of the 3-decenoyl-CoA thiol esters would have to be substrates for the isomerase in vivo, which would still be compatible with the isomerase's substrate selectivity, as described above.

Davidoff and Korn have found 2,3-enoyl-CoA isomerase activity in both *Dictyostelium discoideum*[253] and guinea pig liver mitochondria.[254] Neither isomerase was purified to any significant extent, although heat treatment was found to inactivate contaminating enoyl-CoA hydratase activity that was present in the guinea pig enzyme preparation. Curiously, the guinea pig isomerase was found to equilibrate 2-hexadecenoyl-CoA (presumably the *E* isomer) with *both* the *E* and *Z* isomers of 3-hexadecenoyl-CoA. The reaction was reversible, with all three enoyl thiol esters formed from any one of them. Similar results were obtained with the *Dictyostelium* isomerase, with the exception that 3-hydroxyhexadecanoyl-CoA was the predominant product of this rather crude enzyme preparation. It was hypothesized that these isomerases might be involved in fatty acid catabolism. Specifically, such a versatile isomerase could participate in the degradation of unsaturated fatty acids having either *E* or *Z* double bonds.

The fact that the *Dictyostelium* and guinea pig liver 2,3-enoyl-CoA isomerases are able to produce *both* (*E*)- and (*Z*)-3-hexadecenoyl-CoA is especially interesting if one considers the architecture of the active site (Figure 4). The enzyme would have to recognize and bind tightly to just the thiol ester moiety and carbons 1 and 2 of the acyl chain of the substrate. The substrate could then rotate about the C-3/C-4 bond in order to assume the two conformations that would lead to the diastereomeric products. Significantly, only two relatively well-defined substrate conformations would be productive, since facile deprotonation at C-4 depends upon orbital overlap between the C-4–H bond and the C-2/C-3 double bond. This mechanistic scenario suggests that the enzyme must remove a different C-4 proton depending on which isomer of 3-decenoyl-CoA is produced. If one were to arbitrarily assume that removal of the *pro*-4*S* proton leads to (*E*)-3-decenoyl-CoA, then the *pro*-4*R* proton would be removed en route to (*Z*)-3-decenoyl-CoA. This would be an interesting point to test experimentally.

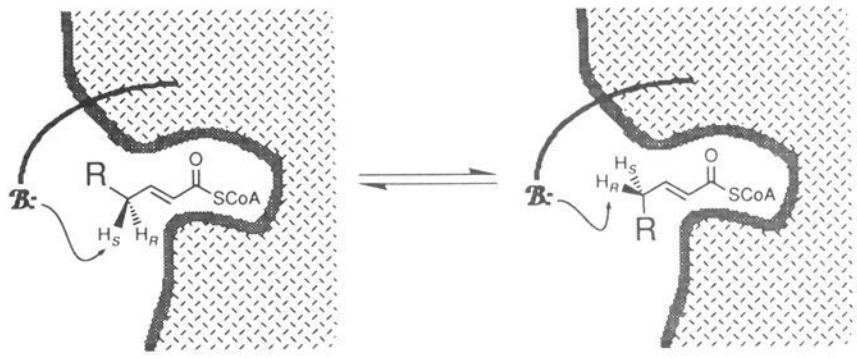


Figure 4. 2,3-Enoyl-CoA isomerase can catalyze the formation of either (*E*)- or (*Z*)-3-hexadecenoyl-CoA from (*E*)-2-hexadecenoyl-CoA (R = *n*-$C_{12}H_{25}$). It is predicted that this is a stereospecific reaction: Removal of the *pro*-4*R* proton leads to one product diastereomer, while removal of the *pro*-4*S* proton gives the other.

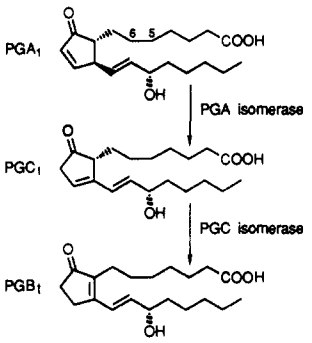
The ability to degrade both unsaturated and saturated fatty acids has also been identified in the yeast *Candida tropicalis*,[255] where β-oxidation has been described as a peroxisomal, rather than a mitochondrial, function. In fact, when *C. tropicalis* is grown on *either* oleate (an unsaturated fatty acid) or myristate (a saturated fatty acid), the expression of all of the enzymes of fatty acid degradation, including the two that are specific to the catabolism of unsaturated fatty acids (i.e. 2,3-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase) is induced. Once again, the product of dienoyl-CoA reductase action on (*E*,*Z*)-2,4-decadienoyl-CoA is (*E*)-3-decenoyl-CoA, which is apparently a physiological substrate for the isomerase. Not surprisingly, the product of the *C. tropicalis* isomerase is (*E*)-2-decenoyl-CoA. These observations lend further support to the generality of the biodegradative pathway[244,246,247] shown in Scheme 28.

E. coli produces a fatty acid degradation multienzyme complex with an $\alpha_2\beta_2$-subunit structure,[256–259] encoded by the genes of the *fadAB* operon.[260] Five different enzyme activities, including 2,3-enoyl-CoA isomerase, are associated with this multi-enzyme complex.[257] The main difference between the role of the bacterial isomerase and that of the corresponding eukaryotic enzyme is a consequence of the different modes of action of the 2,4-dienoyl-CoA reductases from these sources.[251] Whereas in eukaryotes the product of the reductase is (*E*)-3-enoyl-CoA, in bacteria the product is (*E*)-2-enoyl-CoA, formed by a 1,2 rather than a 1,4 reduction. Thus, in bacteria, 2,3-enoyl-CoA isomerase is only needed for the oxidative metabolism of fatty acids with double bonds at odd-numbered positions. There are no other mechanistic data available on the *E. coli* isomerase, aside from the fact that it is sensitive to the sulfhydryl reagent *N*-ethylmaleimide and that protection against inactivation is afforded by acetoacetyl-CoA.[258]

H. Prostaglandin Isomerases

Prostaglandin A (PGA) isomerase has been reported in the serum of a variety of animal species, including rabbit, cat, pig, dog, mouse, and rat (and at very low levels in man, sheep, and guinea pig).[261–264] The enzyme has been partially purified from rabbit[264–267] and cat[263,268] serum. Initial efforts[261,263] to identify the im-

mediate product of the PGA isomerase reaction (i.e., PGC_1[265,268,269]) were complicated by the rapid conversion of PGC_1 to PGB_1. These difficulties were originally attributed to the alleged chemical instability of PGC_1 or to the mistaken impression that PGB_1 is the actual product of the PGA isomerase reaction. Subsequent investigations by Polet and Levine revealed the existence of a second isomerase activity in serum, this one responsible for the conversion of PGC_1 to PGB_1.[262] Since most of the earlier studies utilized rather crude enzyme preparations, the failure to observe PGC_1 may have resulted from contamination of PGA isomerase with PGC isomerase.[262]



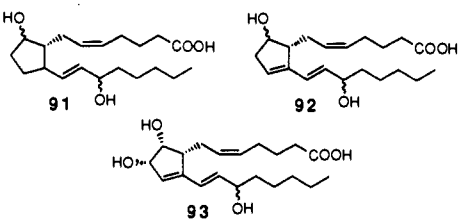
PGA_2, PGC_2, PGB_2 : 5,6-dehydro derivatives

Polet and Levine achieved a 120-fold purification of the enzyme from rabbit serum.[267] The purified protein actually comprised a number of species (possibly isozymes), since in the isoelectric focusing step the isomerase activity was spread over almost a full pH unit. Removal of adhering fatty acids failed to change the isoelectric focusing behavior of the enzyme. The relative molecular mass of the isomerase was estimated at 110 000.

Rabbit PGA isomerase tends to lose activity during concentration, and thiols fail to protect it against inactivation.[267] It is completely inactivated by digestion with pepsin,[264] by treatment with the sulfhydryl reagent *N*-ethylmaleimide,[264,266] or by incubation with 8 M urea[267] (which presumably denatures the enzyme). On the other hand, the isomerase is relatively heat-stable,[263] and low pH does not adversely affect it.[262,264] The K_m for PGA_1 is about 50 μM,[266,267] and that for PGA_2 is 4 μM.[270] The equilibrium constant for conversion of PGA_2 to PGC_2 has been estimated at about 5.[270]

PGA isomerase from cat plasma has been purified by 48-fold.[268] The enzyme is relatively heat-stable, denaturing only after 10 min at temperatures above 50 °C, and it is maximally activity at temperatures approaching 50 °C. The pH-rate curve has its maximum between pH 8 and 9. Five prostaglandins were evaluated as substrates for the cat serum enzyme. Prostaglandins A_1 and A_2 were turned over the fastest, and 19-hydroxy-PGA_1 and 15-epi-PGA_2 were turned over at considerably slower rates. 13,14-Dihydro-PGA_1 was not a substrate, but rather, an inhibitor of PGA isomerase. The mode of inhibition was not determined. Of these five compounds, only the dihydro compound does not occur naturally. The K_m for PGA_1 is approximately 25 μM.[268]

A number of PG analogues, including analogues of PGC_2 have been evaluated as inhibitors of rabbit serum PGA isomerase. Analogues (**91**–**93**) of 2-series prostaglandins were chosen, presumably due to the considerably lower isomerase K_m for PGA_2 relative to PGA_1. All of the compounds tested were competitive inhibitors, and the best of them had K_m/K_i ratios (versus PGA_2) of about 5.[270] The search for inhibitors was initiated because inhibition of PGA isomerase in vivo might help to provide clues as to the physiological role of the enzyme and the isomerization pathway. There appear to have been no reports following up on the results of the original study.



Incubation of PGA_1 with rabbit serum leads to production of PGB_1, whereas when PGA_1 is incubated with 120-fold purified PGA isomerase from rabbit serum only PGC_1 is obtained.[262] This implied the existence of a second isomerase activity, named PGC isomerase. Addition of human serum, human albumin, human mercaptalbumin, rabbit serum, or rabbit albumin to the PGA isomerase preparation also led to the formation of PGB_1, suggesting the presence of PGC isomerase in each protein preparation. Controls were run to demonstrate that PGC isomerase activity did not merely reflect nonspecific protein catalysis, or catalysis by buffer. With rabbit serum, conversion of PGC_1 to PGB_1 was found to be rate-determining in the overall pathway. The opposite trend was observed with human serum. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) and other sulfhydryl blocking reagents including *N*-ethylmaleimide were found to inactivate rabbit serum PGC isomerase, which is even more heat-stable than is PGA isomerase.[262]

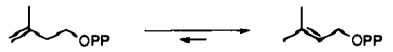
Unfortunately, no detailed mechanistic studies seem to have been carried out on either PGA isomerase or PGC isomerase. Although on the basis of studies of analogous enzymes, one would expect each enzyme to utilize a single active site base and to exhibit a suprafacial stereochemical course, this prediction has not been tested. Some chemical models have been described.[271-273]

III. Relatively Nonacidic Substrates

A. Isopentenyl Pyrophosphate Isomerase

Terpenes comprise a diverse group of metabolically important compounds that are found in virtually all organisms. Even prior to the elucidation of the terpene biosynthetic pathway, it was postulated that they were produced from the condensation of isoprene units.[274] As the details of this pathway became clear, isopentenyl pyrophosphate (IPP) was identified as a key biosynthetic intermediate. Difficulty in formulating a mechanism by which two molecules of IPP could link to initiate terpene biosynthesis led to speculation by Lynen et al.[275] and by Rilling and Bloch[276] that IPP must somehow be "activated" prior to condensation. Subsequently, dimethylallyl pyrophosphate (DMAPP) was identified as the "activated isoprene". DMAPP is formed from IPP by an allylic rearrangement catalyzed

by isopentenyl pyrophosphate isomerase (IPPI).



isopentenyl pyrophosphate (IPP) dimethylallyl pyrophosphate (DMAPP)

Since terpenes are made by so many different organisms, researchers have tended to group all IPPIs together unless experimental results reveal differences. This is the general approach taken in this review, with major distinctions drawn between the enzymes obtained from mammalian versus microbial sources. Our understanding of the enzymology has progressed such that additional differences between IPPIs from various sources within these general classes are now recognized, and an attempt will be made to highlight these differences (as well as notable similarities) when possible.

The work on IPPI prior to 1981 has been reviewed on several occasions,[277-279] and the reader is referred to these articles or to the original reports for in-depth discussion of this material. For comprehensibility, some of the older material is also summarized within this review.

1. Pig and Rat Liver Isomerases

a. Physical Properties. Although rat liver IPPI has never been well-purified, it has been used extensively in mechanistic studies. The available data indicate that the enzyme is soluble and that it is sensitive to sulfhydryl-specific reagents,[280] consistent with the findings for IPPI from other sources.

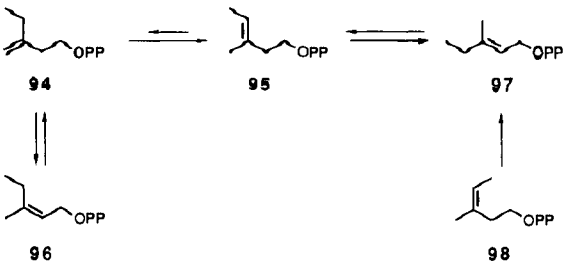
There have been conflicting reports concerning the structure of pig liver IPPI. The most recent study described the enzyme as a monomeric M_r 22 000 protein.[281] This contradicts the descriptions of a dimeric M_r 82 500 structure reported by Banthorpe et al.[282] and an M_r 60 000 structure, as suggested by Shah et al.[283] The origin of these discrepancies is unclear. However, the irreproducibility of several observations reported by Shah et al. has been attributed to the relatively low purity of their enzyme.[284] Also, Bruenger et al.[281] found that mammalian IPPI is especially labile (possibly due to proteolysis), which could explain some of the conflicting results.

The equilibrium position of the reaction catalyzed by pig liver IPPI favors DMAPP by a factor of 8 or 9.[283,284] The K_m value for IPP of 4.0 μM, reported by Holloway and Popják,[284] is in good agreement with the value of 2.7 μM reported by Banthorpe et al.[282] Both groups observed a sharp pH optimum at 6.3 and found that Mn^{2+} or Mg^{2+} will fill the divalent cation requirement, although Mn^{2+} is preferred. Additionally, Shah et al.,[283] Holloway and Popják,[284] and Banthorpe et al.[282] have reported that the isomerase is strongly inhibited by a variety of sulfhydryl-specific reagents, indicating the presence of at least one essential cysteine residue.

b. Substrate Binding Site. Pig liver IPPI is reversibly inhibited by alkyl pyrophosphates. Banthorpe et al.[282] showed that variations in the alkyl chain have little effect on the inhibitory properties of these compounds. However, substitution of phosphate for pyrophosphate makes them far less effective.

Koyama et al.[285] examined the fates of several substrate analogues (Scheme 29). They found that 3-ethyl-3-butenyl pyrophosphate (**94**), when incubated with pig liver IPPI, is converted to a mixture of (*E*)-3-methyl-3-pentenyl pyrophosphate (**95**), (*Z*)-3-methyl-2-pentenyl pyrophosphate (**96**), and (*E*)-3-methyl-2-pentenyl pyrophosphate (**97**). Additionally, it was observed that (*Z*)-3-methyl-3-pentenyl pyrophosphate (**98**) is irreversibly isomerized to **97**.[286] These data were interpreted in terms of a crude topological map of the active site in which there are distinct binding sites for the methyl and pyrophosphate moieties. It was suggested that the methyl binding site will not accept the additional methylene groups of **94–98** without distortion of these compounds, and that such distortion, along with the binding of these compounds in an inverted orientation, could account for their results.

SCHEME 29



c. Mechanism. The mechanism of the allylic rearrangement catalyzed by IPPI has long been debated. Shah et al.[283] conducted a series of studies in which the incorporation of tritium from TOH into IPP and DMAPP was monitored. Several conclusions were drawn from these studies; first, that the protons added to C-4 of IPP and C-2 of DMAPP are ultimately derived from the solvent, since radioactive product and starting material can be isolated; second, that the proton transfer at C-2 is stereospecific, since ca. 95% of the label found in DMAPP was in the C-4 position; third, that the reaction proceeds by way of a carbocation intermediate, since tritium washes into IPP and DMAPP at different rates; finally, that the reaction involves an enzyme-bound intermediate. It has been suggested by Cornforth, however, that the results of Shah et al. that led to the proposal of the covalent intermediate were questionable due to flaws in the experimental design.[277,287] He speculated that the exchange at C-4 of IPP could be explained by multiple isomerizations, and so a concerted mechanism could not be excluded. To add to the confusion, Rose noted that the results obtained by Shah et al. could also be interpreted in terms of a two-base carbanionic mechanism.[288] As it stands, the mechanism of the reaction catalyzed by pig and rat liver IPPI is still uncertain.

While the catalytic mechanism of the allylic rearrangement is still speculative, the stereochemical course of the reaction was determined through an elegant series of experiments. Examination of the isomerization of IPP to DMAPP reveals three questions that required answers: (1) What is the stereochemistry of the proton abstraction from C-2 of IPP? (2) Is the *E*- or *Z*-methyl group of DMAPP derived from C-4 of IPP? (3) Is the double bond of IPP protonated from the *re* or *si* face?

Cornforth and his associates were the first to address the stereochemical course at C-2.[289,290] C-4 of mevalonate becomes C-2 of IPP. Therefore, the researchers fed mevalonate stereospecifically labeled at C-4 with either deuterium or tritium to a rat liver homogenate capable of synthesizing farnesyl pyrophosphate. It was

found that farnesyl pyrophosphate produced from mevalonate labeled at the *pro*-4*S* position was devoid of label. However, mevalonate labeled specifically at the *pro*-4*R* position led to farnesyl pyrophosphate that retained hydrogen isotope. This provided clear evidence that the *pro*-2*R* proton of IPP is stereospecifically removed by farnesyl pyrophosphate synthetase and by IPPI.

The configuration of the double bond in DMAPP with respect to the newly formed methyl group was established by Stone et al.[291] for the rat liver isomerase and by Koyama et al.[285] for the pig liver isomerase. Stone examined the ^1H NMR spectrum of squalene synthesized from mevalonate in D_2O. The spectrum showed that deuterium had been incorporated into the *E*-methyl of DMAPP; therefore, the *E*-methyl of DMAPP is derived from C-4 of IPP. Through a similar series of experiments involving purified pig liver IPPI, Koyama et al. came to the same conclusion. It should be noted that although these groups were the first to resolve this question for the liver isomerases, several other groups had previously observed the same stereochemical course in other systems.[292-294]

The final question to be considered concerns which face of the double bond of IPP is protonated during the isomerization.[277,279,295] Answering this question posed an unusually difficult task in that the addition of a solvent-derived proton to C-4 of IPP results in the formation of a methyl group. By incubating a soluble preparation of pig liver enzymes with either (2*R*,3*R*)- or (2*S*,3*R*)-[2-^3H]mevalonate in D_2O, Cornforth et al.[287,296] produced farnesol in which the ω-*E*-methyl group contained all three hydrogen isotopes. The samples were chemically degraded in such a way that this methyl group was converted into the methyl group of acetate. The configuration of the methyl group was determined enzymatically.[297,298] Cornforth and his coworkers observed that acetate derived from farnesol that had been biosynthesized from (2*R*,3*R*)-[2-^3H]-mevalonate was predominantly (*R*)-[^1H,^2H,^3H]acetate. Acetate obtained by degradation of farnesol biosynthesized from (2*S*,3*R*)-[2-^3H]mevalonate had predominantly the *S* configuration. Consequently, it was deduced that IPPI had added a solvent-derived deuterium atom to the *re* face of the double bond of IPP.

The above results indicate that liver IPPI carries out a 1,3-antarafacial hydrogen shift. This overall stereochemical course is most consistent with a two-base mechanism.[288,296] As alluded to previously, the limited availability and the apparent lability of mammalian liver IPPI has hampered structural investigations of the enzyme; therefore, the nature of these putative functional groups has not been established.

While these studies elegantly established the stereochemistry of the overall reaction, they did little to resolve the controversy surrounding the mechanism of the isomerization. More than a decade passed before researchers began to effectively address this issue.

2. Yeast and Fungal IPPI

a. Physical Properties. The first reported isolation of IPPI was from yeast in 1960 by Agranoff et al.[299]

TABLE III. Inhibition of *Claviceps* and Yeast IPPI by Substrate Analogues

compound	K_i, M: yeast	K_i, M: *Claviceps*	ref
inorganic pyrophosphate	$>1.0 \times 10^{-3}$	7×10^{-6}	281, 303
methyl pyrophosphate	1.0×10^{-4}		300
farnesyl pyrophosphate		3.8×10^{-5}	281
geranyl pyrophosphate		3.8×10^{-5}	281
3-bromo-3-butenyl pyrophosphate	6.0×10^{-5}		300
4-bromo-3-methyl-3-butenyl pyrophosphate	4.0×10^{-5}		300
2-(dimethylamino)ethyl pyrophosphate	$<1.4 \times 10^{-11}$	$<1.2 \times 10^{-10}$	290, 303
2-(dimethylamino)ethyl phosphate	2.0×10^{-3}		300

However, until large quantities of purified enzyme were required for mechanistic studies, researchers largely ignored the yeast enzyme. Subsequently, yeast and the fungus *Claviceps* became valued sources of IPPI.

During the past five years, several IPPI purifications have been described. These schemes provide enzyme from yeast[300-302] and from *Claviceps*[281,303] that is over 90% pure. The most recent purification, by Anderson et al.,[302] affords enzyme of over 95% purity with a specific activity of 12.3 μmol min^{-1} mg^{-1}. This value is 4.6-fold greater than the highest specific activity previously reported.[300] Anderson et al. also cloned the structural gene that encodes the enzyme. Based on the gene sequence, yeast IPPI is a 288 amino acid polypeptide with M_r 33 350.

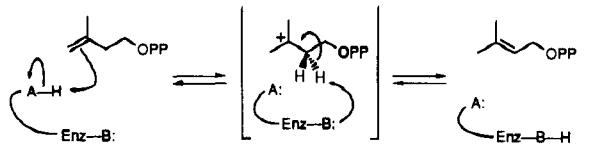
IPPI has also been isolated from *Claviceps purpurea* and *Claviceps* SD58. A relative molecular mass of 35 000 for both enzymes was observed.[281,303] As of this time, no sequence information has been reported on the *Claviceps* isomerases. The enzymes are very labile with half-lives of ca. 30 min at 37 °C in dilute solutions; however, the enzymes are considerably more stable at higher concentrations.[281,303] The same also appears to be true for the yeast isomerase.[300]

There are several notable differences between the *Claviceps* and yeast isomerases. The K_m values for IPP are 2.4 and 5.4 μM for the *C. purpurea* and SD58 enzymes, respectively;[281,303] however, the corresponding K_m for the yeast enzyme is 35.0 μM.[299,300] Each of these enzymes requires a divalent cation for catalytic activity. Although the *Claviceps* enzyme will accept Mg^{2+} or Mn^{2+} (Mg^{2+} is preferred),[281] the yeast enzyme apparently accepts only Mg^{2+}.[299]

b. Substrate Binding Site. Several groups have managed to amass a substantial amount of information concerning the active site and the catalytic mechanism of the yeast and *Claviceps* isomerases.

A variety of alkyl pyrophosphates competitively inhibit the yeast and *Claviceps* isomerases (Table III), as is the case for the hog liver isomerase. Reardon and Abeles[300] found, as did Bruenger et al. with *Claviceps* isomerase,[281] that the enzyme is fairly forgiving when it comes to alterations in the alkyl portions of the inhibitors. The yeast enzyme will readily accept substitutions with little effect on K_i. Similarly, the *Claviceps* enzyme binds farnesyl and geranyl pyrophosphates equally well. Both groups reported that truncation of the alkyl chain does not significantly reduce binding. As expected, the findings for the pyrophosphate moiety were quite different. Reardon and Abeles found that conversion of the pyrophosphate moiety to phosphate

SCHEME 30

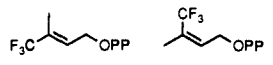


results in dramatic reductions in potency even for the most effective inhibitors of the yeast isomerase. Therefore, the pyrophosphate moiety apparently plays a key role in binding, presumably by complexing with the bound divalent metal ion, while the alkyl chain contributes little to substrate binding.

c. Mechanism. The steric course of the allylic rearrangement catalyzed by yeast and *Claviceps* isomerases has not been investigated; instead, the information gathered from other systems has been assumed to apply to the yeast and *Claviceps* enzymes.

As mentioned, the tritium incorporation patterns observed by Shah et al., as well as the stereochemistry of the transformation catalyzed by the rat and hog liver isomerases, can be explained in terms of a carbocation or a carbanion reaction intermediate, or a concerted mechanism. Rose[288] has noted that detailed kinetic analyses could discriminate between these mechanisms. Ultimately, such studies may be necessary to completely resolve this issue. Still, a number of experiments involving substrate analogues have supported a carbocation mechanism (Scheme 30).

i. Fluorinated Substrate Analogues. The rationale for the preparation and evaluation of fluorinated substrate analogues arose from studies of farnesyl pyrophosphate synthase[304] and dimethylallyltryptophan synthase,[305] in which linear free energy correlations involving model systems provided evidence for carbocation mechanisms. Since electron-withdrawing substituents would be expected to increase the free energy of a carbocation intermediate, the incorporation of fluorine into the isoprene unit could be used as a probe for a carbocation intermediate in the reaction catalyzed by IPPI. The first compounds examined were (*E*)- and (*Z*)-3-(trifluoromethyl)-2-butenyl pyrophosphate, **99** and **100**, respectively.[300] There was no detectable



99 **100**

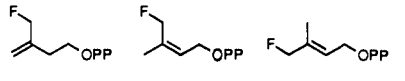
isomerization of **100** to the corresponding homoallylic pyrophosphate. It was therefore concluded that **100** is turned over *at least* 10^6 times more slowly than is the normal substrate. (No analogous data were reported for **99**.) This finding is in good agreement with the 10^7-fold decrease in the rate of solvolysis due to a trifluoromethyl group at C-3.[304] It is also inconsistent with a concerted or a carbanion mechanism and suggests that the yeast IPPI-catalyzed reaction does indeed proceed via a transition state with carbocation character.

Subsequent to the studies of Reardon and Abeles with the trifluoromethyl substrate analogues, Mühlbacher and Poulter prepared a series of monofluorinated substrate analogues for linear free energy studies.[303,306] These compounds included the five allylic fluorides, 3-(fluoromethyl)-3-butenyl pyrophosphate (**101**), (*Z*)-3-(fluoromethyl)-2-butenyl pyrophosphate (**102**), and (*E*)-3-(fluoromethyl)-2-butenyl pyrophosphate (**103**), (*R*)- and (*S*)-2-fluoro-3-methyl-3-butenyl pyrophosphate (**104** and **105**, respectively), as well as the saturated compound 4-fluoro-3-methyl-1-butyl pyrophosphate (**106**).

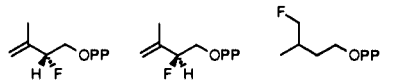
TABLE IV. Kinetic Constants for Irreversible Inactivation of *Claviceps* IPPI by Fluorinated Substrate Analogues

compd	k_{inact},[a] min^{-1}	K_I,[a] μM
101	0.22 ± 0.07	0.09 ± 0.04
102	0.97 ± 0.52	0.97 ± 0.55
103	0.40 ± 0.04	0.59 ± 0.09

[a] Data taken from ref 303.



101 **102** **103**



104 **105** **106**

None of these compounds was a substrate for IPPI. However, **101–103** were irreversible inactivators of *Claviceps* IPPI (Table IV).[303,306] Since they covalently modify IPPI, these compounds may ultimately prove invaluable for identification of active site functionalities.

The structures of the adducts formed between *Claviceps* IPPI and these inactivators have not been reported; however, there is some evidence regarding the general nature of the linkage. The pH rate profile for the normal isomerization is bell-shaped and dependent upon functionalities with apparent pK_a values of 9.3 and 5.5.[303] The pH profile for inactivation of the *Claviceps* isomerase by **101**, the most potent inactivator in this series, is also bell-shaped, with a maximum around pH 7.0, but it decreases sharply above pH 8.0 and below about pH 6.5.[303] The similarity in these profiles suggests that the inactivation involves the same active site functionalities that mediate catalysis. The functional group with the higher pK_a may be a cysteine sulfhydryl, since the rate of inactivation of IPPI by iodoacetamide is dependent upon a functional group with a pK_a of 9.3.[300] (This was shown for the yeast isomerase, which is also inactivated by **101**.[302]) Additionally, partial restoration of activity is achieved by treatment of inactivated IPPI with dithiothreitol.[306] These data have led to speculation that the inactivator may be linked to the enzyme as a thioether. This has not been demonstrated experimentally, however.

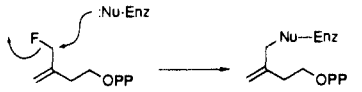
Mühlbacher and Poulter conducted a series of experiments designed to allow determination of the mechanism of IPPI inactivation by **101**.[301] Since the inactivation of IPPI by either ^3H- or ^{32}P-labeled **101** results in the irreversible association of radioactivity with the protein,[303] it is clear that the inactivation does not involve displacement of pyrophosphate. The most likely mechanism of inactivation was thought to be an S_N2 or S_N2' displacement of the allylic fluoride by an active site nucleophile. A ^1H-decoupled ^{13}C NMR spectrum of yeast IPPI inactivated with 3-(fluoromethyl)-3-[4-^{13}C]butenyl pyrophosphate revealed a broad resonance at 119.6 ppm. This signal was not observed in the spectrum of the native enzyme or in that of labeled **101** (which exhibits only a sharp singlet

TABLE V. Kinetic Constants for Irreversible Inactivation of *Claviceps* IPPI by Oxirane Substrate Analogues

compd	k_{inact},[a] min^{-1}	K_I,[a] μM
107	0.13 ± 0.01	0.011 ± 0.004
108	0.17 ± 0.02	0.017 ± 0.001
109	0.24 ± 0.06	8.74 ± 1.83

[a] Data taken from ref 303.

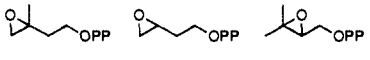
at 117.8 ppm). This demonstrated that C-4 of the inactivator/enzyme adduct remains as an olefinic methylene group, although linked to the enzyme. Since fluoride is known to be lost in the course of inactivation,[301,303] these observations suggest that **101** becomes attached to the enzyme via S_N2 displacement of fluoride ion by an as yet unidentified active site nucleophile.



101

Although there are numerous examples of fluorinated enzyme inactivators,[3,307] in those cases in which carbon/fluorine bonds are broken, it is as a result of elimination, rather than substitution. At first glance, the proposed mechanism of inactivation of IPPI by **101** is remarkable because fluoride is a rather poor leaving group in ordinary S_N2 displacements.[308] Compound **101** is an allylic fluoride, however, and so it should be up to 100 times as reactive as the corresponding saturated compound.[309] Furthermore, the microenvironment of IPPI's active site is critically important, and it is possible that the enzyme is ideally set up to promote the displacement of fluoride. For instance, hydrogen bonding of F to the enzyme would serve to weaken the C–F bond, thus promoting displacement. (The existence of an active site proton donor is supported by the results described below involving oxiranes **107** and **108** and ammonium ion **110**.)

ii. Oxirane Substrate Analogues. A series of oxiranyl substrate analogues, including 3,4-epoxy-3-methyl-1-butyl pyrophosphate (**107**), 3,4-epoxy-1-butyl pyrophosphate (**108**), and 2,3-epoxy-3-methyl-1-butyl pyrophosphate (**109**), have also been found to irreversibly inactivate IPPI (Table V).[303] The first two of these are the most potent inactivators known for IPPI, with inactivation constants in the low nanomolar range. This is not surprising considering the fact that oxiranes have been successfully used as affinity labels in other systems (c.f. section II.A.1.c.iii). Oxiranes are usually activated by protonation of the ring oxygen; this is followed by nucleophilic attack. On the basis of the pH rate profile, this appears to be the mechanism by which IPPI is inactivated by **107**. The rate is maximal up to approximately pH 8.0 at which there is a rapid decrease in the rate of inactivation, presumably due to deprotonation of the putative active site general acid that protonates the epoxide.

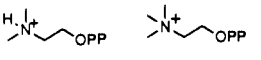


107 **108** **109**

An interesting development occurred when researchers attempted to establish the inactivation stoichiometry for the oxiranes.[303] Compounds **109** and **107** were found to inactivate IPPI with stoichiometries of 0.58 and 0.81 mol of inactivator per mol of isomerase active site, respectively. While the value for **107** may be within experimental error of unity, the value for **109** is not so easily understood. An acceptable explanation is not available at this time.

iii. Reaction Intermediate Analogues. Perhaps the most convincing evidence suggesting that the reaction catalyzed by IPPI proceeds through a carbocation intermediate was provided by Reardon and Abeles,[300,310] who reported the inhibition of yeast IPPI by 2-(dimethylamino)ethyl pyrophosphate (**110**). This compound, designed specifically to mimic the proposed carbocation intermediate, inhibits IPPI irreversibly with pseudo-first-order kinetics. Treatment of the inhibited enzyme with chaotropic agents results in the release of unchanged **110**, indicating that the interaction with the enzyme is not covalent but is probably due to electrostatic interactions. The on rate, k_{on}, was measured under second-order conditions at 2.1×10^6 M^{-1} min^{-1}, and k_{off} was calculated to be less than 3×10^{-5} min^{-1}.[300] Thus, K_i is less than 1.4×10^{-11} M.

The pH profiles for k_{on} and K_i indicate that inhibition is maximal at pH 8.5 and implicate two functionalities, with pK_a values of 7.8 and 9.8. The one with the pK_a of 9.8 is the amino group of **110**. This was demonstrated by comparison to the analogous pH profiles for 2-(trimethylamino)ethyl pyrophosphate (**111**), which retains a positive charge at all pH values. The role of the



110 **111**

group with $pK_a = 7.8$ is unknown. On the one hand, it might not appear to be involved in catalysis, since the pH–V_{max} profile for the isomerization of IPP implicates a group with a pK_a of 9.4. On the other, these groups could be one and the same if its pK_a were influenced by the binding of **110**. Such could be the case if this group were an essential sulfhydryl. Deprotonation of this –SH group upon binding of **110** then would lead to the thermodynamically favorable formation of an ion pair in a hydrophobic active site environment.

Shortly after Reardon and Abeles' initial report on yeast IPPI, Mühlbacher and Poulter published similar findings for inhibition of the *Claviceps* enzyme by **110**.[306] Mühlbacher and Poulter's K_i value ($\leq 1.2 \times 10^{-10}$ M) is in good agreement with the one determined by Reardon and Abeles. Although more stringent conditions were required for denaturation of *Claviceps* IPPI than for denaturation of the yeast system, **110** could also be released intact from inhibited *Claviceps* isomerase.

Qualitative comparisons of the pH-inactivation and pH-activity curves for the yeast and *Claviceps* enzymes indicates that the enzymes behave similarly. This suggests that both enzymes use the same or similar ionizable active site functionalities.

iv. Chemical Modification Studies. The binding sites of yeast and *Claviceps* IPPI apparently differ somewhat. Saturating levels of IPP protect the yeast enzyme from inactivation by the sulfhydryl-specific reagent *S*-methyl methanethiosulfonate (MMTS); however, the competitive inhibitor methyl pyrophosphate does not protect the enzyme against inactivation.[300] Reardon and Abeles also found that

TABLE VI. Summary of Physical Properties of IPPI from Various Sources

enzyme source	M_r[a]	K_m, μM	metal dependence[b]	metal dependence
chicken liver	nd[c]	11.2	Mg^{2+} (Mn^{2+})[b]	311, 312
silkworm (*Bombyx mori*)	35000	20	Mn^{2+} (Mg^{2+})[b]	313
E. coli	nd	5.0	Mn^{2+} (Mg^{2+})[b]	314
soybean (*Soja max*)	nd	nd	nd	294
Penicillium griseofulvum	nd	nd	nd	292
Monterey pine (*Pinus radiata*)	nd	nd	(Mg^{2+})[d]	315
orange (*Citrus sinensis*)	nd	nd	(Mg^{2+})[d]	315
grapefruit (*Citrus paradisii*)	nd	nd	none (?)	316
pumpkin (*Cucurbita pepo*)	nd	nd	(Mg^{2+})[d]	317
tomato (fruit plastids; *Lycopersicum* sp.)	34000	5.7	Mn^{2+} or Mg^{2+}	318
red pepper (chromoplasts; *Capsicum annum*)	33500	6	(Mg^{2+})[d]	319
cotton (*Gossypium hirsutum*)	nd	74	(Mg^{2+})[d]	320

[a] Determined by gel filtration. [b] The metal ion in parentheses leads to lower enzyme activity. [c] Not determined. [d] The metal ion requirement was inferred from the assay conditions used. Evaluation of alternative metal ions was not reported.

MMTS-inactivated yeast IPPI does not bind 110.[300] On the other hand, Bruenger et al.[281] discovered that saturating levels of IPP fail to protect the *Claviceps* enzyme from inactivation by iodoacetamide. Moreover, iodoacetamide-inactivated *Claviceps* isomerase still binds 110.[303]

From these observations, it appears that in the yeast isomerase there is an active site sulfhydryl group in the vicinity of the alkyl chain binding region and that it is inaccessible to the medium when substrate is bound. The *Claviceps* enzyme also appears to possess an active-site sulfhydryl group; however, this sulfhydryl group remains accessible to the solvent even with substrate bound. It is still unclear whether these cysteines are evolutionarily conserved residues and if they play analogous roles in catalysis by the two enzymes, or are functionally distinct.

3. Other Isomerases

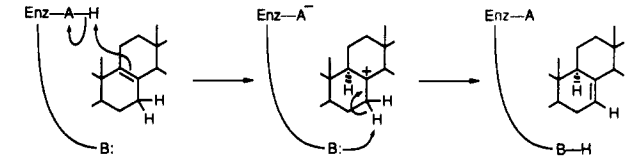
Through the years, there have been a number of reports on the isolation and partial characterization of IPPI from a variety of other sources, as summarized in Table VI.

Some of the earliest stereochemical studies were carried out on extracts of soybean[294] and *Penicillium griseofulvum*[292] and provided the first evidence that the new methyl group of DMAPP is in the *E* configuration. In addition, chicken liver,[311] *Pinus radiata*,[315] and *Citrus sinensis*[315] isomerases have been found to remove the *pro*-2*R* proton of IPP. It is reassuring that these observations are consistent with one another and with those for the mammalian liver isomerases in light of the incomplete characterization of any one of these isomerases.

As mentioned, studies of the pig liver isomerase have led to inconsistent findings with regard to several physical properties. Similarly, Sagami and Ogura[311] have reported multiple forms of chicken liver IPPI, differing slightly in several respects. Bruenger et al.[281] have reported that the freshness of the liver tissue from which the enzyme is isolated can account for the appearance of multiple forms of IPPI, and that fresh chicken liver yields only a single form.

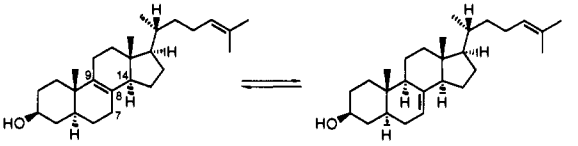
All of the isomerases examined require divalent metal ions for activity, except that from *Citrus paradisii*.[316] There are few experimental details available from the latter studies except that the enzyme source was a soluble extract from a grapefruit exocarp homogenate, and that 10 mM EDTA had no inhibitory effects on the IPP isomerase activity. Owing to a paucity of experimental detail in the report, caution should be taken in its interpretation.

SCHEME 31



B. 7,8-Sterold Isomerase

Steroid 7,8-isomerase is an essential enzyme of sterol biosynthetic pathways in animals, plants, yeasts, and fungi. It catalyzes a 1,3-allylic rearrangement in which a double bond is moved from the 8(9)-position to the 7(8)-position, as illustrated below. The isomerase is



5α-cholesta-8,24-dien-3β-ol (zymosterol): **112**

5α-cholesta-7,24-dien-3β-ol: **113**

membrane-associated (the rat liver enzyme is found in both the smooth and rough endoplasmic reticulum[321]), which has sometimes led to complications in experimental design and in the interpretation of data. For this reason, structural and mechanistic insights have evolved slowly.

The 7,8-isomerases from various sources have been shown to accept a wide variety of sterol substrates with 8(9)-double bonds. This has been demonstrated by using both a mutant strain of *Saccharomyces cerevisiae*,[322] as well as yeast, fungi, and plant cell cultures treated with 7,8-isomerase inhibitors, and animals.[323-332]

Many early mechanistic studies on the 7,8-isomerase focused on the retention or loss of hydrogen atoms and on the stereochemical course of the allylic rearrangement. These sorts of experiments were accessible since they often do not require the use of purified enzymes.

The rearrangement could be envisioned as a hydride shift or a thermally forbidden sigmatropic rearrangement. Such mechanisms would require that there be no exchange of substrate C-7 (or product C-9) hydrogens with the aqueous medium. An alternative is a one-base carbanion-type mechanism. Such a mechanism (which seems unlikely due to the low acidity of the allylic protons) could be accompanied by complete

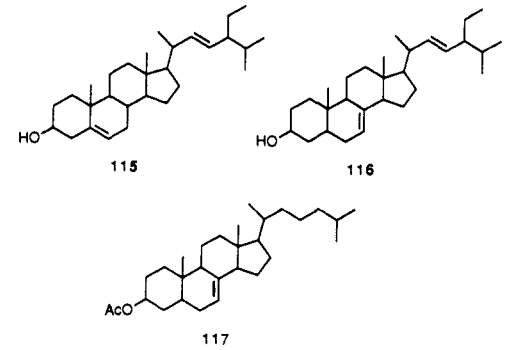
exchange, no exchange, or partial exchange, depending upon the accessibility of the proton in flight to the solvent. On the other hand, a carbocation mechanism (Scheme 31) would require initial protonation at C-9, followed by removal of a C-7 proton from the resulting allylic cation. This mechanism would require that a solvent proton be incorporated at C-9, and that a substrate proton be lost from C-7. Akhtar and Rahimtula, using a rat liver extract, observed the loss of one hydrogen from C-7 and the incorporation of tritium (from a tritiated reaction medium) at C-9 in the aerobic conversion of lanosterol to cholesterol.[333] Schroepfer, using rat liver microsomes anaerobically, found that rearrangement of 8-cholestene-3β-ol involves the incorporation of one proton from the medium.[334] These groups' findings were confirmed by Canonica et al.[335] A carbocation reaction intermediate is therefore implied.

Several groups have observed isotope exchange in the reverse of the 7,8-isomerase reaction, consistent with the mechanism in Scheme 31. Wilson et al. incubated 7-cholesten-3β-ol with rat liver enzyme preparations (both crude extract and microsomes) in tritiated water under anaerobic conditions. The reisolated substrate contained as much 0.67 g-atom of tritium per mol of sterol.[336] Scala et al. performed the complementary experiment, incubating 5α-[4-^{14}C,9α-^{3}H]cholest-7-en-3β-ol (114) with a crude rat liver homogenate both anaerobically and aerobically.[337] (Since there are numerous oxidative transformations in sterol biosynthesis, aerobic conditions are closer to those encountered physiologically.) In the anaerobic incubation, the reisolated 114 had exchanged completely. In the aerobic incubation, the cholesterol that was isolated had lost about one-third of the tritium present in the precursor. Yabusaki et al. demonstrated the reversibility of the 7,8-isomerase reaction in yeast by incubation of 8-cholesten-3β-ol with an acetone powder extract, in TOH. Tritium incorporation into both recovered 8-cholesten-3β-ol as well as 7-cholesten-3β-ol (lathosterol) was noted.[338] These findings are all consistent with the reversibility of the isomerization.

It had been reported that sterols with 14α-methyl groups are not substrates for the 7,8-isomerase from rat liver microsomes.[339] Pascal and Schroepfer demonstrated, however, that under certain conditions (e.g., 20 nmol of substrate with 20 mL of rat liver microsome suspension, under anaerobic conditions, for 3 h) these sterols can be turned over by the enzyme.[340] Small amounts of radiolabeled 14α-methyl-5α-cholest-8-en-3β-ol and 14α-(hydroxymethyl)-5α-cholest-8-en-3β-ol, produced from the corresponding tritiated Δ^7-compounds, could be trapped by cocrystallization.

A number of groups have investigated the stereochemical course of the 7,8-isomerase reaction in rat liver,[341-345] yeast,[345,346] and plants.[347-350] Caspi et al. isolated labeled cholesterol from the incubation of (2*R*,3*R*)-[2-^{14}C,2-^{3}H]mevalonic acid (MVA) with a rat liver enzyme preparation. (This isotopomer of MVA is known to afford lanosterol that is tritiated in the 7α-position.[290]) Analysis of substrate and product ^{3}H/^{14}C ratios indicated that the tritium at C-7 in lanosterol had been retained in the labeled cholesterol. Hence, the 7β-hydrogen had been removed. Since C-9 of cholesterol (and by implication, the immediate product of the allylic rearrangement) bears hydrogen in the α-configuration, the allylic rearrangement must have been a net antarafacial process. Analogous results were obtained by Gibbons et al.[342] and by Canonica et al.,[343,344,351] using essentially the same experimental protocols. Subsequently, Akhtar et al. incubated synthetic [26,27-^{14}C,7α-^{3}H]lanosterol with a rat liver enzyme preparation and observed little loss of tritium during the incorporation of this material into cholesterol.[345]

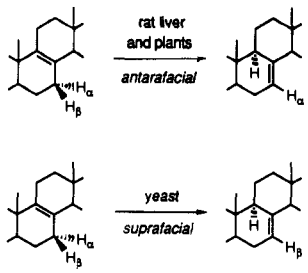
The same outcome is observed in plants. Smith et al. fed (2*S*,3*R*)- and (2*R*,3*R*)-[2-^{14}C,2-^{3}H]MVA to *Ochromonas malhamensis* and isolated labeled poriferasterol (115). By degradation, it was shown that the 7β-hydrogen of the precursor had been removed in the course of biosynthesis of 115.[347] Analogous experiments were performed and similar outcomes obtained both by Sharma with *Camellia sinensis*, which makes α-spinasterol (116),[348] and by Sliwowski and Kasprzyk, who looked at several sterols from *Calendula officinalis* flowers.[350]



115 116 117

A different mechanism is observed in yeast. Caspi and Ramm[346] fed (2*S*,3*R*)- and (2*R*,3*R*)-[2-^{14}C,2-^{3}H]-MVA to a cell-free extract of *Saccharomyces cerevisiae* and isolated (among other compounds) a mixture of yeast sterols of unknown structure. This mixture was converted chemically to 3β-acetoxy-5α-cholest-7-ene (117), which in each feeding retained four of the original six tritium atoms. (The C-4 methyl groups of lanosterol, one of which is derived from C-2 of MVA, are missing in 117.) Degradation of labeled 117 from the (2*R*,3*R*)-[2-^{14}C,2-^{3}H]MVA feeding (which labels the 7α-hydrogen of lanosterol) showed that there was *no* tritium at C-7. The same degradation was performed on steroid from the (2*S*,3*R*)-[2-^{14}C,2-^{3}H]MVA feeding; this time, tritium was shown to reside at C-7. Accordingly, in yeast, Caspi and Ramm concluded that the 7α-hydrogen of the precursor is lost in the course of the allylic rearrangement. Akhtar also investigated sterol biosynthesis in yeast.[345] Synthetic [26,27-^{14}C,7α-^{3}H]-lanosterol was incubated with whole cells of *S. cerevisiae*, and ergosterol that bore relatively little tritium was isolated from the reaction mixture. This confirms that the 7α-hydrogen of lanosterol is lost en route to ergosterol.

The dichotomous stereochemical findings are especially interesting since these enzymes catalyze the same reaction, but utilize different mechanisms. The yeast isomerization is an exception to the trends noted by Schwab and Klassen,[181] since it is a suprafacial rearrangement of a substrate with an isolated allylic system. Since it is suprafacial, Hanson and Rose's empirical rules[123,124] predict that the enzyme uses a single catalytic



acid/base group. If a carbocation intermediate is involved, then in the resting yeast isomerase this acid/base group must be protonated.

A critical breakthrough in the study of the 7,8-isomerase was made recently by Gaylor's group, who optimized the conditions for solubilizing the rat liver enzyme.[352] Although in preliminary experiments the nonionic detergent Triton WR-1339 had been used to solubilize the crude microsomal enzyme,[353] efforts to purify the 7,8-isomerase in the presence of polymeric nonionic detergents were unsuccessful. An extensive set of trials finally led to the use throughout the purification procedure of a 3:1 mixture of *n*-octylglucoside and sodium taurodeoxycholate. The purification protocol involved selective precipitation of the 7,8-isomerase with polyethylene glycol, ion-exchange chromatography, and affinity chromatography on agarose/ADP (to remove contaminating pyridine nucleotide-dependent enzymes). This led to a 21-fold purification of the 7,8-isomerase. Finally, addition of phospholipid nearly doubled the enzyme's specific activity. Although not useful for preparative purification of the 7,8-isomerase, HPLC/gel filtration gave an M_r of 80 000.[352]

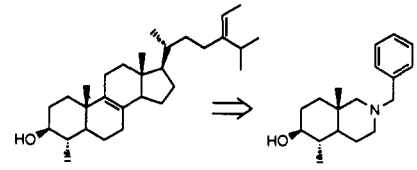
Various properties of 21-fold-purified 7,8-isomerase were compared with those of the crude microsomal isomerase, and the two forms of the enzyme were found to behave similarly.[352] Both are inhibited by 3 mM $HgCl_2$, and the inhibition is reversed by 10 mM glutathione. They are also both inhibited to the extent of about 40% by the fungicide AY-9944 (a known inhibitor of sterol biosynthesis) at 1.2 μM. On the other hand, the microsomal isomerase appears considerably more sensitive to the drug triparanol. This finding was rationalized on the basis of the hydrophobicity of triparanol, which should efficiently partition into the microsomal lipid environment. Neither NADPH nor NADH had any effect on either the soluble or the microsomal 7,8-isomerase.[352] (This is noteworthy since the mammalian microsomal Δ^5-3-ketosteroid isomerase is activated by pyridine nucleotides, as described above.) Both forms of the 7,8-isomerase are most active at pH 7.4, and they have K_m values for **112** of 52–55 μM, with a V_{max} of 4-5 nmol min^{-1} mg^{-1} of protein.[352,353]

Historically, studies on 7,8-isomerase substrate specificity, reaction equilibria, and kinetics have been complicated due to the substantial endogenous sterol content of microsomal lipids. By use of solubilized, partially purified 7,8-isomerase, however, along with sensitive analytical techniques such as capillary GC and HPLC, a number of questions were addressed.[352] Zymostenol (24(25)-dihydro-**112**) is isomerized at only 17% of the rate at which **112** is turned over. Various sterols with 4,4-dimethyl substituents, or double bonds in the 8(14) (rather than 8(9)) position are not 7,8-isomerase substrates. This is consistent with earlier predictions[339] that C-4 demethylation precedes isomerization in the biosynthetic conversion of lanosterol to cholesterol. It also gives some indication as to the specificity of the protonation that presumably initiates the enzymatic isomerization.

Using their chromatographic assay and the partially-purified enzyme, Gaylor et al. not only confirmed that the 7,8-isomerase reaction is reversible, but they also estimated the equilibrium constant and observed the kinetics of the forward and back reactions.[352] Thus, with **114** as the substrate, the reaction equilibrium is ca. 95% in favor of the Δ^7-sterol. The rate of isomerization of **113** is 3.6 nmol h^{-1} mg^{-1} of protein.

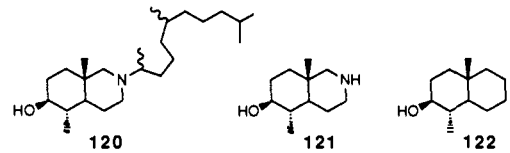
Even with purified protein now available, not much is known about the molecular properties of isomerase and its active site.

Compelling evidence in support of a cationic reaction intermediate has come from studies of a group of rationally designed inhibitors of the 7,8-isomerase. This is part of a larger effort to develop amine-based mimics of carbocationic reaction intermediates, as inhibitors of various enzymes of sterol biosynthesis.[354,355] Preliminary studies identified 4α-methylstigmasta-(8*Z*)-24-(28)-dien-3β-ol (**118**) as a good substrate for the 7,8-isomerase from bramble (*Rubus fructicosus* L.) and marine (*Zea mays* L.).[327] On the basis of this and the fact that amines are positively charged at physiological pH, *N*-benzyl-8-aza-4α,10-dimethyl-*trans*-deca-3β-ol (**119**) was designed as an analogue of the proposed 7,8-isomerase reaction intermediate.[356] Compound **119** is a very potent inhibitor of the isomerase in bramble suspension cultures. At 1 mg mL^{-1}, it inhibits the biosynthesis of Δ^5-sterols by about 90%.



118 **119**

Similarly effective inhibition by **119** of the microsomal 7,8-isomerase from maize seedlings was observed in in vitro assays.[356] From the inhibition curve, an IC_{50} value of 0.13 μM was calculated for **119**. This can be compared to a K_m of 100 μM for **118**. Under the experimental conditions used, it could be inferred that the 7,8-isomerase's affinity for **119** is about 2–3 orders of magnitude greater than for its endogenous substrate.[356] The steroid-like **120** inhibits the isomerase in maize seedling microsomes, with an IC_{50} of 0.2 μM.[355,357] It also is an extremely potent inhibitor of cycloeucalenol-obtusifoliol isomerase (COI), the enzyme that opens the 9(10)-cyclopropyl ring of a prior metabolite, via a carbocationic intermediate (this one at C-9).[355] (Since **120** is such an effective inhibitor of COI, it has not been possible to test it as an inhibitor of the 7,8-isomerase in vivo.[357]) For comparison, the intermediate analogue **121** had an IC_{50} of 10 μM, and its deaza analogue, **122**, did not inhibit the 7,8-isomerase at all.[356]



120 **121** **122**

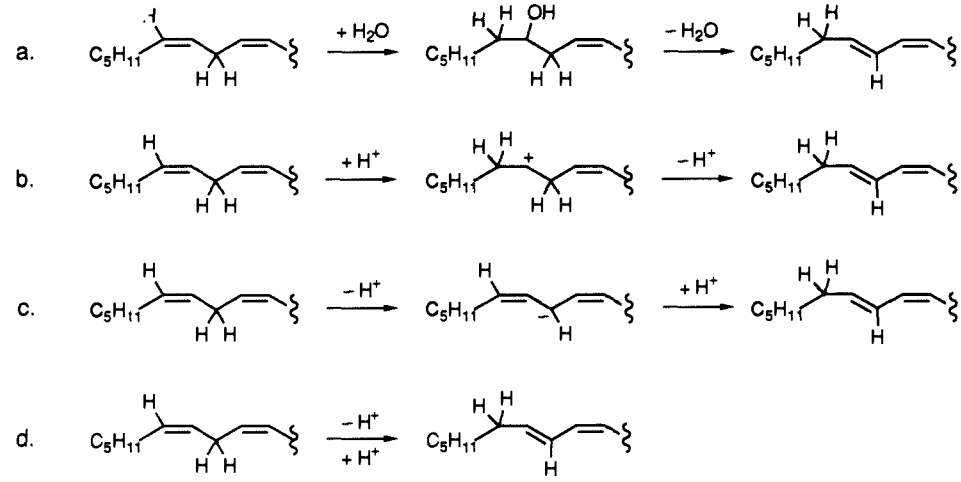


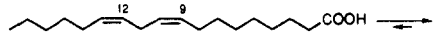
Figure 5. Possible mechanisms for the allylic rearrangement catalyzed by linoleic acid isomerase: (a) hydration/dehydration; (b) carbocation intermediate; (c) carbanion intermediate; and (d) concerted.

Finally, Gerst et al. have found that compound **120** is a potent inhibitor of cholesterol biosynthesis in 3T3 fibroblasts (IC_{50} ca. 20 nM). It acts primarily by shutting down oxidosqualene cyclase, but there is reason to believe that the 7,8-isomerase is a secondary target.[358]

In summary, now that methods have been developed to separate the 7,8-isomerase from the other enzymes of sterol biosynthesis, there are tremendous opportunities to fully characterize the structure of this enzyme. It will be especially interesting to determine how the isomerase stabilizes the carbocation reaction intermediate and to learn why the yeast isomerase catalyzes the rearrangement via an aberrant stereochemical course.

C. Linoleic Acid Isomerase

In 1966 Tove and his co-workers published the first in a series of reports dealing with the biohydrogenation of linoleic acid ((*Z*,*Z*)-9,12-octadecadienoic acid; **123**) to stearic acid by the anaerobic rumen microorganism *Butyrivibrio fibrisolvens*.[359] The actual substrate for the reductase[359] is (*Z*,*E*)-9,11-octadecadienoic acid (**124**),[360] which is produced by the action of linoleic acid isomerase.[359,361] The functional importance of this pathway to the organism remains unknown.



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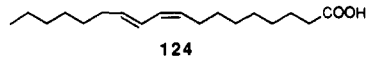


124

The isomerase has been isolated in the membrane fraction of *B. fibrisolvens* by differential centrifugation.[360] Efforts to stabilize the enzyme were unsuccessful, and so all further studies were carried out on this crude preparation.

Linoleic acid isomerase is an oxygen-insensitive enzyme with a relatively narrow range of maximal activity between pH 7.0 and 7.2. The K_m values for **123** and linolenic acid ((*Z*,*Z*,*Z*)-9,12,15-octatrienoic acid) were estimated at 12 μM and 23 μM, respectively. (Substrate inhibition was observed at linoleic acid concentrations above 50 μM.) The equilibrium constant is about 60, thus favoring the conjugated diene system.[360] The isomerase is sensitive to sulfhydryl modification reagents, particularly *p*-hydroxymercuribenzoate, which at 1 μM completely kills the enzyme.[362] Although the isomerase apparently does not require exogenous cofactors,[360] it is reversibly (noncompetitively) inhibited by the metal ion chelators *o*-phenanthroline and EDTA.[362]

When **123** was incubated with the isomerase in D_2O, **124** that was produced had incorporated a single deuterium atom, at position 13.[360] Subsequently, it was determined that a solvent proton is incorporated into the *pro*-13*R* position of the product. A kinetic isotope effect of 2.7 accompanies the isomerization of [11,11-$^2H_2$]**123**.[363] Unfortunately, the steric course of the deprotonation at C-11 (and therefore the overall steric course of the isomerization) remains unknown.

Linoleic acid isomerase has a fairly narrow substrate specificity. It isomerizes only fatty acids with *Z* double bonds in the 9- and 12-positions. Substrates include **123**, linolenic acid, and γ-linolenic acid ((*Z*,*Z*,*Z*)-6,9-12-octadecatrienoic acid).[362,364] The optimal substrate chain length is 18 carbons. (*Z*,*Z*)-9,12-Hexadecenoic acid is turned over at only 16% the rate of turnover of **123**, and (*Z*,*Z*)-9,12-eicosadienoic acid is not isomerized at all.[363] Only carboxylic acids are isomerized; alcohol, amine, methyl ether, methyl ester, ketone, aldehyde, amide, hydroxamate, hydrazide, triglyceride, and oxime derivatives are not substrates. Several linolenic acid derivatives have also been tested, with analogous results.[362]

The isomerase is competitively inhibited by a variety of unsaturated fatty acids and derivatives of unsaturated fatty acids. Of these inhibitors, the most effective, by far, are those that can act as hydrogen-bond donors. The configurations of the double bonds appear unimportant, and even cyclopropyl fatty acids (but not straight-chain saturated fatty acids) inhibit.[362] The K_i values for some of the hydrogen bond donating derivatives fall in the range 7–15 μM (i.e., close to the K_m for **123**).[363]

Several mechanisms of action for the isomerase have been considered (Figure 5).

Mechanism (a) was deemed unlikely on the grounds that 12-hydroxy-(*Z*)-9-octadecenoic acid (ricinoleic acid) is neither turned over[363] by nor does it inhibit[362] linoleic acid isomerase. An isotope trapping experiment failed to detect ricinoleic acid as an intermediate, although it was acknowledged that if it were actually an inter-

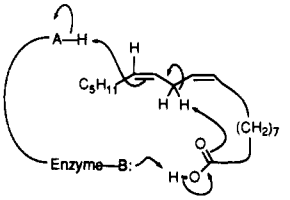
mediate, it might be too tightly bound by the isomerase to exchange with exogenous ricinoleic acid.[363] It is now known that the vast majority of enzyme-catalyzed hydration/dehydration reactions involve substrates with carbon/carbon double bonds that are conjugated to carbonyl groups (or the equivalent). Thus, linoleic acid is an unlikely substrate for such a reaction.

The carbocation mechanism, mechanism (b), was ruled out by the kinetic deuterium isotope effect for C–H bond breaking at C-11, and the claim that under this mechanism, protonation at C-13 ought to be rate-limiting.[363] The logic of this argument is questionable, however, and so a carbocation mechanism must still be considered.

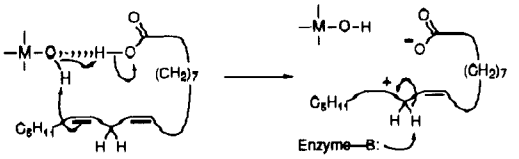
Solvent exchange experiments were carried out in an attempt to demonstrate an enzyme-bound carbanion (mechanism (c)). Linoleic acid was incubated with the isomerase for various time intervals. It was stated that "no exchange in excess of that expected from the equilibrium of the isomerization reaction was observed".[363] Given that rearrangement and protonation of the putative carbanion at C-13 would be energetically favorable (since a conjugated diene system would be formed), any exchange at C-11 not accompanied by rearrangement would be surprising. Also, [11,11-$^2H_2$]oleic acid was incubated with the isomerase for 3.5 h, and again, no exchange could be detected.[363] This negative outcome is actually consistent with the carbanion mechanism, since the C-11 carbanion derived from linoleic acid would be doubly allylic (hence, better stabilized), whereas that derived from oleic acid would only be stabilized by the C-9 double bond. The enzyme may be incapable of removing a proton from a substrate that has C-11 protons that are less acidic than those of linoleic acid.

Finally, there is no evidence either way in regard to concerted mechanism (d).

Kepler et al. made the attractive suggestion that the substrate's carboxyl group may be directly involved in the rearrangement mechanism. This suggestion was based upon (1) the fact that while linoleic and linolenic acids have quite different extended conformations, they have similar "looped-back" conformations, and (2) the absolute requirement for substrates to have carboxyl groups.[363] The proposal of Kepler et al. was presented in the context of their preferred mechanism, the concerted one.



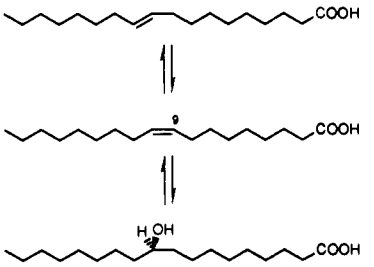
Similar roles for the carboxyl group can also be envisioned in the context of the carbanion and carbocation mechanisms. In particular, the carbanion mechanism merely requires that deprotonation precede protonation, and the carbocation mechanism requires the reverse. A variation on the carbocation mechanism that also includes a role for a metal ion (implied on the basis of the inhibition of the isomerase by metal chelators) is shown below. In fact, it is conceivable that the negatively charged carboxylate group could provide stabilization to the positive charge of the carbocation.



Subsequent to these reports from Tove's group, Garcia et al. reexamined the isomerization of octadecadienoic acids by a cell-free extract of *B. fibrisolvens*,[365] analogous to that which had been used in Tove's studies. They systematically tested all of the methylene-interrupted *Z,Z*-octadecadienoic acids as inhibitors and as substrates. Every acid tested was an inhibitor of the isomerase except for linoleic acid ((*Z,Z*)-9,12-octadecadienoic acid) and (*Z,Z*)-2,5-octadecadienoic acid, which is isomerized at about one-quarter the rate of turnover of linoleic acid. (*Z,Z*)-2,5-Octadecadienoic acid is converted to the 3,5 (rather than the 2,4) isomer, apparently with the *E,Z* configuration. The reason that only these two octadecadienoic acid isomers are isomerase substrates and how this observation fits into a scheme for substrate binding and catalysis remain puzzling questions.

D. Oleate Isomerase/Hydratase

A soluble enzyme preparation from *Pseudomonas* sp. NRRL B-3266 catalyzes the interconversion of (*Z*)-9-octadecenoic (oleic) acid, (*E*)-10-octadecenoic acid, and (*R*)-10-hydroxyoctadecanoic acid.[366-368] This same enzyme preparation also hydrates (*E*)- and (*Z*)-9,10-epoxyoctadecanoic acid.[369] As is the case with β-hydroxydecanoyl thiol ester dehydrase, the isomerization is independent of the olefin hydration reaction; (*Z*)-9- and (*E*)-10-octadecenoic acid are directly interconverted, and only (*Z*)-9-octadecenoic acid is a substrate for the hydration reaction.



Niehaus has cited several lines of evidence that point to the association of all three activities (isomerase, alkene hydratase, and epoxide hydrolase) with a single enzyme.[370] (1) All of the activities are induced simultaneously when the organism is grown in the presence of oleate. (2) All three activities undergo thermal inactivation at the same rate, and the hydratase and isomerase activities are equally sensitive to inhibition by detergents. (3) The three activities remain associated when subjected to salt fractionation, ion-exchange chromatography, density-gradient centrifugation, polyacrylamide gel electrophoresis, or isoelectric focusing. (4) The relative proportions of activities do not change following extensive preparative purification. (5) The stereospecificities of the various reactions (as described below) are consistent with a single substrate binding site.

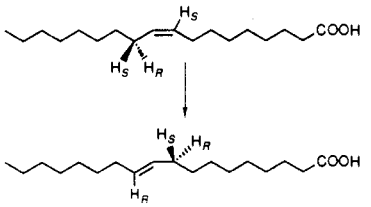
Unfortunately, the highly purified form of the isomerase/hydratase has only been described in Niehaus's

review,[370] and there are no published accounts of its detailed characterization. The mechanistic experiments reported to date have all utilized crude soluble enzyme preparations.

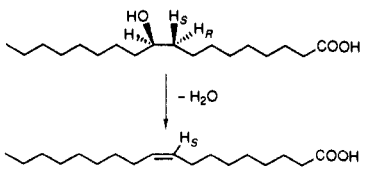
After (*E*)-10-[1-^{14}C,9-^{3}H]octadecenoic acid and (*Z*)-9-[1-^{14}C,^{3}H]octadecenoic acid were equilibrated (separately), the $^{3}H/^{14}C$ ratios of the recovered substrates had increased over their original values. Thus, C–H bond breaking is rate-determining in both isomerization directions.[368] Although solvent isotope effect data have been published for both the isomerization and the hydration,[368] the data are uninterpretable, presumably because of competing enzyme activities in the crude protein preparation used for these assays. The concentrations of the substrates at equilibrium are reported to be 67% 10-hydroxyoctadecanoic acid, 8% (*Z*)-9-octadecenoic acid, and 25% (*E*)-10-octadecenoic acid.

The substrate specificities of the isomerase and hydratase activities are similar though not identical.[368] The *Z*-9-monounsaturated fatty acids from C_{16} to C_{19} are substrates for both reactions. In addition, (*Z*)-9-eicosanoic acid, linoleic acid ((*Z*,*Z*)-9,12-octadecadienoic acid), and (*Z*)-8-heptadecenoic acid are hydration substrates. The best isomerization substrate is (*Z*)-9-octadecenoic acid. The best hydration substrate is (*Z*)-9-hexadecenoic acid; (*Z*)-9-eicosanoic acid and (*Z*)-8-heptadecenoic acid are the worst. (*E*)-9-Octadecenoic acid and 9- and 10-octadecynoic acid are substrates for neither the hydration nor the isomerization.

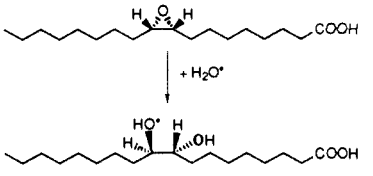
The three enzyme activities exhibit stereospecificities that point toward common mechanistic features. The isomerization of oleic acid involves the loss of the *pro*-11*S* hydrogen.[363] The *pro*-9*R* hydrogen of (*E*)-10-octadecenoic acid comes from the medium.[368] Thus, the overall stereochemical course of the reaction is antarafacial.



The dehydration is a net anti elimination, in which the *pro*-9*R* hydrogen is lost[368] from (*R*)-10-hydroxyoctadecanoic acid.[367]



The epoxide hydrolase converts (9*R*,10*S*)-9,10-epoxyoctadecanoic acid[369] (misidentified as the 9*R*,10*R* isomer in ref 370) to (9*R*,10*R*)-9,10-dihydroxyoctadecanoic acid.[370] Hydrolysis of the *E* isomer is also regio- and stereospecific.



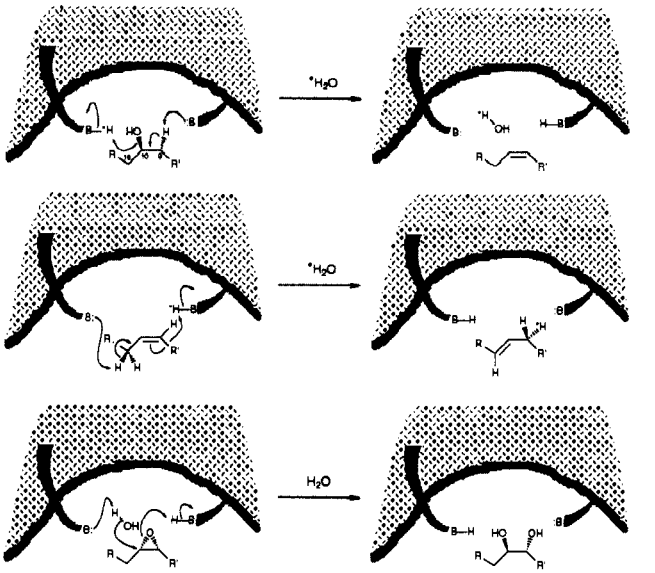


Figure 6. Proposed mechanism of action for *Pseudomonas* oleate isomerase/hydratase. A two-base mechanism is consistent with the observed reaction stereochemistry.

The stereochemical findings just described are consistent with the hypothesis that all three of the reactions catalyzed by the *Pseudomonas* oleate isomerase/hydratase utilize the same two active site acid/base groups. The one that accesses events at C-10 and C-11 is in front of the substrate (Figure 6), and the one that mediates protonations and deprotonations at C-9 is to the rear.

IV. Conclusions

Of all of the isomerases discussed in this review, the one that is by far the best understood in terms of the relationship between its structure and its mechanism is *Pseudomonas testosteroni* Δ^5-3-ketosteroid isomerase. Active-site labeling, NMR, and X-ray crystallography studies have provided a wealth of detailed information. Analogous studies of some of the other isomerases are proceeding. β-Hydroxydecanoyl thiol ester dehydrase has been sequenced and crystallized, and crystallographic studies are under way. Muconolactone isomerase has been sequenced. There are reports that it, too, has been crystallized and that a low-resolution electron-density map has been generated. Yeast isopentenyl pyrophosphate isomerase has also been sequenced, and it is rumored to have been crystallized. Five years from now it may be possible to make sweeping statements regarding general structure/function relationships among allylic isomerases. For now, our ability to do so is limited.

The only basis upon which most of the allylic isomerases discussed here may be compared directly is overall reaction stereochemistry. In this regard, the general stereochemical/mechanistic trends noted by Schwab and Klassen[181] have held up well. In general, those isomerizations that procede via hypothetical (or real) carbanion intermediates in which the carbanion is resonance-stabilized through an adjacent ketone or thiol ester carbonyl group (or the equivalent) proceed suprafacially, indicative of a single active site acid/base group. Those isomerizations that could not proceed via especially stable carbanion intermediates tend to proceed antarafacially, suggesting that both an acidic and

a basic group are required for catalysis. (A more general version of this rule has been stated by Benner, who has considered the stabilities of hypothetical carbocation intermediates, as well.[4]) There appears to be only one exception to these "rules": yeast 7,8-steroid isomerase, which catalyzes a suprafacial allylic rearrangement.

While this exception is indeed noteworthy, the evolutionary relationships among the enzymes that conform to the rules are probably more significant. Are the enzymes within each class related structurally as well as functionally? Is the conservation of stereochemical course (hence, mechanism) a result of convergent or divergent evolution? How finely tuned are the enzymes' mechanisms? Do they approach "evolutionary perfection", as defined by Albery and Knowles?[37] Does the free energy profile for the yeast 7,8-steroid isomerase suggest that this enzyme is much less highly evolved?

For the present, there are no answers to these questions. However, by using engineered expression systems it is now possible to efficiently produce many proteins. This, in turn, simplifies the task of protein purification. The availability of large quantities of highly purified isomerases will facilitate the study of these enzymes and allow many of the outstanding questions to be answered.

Acknowledgments. We thank Drs. P. Talalay, R. M. Pollack, A. S. Mildvan, C. D. Poulter, M. A. Levy, and W. G. Niehaus, Jr., for preprints, for sharing some of their unpublished observations, and for comments on some of the work described here. We also express our sincere gratitute to Dr. Catherine M. Jackson for preliminary literature work on certain sections of this review. Finally, we gratefully acknowledge the National Institutes of Health for their generous support of our own work (as described herein) via Grants GM26074 and GM36286.

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