High-Efficiency Incorporation in Vivo of Tyrosine Analogues with Altered Hydroxyl Acidity in Place of the Catalytic Tyrosine-14 of Δ^5-3-Ketosteroid Isomerase of *Comamonas (Pseudomonas) testosteroni*: Effects of the Modifications on Isomerase Kinetics†

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ABSTRACT: Versions of the Y55F/Y88F modified form of Δ^5-3-ketosteroid isomerase in which the active-site tyrosine-14 is replaced by 2-fluorotyrosine, 3-fluorotyrosine, and 2,3-difluorotyrosine, amino acids having progressively greater acidity of their phenolic hydroxyls, have been expressed in an *Escherichia coli* host and purified to high homogeneity. The steady-state kinetic properties of Y55F/Y88F KSI and its fluorotyrosine modified forms have been determined. The mechanistic implications of the results are presented and discussed.

Investigations of the mechanism of Δ^5-3-ketosteroid isomerase, EC 5.3.3.1 (KSI),[1] from *Comamonas* (*Pseudomonas*) *testosteroni* and *Pseudomonas putida* have recently focused on the role of tyrosine-14 (tyrosine-16 in the *P. putida* enzyme) in catalyzing the allylic C4−C6 proton shift in Δ^5-3-ketosteroid substrates (*1*−*17*). New structural studies of KSI in solution (*18*−*20*) and in crystals (*21*) place the tyrosine hydroxyl in close proximity to the 3-ketone oxygen of bound substrate. Two early ideas about the mechanistic role of this residue were that the phenol proton is either fully transferred from the Y14 hydroxyl oxygen to the substrate carbonyl oxygen to form a dienol intermediate (general acid catalysis) or that no transfer took place at all (hydrogen-bonding catalysis). In the latter case, catalysis is thought to occur through electrostatic stabilization of a dienolate intermediate by hydrogen bonding from tyrosine 14. A third hypothesis is based on the work of Gerlt and Gassman (*22*, *23*) and others (*24*) who have postulated a mechanism for KSI that involves a low-barrier hydrogen bond of enhanced strength between Tyr-14 and a dienolate-like intermediate. The nature of the intermediate in the isomerization reaction has been investigated by spectroscopic studies of complexes between KSI and A-ring phenolic steroids thought to be good mimics of the dienol/dienolate intermediate of the reaction. Both UV absorption and fluorescence spectroscopies (*4*, *8*, *25*) suggest that these intermediate mimics are bound as phenolate anions, presumably hydrogen-bonded to one or more protein hydrogen-bond donors. The study of Holman and Benisek (*26*) indicated that the Bronsted β for proton abstraction from C4 of the substrate is well-advanced (β = 0.66−0.75) in the enolization transition state. These studies have been interpreted as supporting catalytic mechanisms in which a dienolate intermediate is involved, although they do not rule out the possibility that the intermediate is a dienol.

In addition to these studies, ultraviolet spectroscopic changes accompanying the slow isomerization reaction of 5-androstene-3,17-dione catalyzed by the D38N form of KSI are consistent with a mechanism in which initial formation of a dienolate intermediate is followed by a slower conversion of the intermediate to the Δ^4-3-ketone product (*1*, *27*).

A crucial assumption implicit in all the proposals is that the two oxygens are positioned closely together in the KSI−steroid complex, possibly less than the sum of their van der Waals radii in the case of the low-barrier strong hydrogen-bond proposal. Recent NMR-determined solution structural studies of the KSI from *Comamonas testosteroni*, which include NOE-determined steroid/protein proximities (*18*, *20*) and a computer docking of steroid into the active site (*19*), support the assumption of a close approach of the steroid C-3 oxygen to the Y14 hydroxyl. A very recent crystallographic study of the complex between the reaction intermediate analogue, equilenin, and the sequentially homologous KSI from *P. putida* (*21*) validates this close interaction. In addition, these studies also revealed a close proximity of the carboxyl oxygen of D99 to the bound steroid's C-3 oxygen, suggesting that an additional hydrogen bond might exist between a protonated D99 carboxyl and the dienolate intermediate (*19*, *21*). Thus, stabilization of the negative charge on the dienolate C-3 oxygen is proposed

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[1] Abbreviations: KSI, Δ^5-3-ketosteroid Isomerase (EC 5.3.3.1); MS, mass spectrometry; MES, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl thiogalactoside; STE, 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 1 mM EDTA; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tricine, *N*-tris(hydroxymethyl)methylglycine; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

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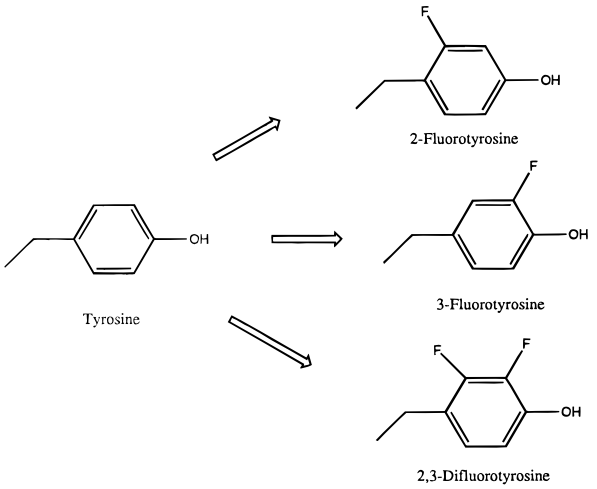
Scheme 1



to be provided by hydrogen bonds from two donors, Y14 hydroxyl and D99 carboxyl. However, a plausible alternative hydrogen-bond network involving these residues and bound steroid has very recently been proposed (*20*). An assumption of the strong hydrogen-bond mechanism is that the −OH of Tyr-14 and the dienol form of the putative KSI intermediate have similar pK_as. This would permit formation of a hydrogen bond of sufficient strength to overcome the endergonic process of transfer of a proton from C-4 of the substrate (pK_a = 12.72) to the carboxylate of D38 (pK_a = 4.7) (*24*, *28*).

Here we report the results of an exploratory study of the effect of alteration of the pK_a of Y14 hydroxyl on the kinetic constants of the Y55F/Y88F form of the KSI from *Comamonas testosteroni*. This KSI construct contains only one tyrosine residue, Y14. Thus, forced biosynthetic incorporation in an *Escherichia coli* expression host of tyrosine analogues having altered hydroxyl pK_as into this single site was achieved, following our earlier method (*17*) based on the previous work of Lu et al. (*29*). We now report not only that 3-fluorotyrosine is incorporated at high efficiency in place of tyrosine-14 but also that 2-fluorotyrosine and 2,3-difluorotyrosine can be substituted at high efficiency by this method. Although it has long been known that in vivo incorporation of 3-fluorotyrosine in place of tyrosine can be achieved in *E. coli* (*29*−*32*), the present work is, to our knowledge, the first report of successful in vivo incorporation in a procaryotic cell of 2-fluorotyrosine and in any cell of 2,3-difluorotyrosine. The possibility of success in achieving these modifications was foreshadowed by a report of incorporation of 2-fluorotyrosine into rat brain protein (*33*) and in vitro incorporation work using the amber codon suppression strategy (*34*).

The structural alterations to tyrosine-14 that have been achieved are shown in Scheme 1. The KSIs containing these site-specific modifications have been expressed in *E. coli* and sufficient quantities for kinetic studies have been purified to high homogeneity. The steady-state kinetic properties at pH 6.0 of these four forms of Y55F/Y88F KSI are reported and the implications for the catalytic mechanism are presented.

EXPERIMENTAL PROCEDURES

Materials. 2-Fluoro-L-tyrosine and 2,3-difluoro-L-tyrosine were synthesized from the corresponding fluorophenols and pyruvic acid by using tyrosine−phenol lyase (EC 4.1.99.2) from *Citrobacter freundii* as described previously (*35*, *36*). The purified amino acids were free of ninhydrin-reacting impurities as determined by ion-exchange amino acid analysis using a Beckman 6300 amino acid analyzer. Other materials were the same as described previously (*17*, *26*).

Expression and Purification of KSI Forms. All purification procedures were carried out at 4 °C unless otherwise stated. Bacterial growth, expression, and purification of KSI containing 3-fluorotyrosine was as described previously (*17*). Growth and expression of KSI containing tyrosine was as described previously (*17*); its purification is described below. The strategy for expressing KSI containing 2-fluorotyrosine or 2,3-difluorotyrosine was similar to that used for 3-fluorotyrosine. The cells were inoculated with IPTG (0.5 mM final concentration), fluoro-Y, F, and W (1 mM each amino acid, final concentration) at a culture absorbance of 0.2−0.25 at 550 nm and then were allowed to grow overnight.

The overnight cultures were harvested by centrifugation followed by washing the pellet twice in either STE buffer or water. The washed pellets were frozen and thawed at least once at either −85 °C (usually) or at −20 °C. The cells were resuspended in STE buffer and then opened by sonication on ice for a total of 2−2.5 min (cumulative on time) in 5 s pulses interspersed between 5 s rest periods. Microscopic examination showed no more than about 10% intact bacteria by this sonication regime. An equal volume of 95% ethanol was added and the preparations were allowed to incubate overnight at 4 °C. The next day we centrifuged the mixtures at 12000*g* for 1 h, resuspended the pellets in 95% ethanol/STE (50:50 v/v), and centrifuged them again. Supernatants were combined and the pellets were discarded. Ethanol (95%), $MgSO_4$, and EDTA were added to final concentrations of 80%, 5 mM, and 1.5 mM, respectively. The preparations were allowed to incubate for a few days to precipitate the KSI, after which time they were centrifuged at 4000*g* for 15 min to recover the enzyme. We dissolved the pellets in 10 mM potassium phosphate, pH 7.5, removed insoluble materials by centrifugation, and then brought the supernatants to 0.4 M potassium phosphate, pH 7.5. Affinity chromatography was done as previously described (*17*). As a final step we crystallized the pure KSI forms from a high concentration of ammonium sulfate in 50 mM potassium phosphate, pH 7.5. During this work a crystallization procedure as the final purification step was developed. Consequently, no consistent crystallization regime was used for all the preparations. Generally, when the protein concentration was brought to at least 1 or 2 mg/mL in 50 mM potassium phosphate buffer, pH 7.5, and then a saturated solution of ammonium sulfate was added at room temperature to a final concentration of 25−50% of saturation or even higher, crystallization began within 15 s. We were able to observe further growth of the long, needlelike crystals for weeks afterward at room temperature, but the majority of the enzyme crystallized very rapidly. The crystalline enzyme was recovered by centrifugation and the crystals were dissolved and stored in 50 mM potassium phosphate, pH 7.5, and 0.02% NaN_3 at 4 °C at a protein concentration of at least 0.67 mg/mL.

Assessment of Purity and Measurement of KSI Concentration. Stock solutions of the purified proteins were analyzed for purity and concentration as described previously (*17*) by

SDS−PAGE and quantitative amino acid analysis except the SDS−PAGE was done in the absence of urea.

Fluorotyrosine Content Measured by Electrospray Mass Spectroscopy. Because 2-fluorotyrosine and 2,3-difluorotyrosine coelute with tyrosine and 3-fluorotyrosine is only partially resolved from tyrosine on the Beckman 6300 amino acid analyzer, no attempt was made to measure the extents of incorporation of these amino acids by this method. Instead, electrospray mass spectrometry was used to determine the extents of incorporation of the fluoro amino acids. The purified KSIs were dialyzed extensively against water that was purified through the Millipore ion-exchange process. Ten microliters of dialyzed KSI was mixed with 10 μL of acetonitrile to give a solution having a protein concentration of about 50 μM. Electrospray mass spectra were generated with a Quattro-BQ mass spectrometer (VG Biotech, Altrincham, U.K.). Protein samples dissolved in acetonitrile/water (50:50 v/v), which also served as the carrier solvent, were delivered at 5 μL/min by an Isco μLC-500 syringe pump. Proteins were analyzed by direct flow injection of 10 μL. Spectra were obtained in the positive ion mode using a capillary voltage of +3.5 kV, a source temperature of 70 °C, and a cone voltage of +50 V. Mass calibration was performed with horse heart myoglobin (Sigma) as reference. Mathematical transformations of electrospray spectra to true mass scale were attained with the MaxEnt algorithm (Masslynx software) at 0.5 Da/point. The peaks corresponding to the fluoro and hydrogen forms of KSI were identified by molecular weight. The observed molecular weights were within about 0.01% of the expected values. In all MS spectra the only significant species of KSI observed were either Y55F/Y88F/Y14 or Y55F/Y88F/fluoroY14. The MS software provided values for the areas of the peaks, from which the percentage of fluorotyrosine incorporation was calculated by dividing the area of the fluorotyrosine KSI peak by the sum of the fluorotyrosine and tyrosine KSI peaks.

Kinetic Methods. Enzyme assays and protein concentration measurements were carried out as previously described (*17*, *37*) except all assays were done at pH 6.0 in 34 mM MES, 2.5 mM EDTA, and 3.3% (v/v) methanol. k_{cat} and K_m values were determined from linear least-squares fits of Lineweaver−Burk plots with DeltaGraph on a Macintosh computer.

RESULTS AND DISCUSSION

Protein Homogeneity. The final enzyme preparations were virtually homogeneous even when overloaded on SDS−PAGE. In some of the preparations we observed a faint band of 26−29 kDa, estimated at less than 1% of the total protein concentration.

Efficiency of Incorporation of Fluorotyrosines. From the electrospray mass spectra for the fluorotyrosine-containing KSIs, as described in Experimental Procedures, the measured incorporations of the fluorotyrosines were 2-fluorotyrosine, 95%; 3-fluorotyrosine, 95%; and 2,3-difluorotyrosine, 75%.

pK_as of Tyrosines. We measured the pK_a in free solution of tyrosine and the fluorotyrosines to be 10.05 ± 0.04 for tyrosine, 9.04 ± 0.03 for 2-fluorotyrosine, 8.54 ± 0.03 for 3-fluorotyrosine, and 7.53 ± 0.005 for 2,3-difluorotyrosine, consistent with the known or predicted values of 10.0, 9.3, 8.5, and 7.9, respectively, for these compounds (*38*). The pK_as were determined spectrophotometrically as a function of absorbance (294 nm for tyrosine, 290 nm for 3-fluorotyrosine, 288 nm for 2-fluorotyrosine and 279 nm for 2,3-difluorotyrosine) vs pH in buffers containing Tricine (tyrosine), MOPS and CAPS (2-fluorotyrosine), or MES and Tricine (2,3-difluorotyrosine) at an ionic strength of 0.2.

Table 1: Kinetic Properties for KSI (Y55F/Y88F) Forms Modified at Residue 14[a,b]

residue 14	pK_a of free amino acid	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
tyrosine	10.0	6300	100	6.3×10^7
2-fluorotyrosine	9.04	12100	150	8.1×10^7
3-fluorotyrosine	8.5	2100	80	2.6×10^7
2,3-difluorotyrosine	7.53	7500	110	6.8×10^7

[a] Kinetics were measured at pH 6.0 in 34 mM MES buffer containing 2.5 mM Na_2EDTA and 3.3% (v/v) methanol. Temperature = 25 °C. The kinetic runs were performed in triplicate at each substrate concentration. [b] The kinetic data for the fluoro- and difluorotyrosine-14 forms of Y55F/Y88F KSI have been corrected for the contribution of the tyrosine form determined by the mass spectrometry data (see Results and Discussion, Efficiency of Incorporation of Fluorotyrosines), as described previously (*17*).

Kinetic Constants of Y55F/Y88FKSI and Derivatives Containing Fluorotyrosines. The steady-state kinetics of Y55F/Y88F KSI and its fluorotyrosine derivatives were measured at pH 6.0 to reduce the likelihood that the fluoro-Y residues in the free enzyme would be significantly ionized under the conditions of the kinetic analyses. The kinetic results are summarized in Table 1. k_{cat} varies in a complex way as the pK_a of the free amino acid decreases. Experiments (not shown) showed the activity of Y55F/Y88F KSI measured at pH 6.0 was 67% of the corresponding value at pH 7.0. For Y55F/Y88F KSI the kinetic values in this study (Table 1) are consistent with the values reported previously at pH 7 (*17*) after application of the above correction. Our previous value of k_{cat} for 3-fluorotyrosine-containing KSI was based on incorporation data derived from amino acid analysis. The accuracy of this earlier analysis is compromised by the marginal chromatographic resolution of 3-fluorotyrosine from tyrosine on our amino acid analyzer. In the present work we used electrospray mass spectroscopy to determine the incorporation data for all KSI forms more accurately. The revised k_{cat} for 3-fluorotyrosine KSI reported earlier agrees with our present results after application of the above correction for pH differences.

For a mechanism involving a dienol intermediate, the dependence of k_{cat} on pK_a of Y14 can be qualitatively predicted. The predicted dependency depends on which step of the mechanism, enolization or ketonization, is rate-limiting. Divergent conclusions on this point are present in the published literature. Mildvan and co-workers have concluded from kinetic isotope effects that enolization is rate-limiting for both wild-type and Y55F KSI (*39*). Thus, the acidity of Y14 functioning as a general acid catalyst will dictate the pK_a dependency such that k_{cat} should increase as pK_a decreases, contrary to what we find in the present study. Pollack and co-workers have concluded from kinetic studies of isomerization of externally generated dienol that both enolization and ketonization are partially rate-limiting for wild-type KSI and its D38E form (*40*−*42*). For this situation, k_{cat} would be expected to increase to a maximum and then decrease as pK_a decreases. This is expected because

as enolization increases with decreasing pK_a it eventually becomes non-rate-limiting. Simultaneously, ketonization slows down and becomes rate-limiting as pK_a decreases (as tyrosinate anion becomes a weaker base). However, our results do not follow this prediction. Consequently, we conclude that mechanisms based solely on a dienol intermediate appear to be inconsistent with our kinetic data (however, see below).

Therefore, in trying to explain the results summarized in Table 1 we provisionally assume that the dienolic intermediate in the KSI mechanism for the Y55F/Y88F KSI having Y at position 14 is a 4,6-dienolate anion stabilized by hydrogen bonding of the steroid's C3 oxygen to the hydroxyl of the enzyme's Y14 residue. This hydrogen bond may be of the ordinary type or of the more recently recognized strong low-barrier type. In either instance the strength of the hydrogen bond will be maximal when the pK_a of the donor equals the pK_a of the conjugate acid of the acceptor (*24*, *28*, *43*). Some of the present results can be rationalized qualitatively by these hypotheses. Thus, the increase in k_{cat} in going from tyrosine to 2-fluorotyrosine could be ascribed to a somewhat better match in pK_a for 2-fluorotyrosine and the bound dienol. The pK_a of the dienol in free solution is known to be 10.0 (*44*). As the pK_a of the protein hydrogen-bond donor is lowered further by going to 3-fluorotyrosine, the pK_a of the hydrogen-bond donor may become mismatched to that of the acceptor and the strength of the hydrogen bond may be decreased, with attendant reduction in k_{cat}. However, a dilemma is presented by the result for 2,3-difluorotyrosine, for which the k_{cat} is 3-fold greater than that for 3-fluorotyrosine. One would have expected its k_{cat} to be even lower than that of 3-fluorotyrosine in a hydrogen-bond-mediated mechanism. However, if the pK_a of the hydrogen-bond donor becomes sufficiently low, proton transfer to the dienolate intermediate will be favored enough thermodynamically so that full proton transfer must occur, resulting in a change of mechanism from one involving a dienolate intermediate to one involving a dienol intermediate. Thus, the kinetic parameters for the 2,3-difluorotyrosine form of KSI may not be directly comparable to those of the other forms as that enzyme may follow a different chemical mechanism involving a dienol intermediate.

Another potential explanation for our results relates to the pK_as of the four tyrosine derivatives in the KSI active site. Other than that for Y14 of the Y55F/Y88F form of KSI (*7*) (pK_a = 11.6), these pK_as have not yet been measured. It may be that the relative pK_as in the active site are quantitatively different than those in free solution (Table 1). In fact, this would be expected in going from the high dielectric constant environment of aqueous solution to the much lower dielectric constant environment of the KSI active site, which has been estimated to be 18 (*7*). As the dielectric constant of solvent is lowered, the pK_as of neutral acids increase and the spread of pK_as for a homologous series of acid derivatives also increases—that is, the pK_as of the weaker acids increase more than those of the stronger acids. Thus, a Bronsted analysis based on relative pK_as measured in free aqueous solution (*17*) is not quantitatively correct. To complicate this issue even more, it may be that the order of acidities of the tyrosine derivatives in the low-polarity KSI active site may be different than that in free solution (*45*). For example, Fujio et al. (*46*) have found that the gas-phase acidity of 3-fluorophenol is greater than that of 2-fluorophenol, the reverse of the acidity order found in aqueous solution. If the acidity order in the KSI active site was found to be different than that of the free amino acids, mechanisms involving a dienol intermediate for wild-type KSI could be viable (see above). Clearly, direct pK_a determinations of the phenolic hydroxyls in the KSI derivatives reported in the present study would be of great interest.

A third possibility which might explain our results, that of steric interference to catalysis as a result of the slightly greater van der Waals radius of fluorine compared to hydrogen, is possible but not likely. Phillips et al. (*47*) have documented steric effects of fluorine vs hydrogen in the reactions catalyzed by tyrosine−phenol lyase and tryptophan−indole lyase. Arguing against this explanation for our present results is the similarity of the K_ms for all KSI forms (Table 1) and the reported (K. Y. Choi and B. Oh, personal communication) lack of steric conflict of the *ortho* positions of the homologous catalytic tyrosine of the KSI of *P. putida* with other active-site groups or bound equilenin, a reaction intermediate analogue.

Further research is necessary to distinguish among these potential explanations for our data. Prime avenues for further study would be spectral studies of bound phenolic steroid intermediate analogues to determine their ionization state and direct measurement of the pK_as of the hydroxyls of the modified tyrosines in the various forms of KSI. Due to the recent full retirement of the third author, W.F.B., we will not do these studies.

The virtues of our in vivo method for replacement of tyrosine with various fluorotyrosines are its easy application, once nonessential tyrosines are mutagenized to phenylalanines, and the ability of in vivo expression in *E. coli* to produce enough fluoro protein to permit high-resolution structural studies of the modified forms. Given the potential for production of multimilligram quantities of these modified KSIs by the straightforward method described in this report, NMR and crystallographic studies of their detailed structures should be feasible. The same approach may allow incorporation of other modified tyrosines with more closely spaced pK_as, permitting a more complete display of the pK_a dependency of the kinetics of KSI. Extension of this methodology to study of other proteins having one critical tyrosine should be feasible.

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