Mechanism of the Reaction Catalyzed by Δ^5-3-Ketosteroid Isomerase of *Comamonas* (*Pseudomonas*) *testosteroni*: Kinetic Properties of a Modified Enzyme in Which Tyrosine 14 Is Replaced by 3-Fluorotyrosine†

Bob Brooks and William F. Benisek*

Appendix: Effects of Y14 and D38 pK_a' Values on k_{cat} of Steroid Isomerase†

William F. Benisek*

Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616

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ABSTRACT: Tyrosine 14 of Δ^5-3-ketosteroid isomerase plays an important role in the function of the enzyme, since its replacement by phenylalanine results in a decrease in k_{cat} by a factor of $10^{-4.7}$. This result and the fact that this residue resides in the enzyme's substrate binding site and is in close proximity to C-2 of the bound steroid suggests that it functions as an electrophile in the catalytic mechanism by protonation of or hydrogen bonding to the C-3 carbonyl oxygen of the substrate. In order to obtain more information about the role of tyrosine 14, we have prepared a modified form of the enzyme in which tyrosine 14 has been substantially replaced *in vivo* by exogenously supplied 3-fluorotyrosine, a tyrosine derivative in which the pK_a' of the phenol hydroxyl should be decreased by about 1.5 log units. Site specificity of this modification has been ensured by mutation of the codons for the nonessential tyrosines 55 and 88 to phenylalanine. We find that replacement of tyrosine 14 by 3-fluorotyrosine in the Y55,88F modified form of the isomerase results in a 4-fold decrease in k_{cat}. We interpret this result in terms of a mechanism in which the transition state for enolization is dienolate-like, characterized by relatively little proton transfer from tyrosine 14 in the transition state, and the intermediate in the overall reaction is dienol-like. An alternative mechanism in which the intermediate is stabilized by a short, strong hydrogen bond can also be consistent with the data.

The Δ^5-3-ketosteroid isomerase (KSI)[1] from *Comamonas* (formerly *Pseudomonas*) *testosteroni* (Tamaoka et al., 1987) catalyzes the transfer of the 4β proton of the substrate to the 6β position in the product. Concomitant with this transfer is the migration of the 5,6 double bond of the substrate to the 4,5 position in the product. The reaction is believed to proceed through a dienol or dienolate intermediate, as is shown in Figure 1. A substantial body of evidence has been accumulated which indicates that a single base, D38, abstracts the 4β proton in the enolization step of the reaction and then, with only a little exchange with solvent protons, transfers the abstracted proton to the 6β position of the product in the ketonization step (Pollack et al., 1989; Schwab & Henderson, 1990).

It has been suspected, even in the earliest studies on the KSI mechanism (Malhotra & Ringold, 1965), that the enzyme promotes proton abstraction from C-4 (and C-6 in the reverse direction) by electrophilic polarization of the C-3 carbonyl oxygen with a Brønsted acid group. This group has been postulated either to actually protonate the carbonyl oxygen or to simply hydrogen bond to it while D38 abstracts the 4β proton. With full protonation, proton abstraction would lead to a dienol intermediate, while hydrogen bonding would lead to a dienolate intermediate. In an enzyme active site from which bulk solvent is excluded unusually strong hydrogen bonds can be formed between the electrophilic group and the dienolate anion if the pK_a's of the electrophilic group and the dienol are similar (Hibbert & Emsley, 1990; Gerlt & Gassman, 1993).

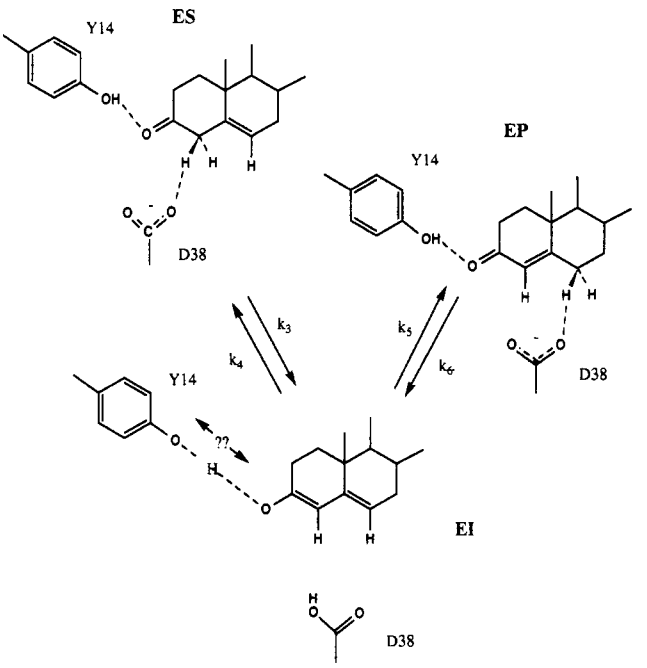

FIGURE 1: Mechanism of the reaction catalyzed by KSI. The structures ES, EI, and EP refer to the enzyme–substrate, enzyme–intermediate, and enzyme–product complexes, respectively. Rate constants k_3–k_6 are defined in eq 1 (Results and Discussion). The uncertainty in the state of proton transfer to the steroid intermediate is indicated in the structure of EI. The overall reaction catalyzed by KSI is given in Figure 1 of Holman and Benisek (1994) (accompanying paper).

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* Author to whom correspondence should be addressed.

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[1] Abbreviations: KSI, Δ^5-3-ketosteroid isomerase; 5-AND, 5-androstene-3,17-dione; fluoroY, 3-fluorotyrosine; IPTG, isopropyl thiogalactoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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A likely candidate for the electrophilic group has been found in the hydroxyl group of tyrosine 14. Replacement of tyrosine 14 with phenylalanine results in a decrease in k_{cat} of about $10^{-4.7}$ (Kuliopulos et al., 1989), and additional studies have supported the critical nature of this residue (Kuliopulos et al., 1990; Austin et al., 1992; Li et al., 1993). In the preliminary crystal structure of wild-type KSI tyrosine 14 is located at the bottom of the pit-like steroid binding site (E. Westbrook, personal communication). Y14 can be positioned to interact with the carbonyl oxygen of a bound substrate via a hydrogen bond if its side-chain conformation is altered upon binding of substrate. Although considerable effort has been expended in trying to determine whether the intermediate, I, in the mechanism shown in Figure 1 is a dienol or a dienolate ion (Xue et al., 1991; Kuliopulos et al., 1989; Eames et al., 1990; Hawkinson et al., 1991a; Bantia & Pollack, 1986; Zeng et al., 1992; Austin et al., 1992; Li et al., 1993), the nature of the transition states for the enolization step and the ketonization step has not yet been clarified.

In the present study, we have modified Y14 of the high-activity Y55,88F form of KSI by replacing the single tyrosine of this enzyme with 3-fluorotyrosine, a tyrosine derivative in which a fluorine is located ortho to the hydroxyl group. Due to the inductive effect of the fluorine, the pK_a' for 3-fluorotyrosine in the enzyme is expected (from data for model compounds) to be approximately 1.5 log units less than the pK_a' of tyrosine. The kinetic constants k_{cat} and K_m for the fluorinated isomerase have been measured, and the change in k_{cat} has been interpreted in terms of Brønsted acid catalysis theory. For a mechanism in which the intermediate is a dienol, the change observed for k_{cat} (compared to the k_{cat} for Y55,-88F) can be accounted for by a Brønsted coefficient for acid catalysis, α, of 0.5 or less, indicating that there is little proton transfer from Y14 to the substrate at the enolization transition state. This conclusion, and the results of Holman and Benisek (1994) which indicate that proton transfer from C-4 of the substrate to D38 is well-advanced in the enolization transition state, when considered together, suggest that the enolization transition state is dienolate-like, and I resembles a dienol.

EXPERIMENTAL PROCEDURES

Materials. *dl*-3-Fluorotyrosine was obtained from Sigma or Aldrich and was used without further purification. When the material was subjected to ion-exchange amino acid analysis, only a single ninhydrin-positive peak was observed. The oligonucleotide used to direct the Y55F mutation was synthesized by the UCD Protein Structure Laboratory by the phosphoramidite method using an Applied Biosystems Model 430A synthesizer. The oligonucleotide used to direct the Y88F mutation was synthesized by Genetic Designs, Inc. The plasmids M13mp19 RF and pKK223-3 were supplied by Bio-Rad and Pharmacia, respectively. We have described the construction of pKSI*tac* previously (Brooks & Benisek, 1992). A deoxycholate–agarose affinity resin was synthesized as described by Linden and Benisek (1986). We synthesized 5-AND by the method of Djerassi et al. (1956). Other chemicals were of reagent grade or better.

Construction of the Y55,88F Modified Form of the KSI Gene. We subcloned the KSI gene from pKSI*tac* into M13mp19 and mutagenized it in a two-step approach. First, the codon for Y55, TAC, was converted to the TTC codon of F using the 18mer oligonucleotide 5′-CGTGAGTTTTTCGC-CAAC-3′ and employing the mutagenesis kit from Bio-Rad, based on the method of Kunkel (1985). Then, after isolation of the single-stranded form of the Y55F gene, the second mutation, Y88F, was introduced using the oligonucleotide 5′-TTCGAGTTTCAGGGCCGC-3′ and the mutagenesis kit from Amersham, which is based on the method of Eckstein (Taylor et al., 1985). We then subcloned the Y55,88F gene from the replicative form of the M13 construct into pKK223-3 to make the expression vector pKSI*tac*.Y55,88F. The complete KSI gene in pKSI*tac*.Y55,88F was sequenced in order to check that only the intended mutations had been introduced.

Expression of Y55,88F KSI. We expressed pKSI*tac*.Y55,-88F in *Escherichia coli* TG1 cells growing in 600 mL of TB media (Tartof & Hobbs, 1987). The culture contained 50 μg/mL ampicillin and 0.5 mM IPTG. The cells were grown overnight at 37 °C in shaking culture. Cells were harvested by centrifugation and washed twice with 20 mM Tris-HCl, pH 7.25 ± 0.25. The washed cells were resuspended in the Tris buffer and opened by sonication for 2 × 45 s on ice. Intact cells and other debris were removed by centrifugation at 12000*g* for 10 min at 4 °C.

Expression of Y55,88F KSI Enriched with FluoroY. Y55,-88F containing 3-fluorotyrosine was produced in *E. coli* TG1 cells transformed with pKSI*tac*.Y55,88F after induction by IPTG. The strategy for maximizing the incorporation of exogenous 3-fluorotyrosine into the Y55,88F polypeptide was adapted from the method of Lu et al. (1976), in which the cells are grown in a defined medium and aromatic amino acid biosynthesis is inhibited by feedback inhibition of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthetase and repression of its synthesis. In our method *E. coli* TG1 cells transformed with pKSI*tac*.Y55,88F (Ausubel et al., 1989) were grown in minimal media (Neidhardt et al., 1974) containing 0.4% glucose and 100 μg/mL ampicillin in shaking culture at 37 °C. When the absorbance of the culture reached 1.0 at 420 nm, IPTG was added (final concentration, 0.5 mM), followed by the addition of a solution of 3-fluoroY, F, and W (final concentrations, 1 mM in each amino acid). After incubation with shaking for 2.25 h, the cells were harvested by centrifugation at 4 °C. The harvested cells were washed twice in ice-cold 10 mM Tris-HCl, pH 8, and stored overnight at −85 °C. The frozen cells were thawed, resuspended in 10 mM Tris-HCl, pH 8, and broken open using a French press. DNAse (approx. 2–3 units/mL) was added to the viscous mixture and allowed to incubate for 30 min at 37 °C.

Purification of KSIs Y55,88F and Y55,88F Enriched with FluoroY. All procedures were carried out at 4 °C, unless noted otherwise. An equal volume of freezing-cold (−20 °C) 95% ethanol was added to the cell extract prepared as described above; the solution was allowed to sit overnight, during which time a precipitate formed. The next day the mixture was centrifuged at 12000*g*, the pellet obtained was washed once with 5 mM Tris-HCl, pH 8, in 50% ethanol, and the wash and supernatant solutions were combined. EDTA, $MgCl_2$, and 95% ethanol were added to the resulting solution to final concentrations of 0.5 mM, 5 mM, and 80% (v/v), respectively. The resulting precipitate was allowed to accumulate for a few days at 4 °C. The settled precipitate was recovered by centrifugation for 15 min at 4300*g* and then extracted with 10 mM potassium phosphate, pH 7.0. Undissolved material was removed by centrifugation. The supernatant solution was brought to 50 mM potassium phosphate, pH 7.0, and the protein was precipitated by adding ammonium sulfate to 60% (Y55,88F) or 80–85% (Y55,88F enriched with fluoroY) of saturation. The precipitate was recovered by centrifugation for 40 min at 12000*g*. The pellet was redissolved in 5–10 volumes of water, and the resulting solution was dialyzed against 0.4 M potassium phosphate, pH 7. The resulting

solution was applied to a deoxycholate affinity column. The column was washed with 0.4 M potassium phosphate, pH 7.0, in order to remove unbound proteins, and the bound proteins were eluted with 1 mM potassium phosphate, pH 7.0, containing 25% ethanol. The fractions exhibiting KSI activity were pooled and dialyzed against 10 mM potassium phosphate, pH 7.0, and the dialyzed solution was concentrated 5-fold by lyophilization (the Y55,88F preparation was lyophilized to dryness, and the residue was redissolved in 50 mM potassium phosphate, pH 7.0). The concentrated enzyme was applied to a Sephacryl S-100 HR column and eluted with 50 mM potassium phosphate, pH 7. The active fractions were pooled and concentrated by ammonium sulfate precipitation (70% saturation). The precipitate was dissolved in 3–5 volumes of water and dialyzed against 10 mM potassium phosphate, pH 7.0 (0.02% NaN_3 was added to the Y55,88F prep), to give a stock solution of the enzyme. The homogeneity of the preparations so obtained was assessed by SDS/6 M urea–PAGE. The purified proteins were also characterized by amino acid analysis of their acid hydrolysates.

Amino Acid Analysis. Proteins were hydrolyzed *in vacuo* for 24 h at 110 °C with 5.7 N HCl containing 0.1% phenol. Control experiments in which 3-fluorotyrosine was subjected to these hydrolysis conditions demonstrated that it was completely stable to this treatment. Amino acid analyses were performed by the UCD Protein Structure Laboratory using a Beckman Model 6300 amino acid analyzer equipped with a System Gold data system. 3-Fluorotyrosine eluted just ahead of tyrosine, using the physiological fluids analysis elution protocol.

Kinetic Methods. The isomerase-catalyzed reactions were carried out at 25 °C in 34 mM potassium phosphate, pH 7.0, containing 2.5 mM EDTA and 3.3% (v/v) methanol, as described previously (Schriefer & Benisek, 1984), using 5-AND as the substrate. The concentration of 5-AND was varied from 30 to 295 μM. No precipitation of the steroid during the course of the reaction was observed. Data were analyzed using the program of Cleland (1977), as adapted for use on an IBM PC-AT microcomputer, or by means of the program ENZFITTER (Elsevier-BIOSOFT).

Protein Concentration. The protein concentration of purified KSI stock solutions used for kinetic studies was determined by amino acid analysis and is based on the amounts of A, L, and K in the hydrolysate and their molar abundances in the KSI polypeptide.

RESULTS AND DISCUSSION

Purification of Y55,88F and Y55,88F Enriched with FluoroY. Using the expression methods and purification protocol described in Experimental Procedures, approximately 0.4 mg of Y55,88F and 0.2 mg of fluoroY-enriched Y55,88F were obtained per gram of wet *E. coli* cells. The purity of the proteins was assessed by SDS/6 M urea–PAGE and by amino acid analysis. Only single bands were visible on the gels when stained with Coomassie Blue. Amino acid analyses of acid hydrolysates of Y55,88F and Y55,88F enriched with fluoroY are given in Table 1. These analyses demonstrate that the intended amino acid replacements had been achieved in Y55,88F. The analysis of the fluorotyrosine-enriched Y55,88F form of KSI showed that Y14 in 78% of the polypeptides had been replaced by fluoroY.

Kinetic Properties of Y55,88F and Y55,88F Enriched with FluoroY. The values of k_{cat} and K_m with 5-AND as the substrate for Y55,88F and Y55,88F enriched with fluoroY and calculated values of these parameters for pure Y55,88F:Y14fluoroY are given in Table 2. The kinetic properties measured for Y55,88F are in good agreement with the results of Mildvan's group (Kuliopulos et al., 1991; Li et al., 1993). Interestingly, replacement of Y14 by fluoroY in Y55,88F results in a decrease in k_{cat} by a factor of 4, with no change in the value of K_m.

Table 1: Amino Acid Compositions of Y55,88F and Y55,88F Enriched with FluoroY[a]

amino acid (AA)	expected AA composition of Y55,88F from gene sequence	obsd AA compositions: Y55,88F	obsd AA compositions: Y55,88F enriched with fluoroY
Asp	12	12.0	12.0
Thr	7	6.4[c]	7.3[c]
Ser	5	4.1[c]	4.2[c]
Glu	12	11.9	12.1
Pro	5	5.0	5.0
Gly	9	9.0	9.0
Ala	21	20.8	20.7
Val	14	13.2[d]	12.5[d]
Met	3	0.4	1.0
Ile	4	3.7[d]	3.5[d]
Leu	8	8.1	8.4
fluoroTyr[b]	0	0	0.8
Tyr	1	1.0	0.2
Phe	10	9.9	9.8
His	3	3.0	2.6
Lys	4	4.1	3.9
Arg	7	7.0	6.7
Cys	0	*e*	*e*
Trp	0	*e*	*e*

[a] Methods for hydrolysis and amino acid analysis are described in Experimental Procedures. Retention times (min) in the Y55,88F enriched with fluoroY analysis were 49.34 for Leu, 50.16 for F-Tyr, and 51.24 for Tyr. Molar ratios are calculated using a factor based on Ala, Leu, and Lys. [b] FluoroY eluted just before Tyr. [c] Not corrected for hydrolytic destruction. [d] Incompletely released from peptide linkage after 24 h of hydrolysis. [e] Not determined.

Table 2: Kinetic Parameters for Y55,88F and Y55,88F:FluoroY KSI[a]

KSI	k_{cat} (s^{-1})	K_m (μM)
Y55,88F	10 800 ± 200	138 ± 5
Y55,88F enriched with fluoroY	4520 ± 70	137 ± 4
Y55,88F:fluoroY[b]	2710	136

[a] Results are ±1 SD for assays done in triplicate. [b] These values are calculated using the relation $k_{cat}(Y55,88F:Y14fluoroY) = [k_{cat}(mixture) - 0.22k_{cat}(Y55,88F)]/0.78$.

Strategy for Alteration of pK_a'. The strategy we have employed to alter the pK_a' of Y14 was to replace it with a tyrosine analogue having an ortho fluoro substituent, which in model compounds reduces the pK_a' of the hydroxyl by about 1.5 log units. The pK_a' of Y14 in the Y55,88F mutant has recently been found to be 11.6 (Li et al., 1993). Since the acidifying effect of the fluorine is inductive by nature rather than operating by a "through-space" mechanism, we would expect that the pK_a' of fluoroY14 in the Y55,88F form of KSI should be close to 10. The replacement of an ortho hydrogen by fluorine is a very minor steric perturbation since the van der Waals radius of fluorine is only 0.15 Å larger than that of hydrogen (Bondi, 1964). Although fluorine possesses unshared electron pairs, carbon-bound fluorine is a very weak hydrogen bond acceptor, similar in this parameter to thioether sulfur (Joesten & Schaad, 1974). Thus, we ascribe any change in the properties of Y55,88F resulting from fluorination to fluorine's acidifying effect on the hydroxyl of Y14 or to its effect on the strengths of hydrogen bonds which the hydroxyl may make with hydrogen bond acceptors (Rochester, 1971).

Calculation of the Brønsted Coefficient, α. The kinetic mechanism for KSI is given by eq 1.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \underset{k_4}{\overset{k_3}{\rightleftarrows}} EI \underset{k_6}{\overset{k_5}{\rightleftarrows}} EP \underset{k_8}{\overset{k_7}{\rightleftarrows}} E + P \quad (1)$$

In this mechanism, S, I, and P refer to substrate, intermediate, and product, respectively. For the wild-type enzyme, Pollack's group has obtained evidence (Hawkinson et al., 1991b) that both chemical steps (rate constants k_3 and k_5) and the product dissociation step (rate constant k_7) are partially rate limiting and that $k_6 \ll k_7$. Thus, k_{cat} is given by eq 2.

$$k_{cat} = \frac{k_3k_5k_7}{(k_3 + k_4 + k_5)k_7 + k_3k_5} \quad (2)$$

If EI is a dienol, then Y14 functions as a Brønsted acid which protonates the substrate's (or product's) C-3 carbonyl oxygen in the forward (or reverse) direction of the overall reaction. This is expected to increase the acidities of the 4β proton of ES and the 6β proton of EP (Gerlt et al., 1991), facilitating their abstraction by the D38 anion in the forward and reverse enolization steps (rate constants k_3 and k_6). In the steps in which EI reverts to ES (rate constant k_4) or proceeds to EP (rate constant k_5), the Y14 anion serves to abstract the dienol proton from EI as the acid form of D38 protonates C-4 or C-6. Thus, according to Brønsted catalysis theory (Maskill, 1985), if the pK_a' of Y14 is decreased by fluorination, k_3 and k_6 will increase, while k_4 and k_5 will decrease.

For such a mechanism the expected effects of acidification of the hydroxyl of Y14 on k_{cat} can be analyzed using Brønsted theory (see Appendix). Equation 3 can be used to determine the value of α which accounts for the 4-fold decrease in k_{cat} due to fluorination of Y14, i.e., for $k_{rel} = 0.25$.

$$k_{rel} = \frac{\left[\frac{(k_3 + k_4 + k_5)k_7}{k_3k_5} + 1\right]r^{\alpha}}{\frac{(k_4 + k_5)k_7}{k_3k_5} + \frac{k_7}{k_5}r + r^{\alpha}} \quad (3)$$

(In using eq 3 we make the assumption that site-specific fluorination of Y14 in wild-type KSI would result in the same k_{rel} as we observe for Y55,88F.) Although the values of k_3 (1.7×10^5 s^{-1}) and k_7 (1.3×10^5 s^{-1}) have been determined by Hawkinson et al., only lower limits for k_4 ($\geq 3.0 \times 10^5$ s^{-1}) and k_5 ($\geq 1.0 \times 10^5$ s^{-1}) have been established, although these workers did find that $k_4/k_5 = 3$ (Hawkinson et al., 1991b).

A graphical method based on eq 3 is employed to estimate α. Curves a–e of Figure 2 are theoretical plots of k_{rel} versus α for values of k_4 and k_5 ranging from $k_4 = 3.0 \times 10^5$ and $k_5 = 1.0 \times 10^5$ s^{-1} (curve a) to $k_4 = 15 \times 10^5$ and $k_5 = 5 \times 10^5$ s^{-1} (curve e). The values of α for which $k_{rel} = 0.25$ for each curve range from 0.22 (curve a) to −1.2 (curve e). Since negative values for α are unlikely for an acid-catalyzed step, it would appear that the maximum values for k_4 and k_5 are 9×10^5 and 3×10^5 s^{-1}, respectively. In this instance $\alpha = 0$ (curve c). Consequently, if the values of k_3 and k_7 determined by Pollack are correct, we find that regardless of the exact values assumed for k_4 and k_5, α must be substantially less than 0.5. We interpret this qualitative result as evidence that in the transition state for enolization in the forward direction of the overall reaction proton transfer from Y14 is not well-advanced.

It is possible that site-specific fluorination of wild-type KSI would produce an isomerase having a k_{rel} different from that

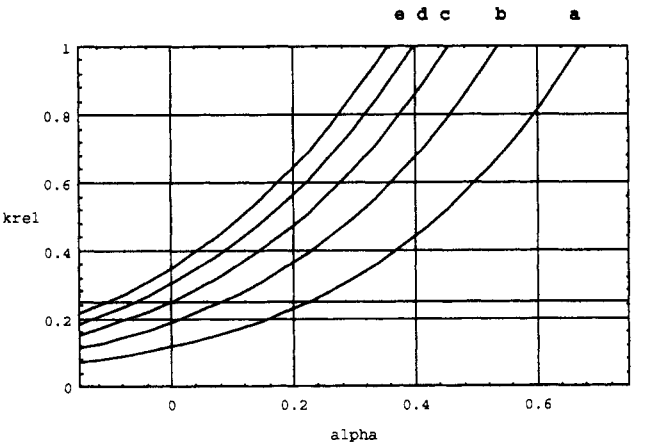

FIGURE 2: Calculated dependence of k_{rel} on α. Curve a: k_{rel} is calculated by using eq 3 and setting $k_3 = 1.7 \times 10^5$ s^{-1}, $k_4 = 3 \times 10^5$ s^{-1}, $k_5 = 1 \times 10^5$ s^{-1}, $k_7 = 1.3 \times 10^5$ s^{-1}, and $r = 32$. Curve b: Same as curve a except that $k_4 = 6 \times 10^5$ s^{-1} and $k_5 = 2 \times 10^5$ s^{-1}. Curve c: Same as curve a except that $k_4 = 9 \times 10^5$ s^{-1} and $k_5 = 3 \times 10^5$ s^{-1}. Curve d: Same as curve a except that $k_4 = 12 \times 10^5$ s^{-1} and $k_5 = 4 \times 10^5$ s^{-1}. Curve e: Same as curve a except that $k_4 = 15 \times 10^5$ s^{-1} and $k_5 = 5 \times 10^5$ s^{-1}. The horizontal line at $k_{rel} = 0.25$ represents the expected k_{rel} for Y14fluoroY.

of Y55,88F:Y14fluoroY. Consideration of the theoretical plots in Figure 2 shows that variation of k_{rel} for the fluorinated derivative would have a marked effect on the value of α obtained. Thus, if k_{rel} for fluorinated wild-type enzyme were 0.1, α would be near zero, whereas if k_{rel} were 0.6, α would be 0.2–0.5, depending on the values assumed for k_4 and k_5. Thus, we must regard our estimate of $\alpha \leq 0.5$ for the wild-type enzyme as provisional, pending the results of additional investigations.

In the accompanying manuscript (Holman & Benisek, 1994) we have obtained evidence that, in the enolization transition state, proton transfer from C-4 to D38 is well-advanced. When considered together, the results of Holman and Benisek and the present results provide a picture of the enolization transition state which is dienolate-like. Since enzyme active sites are more complementary to the transition states than to the substrate or product, our conclusion on the nature of the transition state is consistent with the spectroscopic data of Pollack's group (Zeng et al., 1992), which clearly indicates that the dienol analogue equilenin binds to KSI in its ionized, anionic form.

The Brønsted analysis utilized for estimation of α described above contains the inherent assumption that I is a dienol. If, in fact, I is a dienolate anion, then the method of data analysis which we have employed would need modification. One would need to take into account the fact that the effect of the acidity increase in Y14 due to fluorination would not be fully felt in an increased stabilization of EI.

In the case of a mechanism involving a full proton transfer creating a dienol intermediate it follows that $K_{eq}' = K_{eq}r$, where K_{eq} is the equilibrium constant for conversion of ES to EI by wild-type KSI and K_{eq}' is the equilibrium constant for that conversion by Y14fluoroY KSI, r being the ratio of the acid dissociation constants of fluorotyrosine and tyrosine. In contrast, the relationship between K_{eq} and K_{eq}' for a hydrogen-bonding mechanism (in which proton transfer from Y14 is incomplete in forming EI) could be described by

$$K_{eq}' = K_{eq}r^{\epsilon}$$

in which ϵ measures the extent of proton transfer from Y14 in EI. Thus, ϵ can be regarded as an equilibrium Brønsted

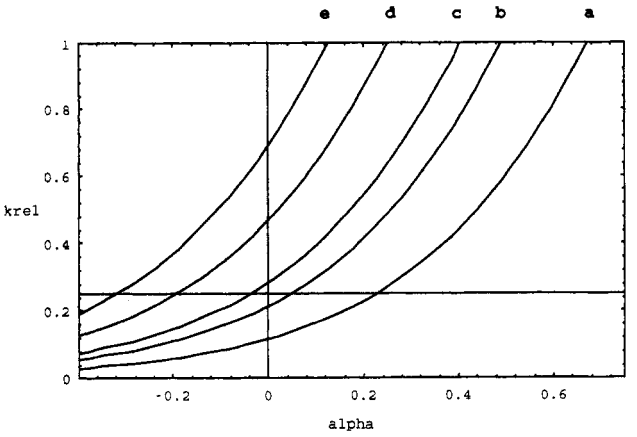

FIGURE 3: Calculated dependence of k_{rel} on α: effects of $\epsilon < 1$. All curves are calculated by using eq 4 and setting $k_3 = 1.7 \times 10^5$ s^{-1}, $k_4 = 3 \times 10^5$ s^{-1}, $k_5 = 1 \times 10^5$ s^{-1}, $k_7 = 1.3 \times 10^5$ s^{-1}, and $r = 32$. Curves a–e: $\epsilon = 1.0$, 0.8, 0.7, 0.5, and 0.3, respectively.

coefficient, analogous to α, the kinetic Brønsted coefficient for proton transfer in the enolization transition states of the k_3 and k_6 steps. Following the derivation leading to eq 3, these relationships lead (Appendix) to eq 4 for k_{rel}.

$$k_{rel} = \frac{\left[\frac{(k_3 + k_4 + k_5)k_7}{k_3k_5} + 1\right]r^{\alpha}}{\frac{(k_4 + k_5)k_7}{k_3k_5} + \frac{k_7}{k_5}r^{\epsilon} + r^{\alpha}} \quad (4)$$

Figure 3 shows plots of k_{rel} versus α according to eq 4 for k_3, k_4, k_5, and k_7 having the values estimated by Hawkinson et al. (1991b), $r = 32$, and ϵ having selected values from 0.3 to 1.0. The plots in Figure 3 show that, in order for α to be greater than zero, ϵ cannot be less than ca. 0.75. Lower values of ϵ would require *negative* values of α in order to account for a $k_{rel} = 0.25$, a condition which would be physically unreasonable for electrophilic catalysis by Y14.

This analysis of our data in terms of a hydrogen-bonding mechanism suggests that even if full proton transfer to the C-3 carbonyl oxygen does not occur, it is, nevertheless, well-advanced in EI ($\epsilon > 0.7$), leading to the qualitative view that EI strongly resembles a dienol even in a hydrogen-bonding mechanism.

Gerlt and Gassman (1993) have very recently proposed that the electrophilic component of KSI catalysis is executed via a strong, short hydrogen bond between the Y14 oxygen and the C-3 oxygen of the dienolic intermediate, I. They point out that in the confines of a substrate-occupied binding pocket from which bulk solvent is excluded [as appears to be the case for the KSI active site (E. Westbrook, personal communication)] conditions may be favorable for formation of a strong, short hydrogen bond. In this environment, when D38 abstracts the 4β proton of the substrate, the dienolate anion created could have a basicity similar to that of the Y14 anion, allowing the possibility of sharing of a proton between these oxygens in a strong hydrogen bond (Hibbert & Emsley, 1990; Gerlt & Gassman, 1992; Cleland, 1992). Consequently, in an active site in which pK_a's of participants in a hydrogen bond are similar, as may be the case for the hydroxyls of Y14 and the steroid dienol (Li et al., 1993; Zeng & Pollack, 1991), perturbation of the pK_a' of one of the participants would weaken the strong hydrogen bond stabilizing transition states leading to EI, thus decreasing the rate constant for those steps. Whether a strong hydrogen bond exists in the KSI mechanism is an interesting question which should be addressed by further experimentation.

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APPENDIX: EFFECTS OF Y14 AND D38 pK_A VALUES ON K_{CAT} OF STEROID ISOMERASE

The steroid isomerase reaction passes through a 3,5-dienol or -dienolate intermediate, I, according to the following mechanism:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \underset{k_4}{\overset{k_3}{\rightleftarrows}} EI \underset{k_6}{\overset{k_5}{\rightleftarrows}} EP \underset{k_8}{\overset{k_7}{\rightleftarrows}} E + P \quad (A1)$$

When the steady-state approximation is applied to ES, EI, and EP, k_{cat} for the overall reaction can be expressed in terms of rate constants for individual steps in this mechanism. Thus,

$$k_{cat} = \frac{k_3k_5k_7}{(k_3 + k_4)k_6 + (k_3 + k_4 + k_5)k_7 + k_3k_5} \quad (A2)$$

In the case of steroid isomerase, $k_6 \ll k_7$, and the above expression for k_{cat} can be simplified to

$$k_{cat} = \frac{k_3k_5k_7}{(k_3 + k_4 + k_5)k_7 + k_3k_5} \quad (A3)$$

The rate constants associated with proton-transfer steps, i.e., k_3, k_4, and k_5, are expected to depend on the pK_a' of the catalytic base, D38, and the pK_a' of the catalytic acid, Y14. Thus, we replace these rate constants by their pK_a'-dependent counterparts, k_3', k_4', and k_5', to give an expression for the pK_a'-dependent k_{cat}, k_{cat}'.

$$k_{cat}' = \frac{k_3'k_5'k_7}{(k_3' + k_4' + k_5')k_7 + k_3'k_5'} \quad (A4)$$

Catalytic Base. If k_3, k_4, and k_5 refer to the wild-type KSI and k_3', k_4', and k_5' refer to a KSI in which the pK_a' of the catalytic base is different from that of wild-type KSI, such as D38ASI, Brønsted theory leads to the following relationships between the primed and unprimed rate constants:

$$k_3' = k_3r^{-\beta} \quad (A5)$$

$$k_4' = k_4r^{1-\beta} \quad (A6)$$

$$k_5' = k_5r^{1-\beta} \quad (A7)$$

In these equations r is the ratio of the dissociation constants of conjugate acids of the catalytic bases and β is the Brønsted coefficient for the proton transfer from C-4 of the substrate to the catalytic base. Substitution of these relationships into eq A4 leads to eq A8, which can be used to evaluate β when experimental values of k_{cat}', k_3–k_7, and r are available.

$$k_{cat}' = \frac{k_3k_5k_7}{(k_3r^{\beta-1} + k_4r^{\beta} + k_5r^{\beta})k_7 + k_3k_5} \quad (A8)$$

Catalytic Acid. In the case of acid catalysis of the isomerase reaction the following relationships between the rate constants for wild-type KSI and those of a modified KSI having an altered pK_a' for the catalytic acid follow from Brønsted theory:

$$k_3' = k_3 r^{\alpha} \tag{A9}$$

$$k_4' = k_4 r^{\alpha-1} \tag{A10}$$

$$k_5' = k_5 r^{\alpha-1} \tag{A11}$$

Substitution of these expressions into eq A4 gives

$$k_{cat}' = \frac{k_3 k_5 k_7}{(k_3 r^{1-\alpha} + k_4 r^{-\alpha} + k_5 r^{-\alpha})k_7 + k_3 k_5} \tag{A12}$$

Dividing eq A12 by eq A3 gives k_{rel}, the ratio of k_{cat}' for the modified KSI to k_{cat} for native KSI. k_{rel} is given by eq A13:

$$k_{rel} = \frac{\left[\frac{(k_3 + k_4 + k_5)k_7}{k_3 k_5} + 1\right] r^{\alpha}}{\frac{(k_4 + k_5)k_7}{k_3 k_5} + \frac{k_7}{k_5} r + r^{\alpha}} \tag{A13}$$

The preceding analysis assumes that proton transfer to the C-3 oxygen of the substrate is complete in the intermediate, I. Since the degree of proton transfer to the intermediate is not yet clear, an extension of this analysis is required. As described in Results and Discussion, the parameter ϵ measures the extent of proton transfer to the intermediate ($\epsilon = 0$ for no proton transfer, and $\epsilon = 1$ for complete proton transfer). Thus, we rewrite eqs A9–A11 as eqs A14–A17.

$$k_3' = k_3 r^{\alpha} \tag{A14}$$

$$k_4' = k_4 r^{\alpha-\epsilon} \tag{A15}$$

$$k_5' = k_5 r^{\alpha-\epsilon} \tag{A16}$$

$$k_6' = k_6 r^{\alpha} \tag{A17}$$

Insertion of these relationships into eq A4 leads to the following expression for k_{rel}:

$$k_{rel} = \frac{\left[\frac{(k_3 + k_4 + k_5)k_7}{k_3 k_5} + 1\right] r^{\alpha}}{\frac{(k_4 + k_5)k_7}{k_3 k_5} + \frac{k_7}{k_5} r^{\epsilon} + r^{\alpha}} \tag{A18}$$

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