Mechanism of Proton Transfer in the Isomerization of 5-Androstene-3,17-dione by 3-Oxo-Δ^5-steroid Isomerase and Its D38E Mutant†

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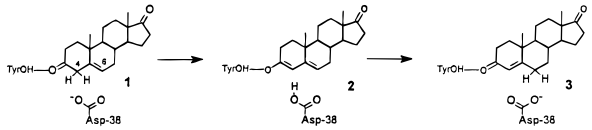
ABSTRACT: The stereochemistry of proton transfer in the isomerization of [4β-^{2}H]-5-androstene-3,17-dione (**1d**) to 4-androstene-3,17-dione (**3**) catalyzed by 3-oxo-Δ^5-steroid isomerase (KSI) has been reinvestigated. In H_2O, approximately 65% of the label is retained in the product (**3**); of this, one-third is at C-4 and two-thirds at C-6β. When the same reaction is catalyzed by the D38E mutant of KSI, ca. 60% of the label is retained in the product, but almost all of it is at C-4. These reactions run in deuterium oxide result in 13% incorporation of a second deuterium with the wild type (WT) enzyme and 75% incorporation with the D38E mutant. When unlabeled **1** is isomerized in D_2O, there is little incorporation of deuterium with WT (ca. 5 at. %) but substantial incorporation with D38E (130 at. %). These results are consistent with competitive abstraction of both the C-4α and C-4β protons, as proposed by Viger et al. [(1981) *J. Am. Chem. Soc. 103*, 4151], and demonstrate that the KSI reaction is not completely stereospecific. A mechanism is proposed to account for these observations.

The mechanism of proton transfer in enzymatic reactions may be probed by monitoring both the stereochemistry of the reaction and the extent of exchange of substrate protons with solvent, using either deuterated substrate or solvent. Thus, intermediates may be detected by the occurrence of proton exchange with solvent, and the stereochemistry of proton transfer can lead to conclusions concerning the number of active site bases in the reaction. Enzymes that catalyze 1,2- or 1,3-proton transfers, such as triose-phosphate isomerase (Bloom & Topper, 1956; Reider & Rose, 1956, 1959; Rose, 1958), glucose-phosphate isomerase (Topper, 1957; Rose & O'Connell, 1960), aconitate isomerase (Klinman & Rose, 1971), and vinylacetyl-CoA isomerase (Hashimoto et al., 1973), preferentially abstract one of a pair of prochiral protons as part of the reaction mechanism. This evidence, coupled with the generality of suprafacial proton transfer, has led Creighton and Murthy (1990) to conclude that these processes are usually mediated by a single enzyme base.

The situation, however, is more complicated with 3-oxo-Δ^5-steroid isomerase (KSI). KSI catalyzes the conversion of a variety of 3-oxo-Δ^5-steroids, such as 5-androstene-3,-17-dione (**1**), to their conjugated Δ^4-isomers through an intermediate dienolate (**2**) formed by abstraction of a proton from C-4 of the substrate by Asp-38 (Ogez & Benisek, 1977; Benisek et al., 1980; Bounds & Pollack, 1987; Kuliopulos et al., 1987, 1990). Electrophilic assistance by the phenolic hydrogen of Tyr-14 enhances catalysis by hydrogen bonding to the oxygen at C-3 (Zeng et al., 1992; Brooks & Benisek, 1994; Zhao et al., 1995). Subsequent reprotonation of the dienolate at C-6 leads to product [Scheme 1; for reviews on the mechanism of action of KSI and related enzymes, see Pollack et al. (1989a), Schwab and Henderson (1990), and Creighton and Murthy (1990)].

Scheme 1



In early work using **1** that was deuterated at C-4β (**1d**) in H_2O, Malhotra and Ringold (1965) reported that 67% of the deuterium is transferred from C-4β to C-6β, with the rest of the label lost to solvent. They concluded that proton transfer is stereospecific from 4β to 6β. These results, however, were later questioned by Viger and co-workers (Viger & Marquet, 1977; Viger et al., 1981), who observed that 25% of the deuterium at 4β is retained at C-4 after enzymatic isomerization of **1d** in H_2O at 25 °C, suggesting that there is some labilization of the 4α proton as well as the 4β proton. Similar experiments led to the conclusion that nonstereospecific labilization of the C-4 protons also occurs with 5(10)-estrene-3,17-dione, whereas with 5-pregnene-3,20-dione, only the 4α proton is abstracted (Viger et al., 1981).

As part of our studies with KSI, we have investigated the effect of modification of the active site base (Asp-38) by converting it to a glutamic acid residue. Replacement of Asp-38 by Glu (D38E) results in a significant decrease in the rate of isomerization. For example, k_{cat} for the isomerization of **1** catalyzed by D38E is less than 1% of that for the wild type (WT) enzyme (Zawrotny et al., 1991). Analysis of the free energy profiles for WT (Hawkinson et al., 1991, 1994) and D38E (Zawrotny & Pollack, 1994) shows that the effect of the mutation is to decrease the rates of the chemical steps of the reaction without any change in the relative energies of bound substrate and intermediate.

In the present work, we reexamine the stereochemistry of the proton transfer catalyzed by WT KSI with [4β-^{2}H]-5-

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androstene-3,17-dione (**1d**). In addition, we report the determination of the stereochemistry of the reaction with the D38E enzyme. We find that, although the 4β deuterium atom is preferentially removed by the wild type enzyme, the D38E mutant primarily abstracts the 4α hydrogen. Furthermore, most of the deuterium atoms abstracted by WT are transferred to C-6, with little washout to solvent, but ca. 90% of the deuterium abstracted from C-4 by D38E is exchanged with solvent. When **1d** is isomerized in D_2O, incorporation of a second deuterium occurs with both WT (13%) and D38E (75%). These results are discussed in terms of a detailed mechanism for proton transfer at the active site.

MATERIALS AND METHODS

Materials. 5-Androstene-3,17-dione (**1**) was prepared as before (Pollack et al., 1989b). 4-Androstene-3,17-dione content was estimated at <5% by determining the absorbance at 248 nm prior to and following isomerization of **1** with KSI. 4-Androstene-3,17-dione (**3**) was purchased from Sigma and purified by recrystallization from ethyl acetate and methanol, mp 173−174 °C. [4β-^{2}H]-5-Androstene-3,-17-dione (**1d**) was synthesized according to a previously published procedure (Brothers et al., 1995). The 4β hydrogen content of **1d** was estimated at less than 3% from the NMR spectrum.

All deuterated solvents and reagents were ≥99 at. % deuterium. Bovine serum albumin (BSA) was purchased from Calbiochem and used without further purification. Other reagents were reagent grade or better and used as obtained. WT and D38E were available from previous investigations (Eames et al., 1989; Zawrotny & Pollack, 1994).

Measurement of Deuterium Exchange by Mass Spectrometry. A 20 mL phosphate solution (34 mM, pH 7.0, 2.1% MeOH) of KSI (0.05 μM for WT, 6 μM for D38E) was stirred at 20 °C during rapid addition of 250 μL of **1** or **1d** (ca. 5 mg/mL in methanol). The final solution had pH 7.0 and 3.3% methanol cosolvent. The solution was incubated for the time necessary to allow the isomerization to go to >99% completion (5−20 s) and then quenched into 5 mL of chloroform in a separatory funnel. The chloroform layer was dispensed into a separate container, and the remaining aqueous layer was extracted twice more with 5 mL of chloroform. The chloroform extracts were combined and dried over magnesium sulfate for at least 60 min. The magnesium sulfate was removed by filtration through a small plug of glass wool, and the chloroform was removed by rotary evaporation. (Because of the high concentration of enzyme when D38E was used, there was considerable foaming when the separatory funnel was shaken. Most of the aqueous and foam layers were removed with a Pasteur pipette, and 3 mL of chloroform was added. Removal of the aqueous/foamy layers, followed by the addition of more chloroform, gave a well-separated two-phase mixture.) Thin-layer chromatography (silica, 2:1 hexane:ethyl acetate) showed a single spot that comigrated with an authentic sample of **3**. Most lanes, including the control (**3**), showed one or two other small spots. CH_3OD was used to minimize introduction of ^{1}H into reactions run in D_2O. For reactions run in D_2O, the enzyme (initially in aqueous phosphate buffer, pH 7.0) was lyophilized and then redissolved in D_2O prior to use.

Samples were analyzed by mass spectrometry (electron impact) using either the direct insertion or LC ports on a Hewlett-Packard 5988A quadrupole mass analyzer. Samples were dissolved in either chloroform or acetonitrile prior to analysis by LC/MS. Data were collected by selective ion monitoring of the peaks with *m/z* from 285 to 289. Deuterium content was calculated after correction for ^{13}C content. No correction was made for incomplete deuteration of the substrate.

Measurement of Deuterium Exchange and Localization of Deuterium by NMR. Exchange reactions were run as described above, except that volumes of all solutions were increased 2−4-fold. Sample workup was also performed as above except that the residue was redissolved in an 80% MeOD/20% D_2O solution immediately prior to acquisition of NMR spectra on a General Electric GN-500 spectrometer. Data were collected at room temperature without sample spinning. Each spectrum was an average of 16 scans with a 5 s delay time between each acquisition. The C-6β proton has a chemical shift of 2.5 ppm and overlaps with the protons at C-2β and C-16β (Zeng et al., 1990). However, the 2β and 16β protons can be removed by exchange with deuterium in D_2O under moderately basic conditions with minimal loss of the 6β proton (Pollack et al., 1989b). After a spectrum was collected in the CD_3OD/D_2O solution (500 μL), 50 μL of a 1 M NaOD solution was added (final NaOD concentration was 0.09 M) and a second spectrum collected 30 min after the addition of the NaOD.

RESULTS

The stereochemistry of proton transfer for wild type was probed by reaction of unlabeled **1** in D_2O and of **1** specifically deuterated at C-4β (**1d**) in both H_2O and D_2O. Reactions were run in either 34 or 800 mM phosphate (pH or pD 7.0, 20 °C). The product (**3**) was analyzed by mass spectrometry in order to determine the extent of incorporation of isotope and by ^{1}H NMR to localize the deuterium in the product (Table 1). Agreement between the two methods with regard to total mole fraction deuterium in each sample is generally quite good. The mass spectral results are assumed to be more accurate, and these values are used for total deuterium incorporation. The NMR results are used for localization of the deuterium.

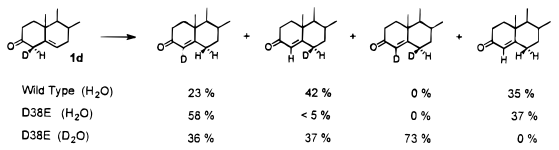
Isotopic analysis by NMR is complicated by spectral overlap, with the C-6 protons appearing as part of two multiplets. The C-6β protons show up as part of a multiplet at 2.45−2.57 ppm, which also contains the signals due to the C-2β and C-16β protons. The C-6α protons appear at 2.28−2.40 ppm, along with the C-2α protons (Zeng et al., 1990). However, since the C-2β and C-16β protons rapidly exchange with deuterium in 0.1 N $NaOD/D_2O$, with little exchange of the C-6β protons, an estimate can be made of the isotopic composition at C-6β (Pollack et al., 1989b). Deuterium content and position were determined from integration of the peaks at 5.75 ppm (C-4) and 2.5 ppm (C-6β), referenced against the corresponding peaks from parallel samples of pure **3**. Peak areas were referenced against the peaks at 0.93 ppm (C-18Me) for reactions run in D_2O. Reactions run in H_2O were referenced against the peaks at 2.3 ppm (C-2α and C-6α), 1.9−2.1 ppm (C-1β, C-7β, C-15α, and C-16α), and 1.4−1.9 ppm (C-1α, C-8, C-11α, C-11β, C-12β, and C-15β) and the resulting protium/deuterium contents averaged. The deuterium content at C-4 was determined from integration of the 5.75 ppm peak for the spectra collected prior to addition of NaOD; deuterium

Table 1: Incorporation of Solvent Protons during the Isomerization of 5-Androstene-3,17-dione by KSI[a]

enzyme	substrate	[phosphate] (mM)	solvent	% D_0 (MS)[a,b]	% D_1 (MS)[a,c]	% D_2 (MS)[a,d]	m_f of D (MS)[a,e]	% D at C-4 (NMR)[e]	% D at C-6β (NMR)[e]	m_f of D (NMR)[e]	temp (°C)
WT	**1**	34	D_2O	94.3	5.7	0	0.057				20
	1	34	D_2O	97.3	2.7	0	0.027				3
	1	800	D_2O	94.4	5.6	0	0.056				21
	1d	34	H_2O	35.0	65.0	0	0.650	26	49	0.75	20
	1d	34	D_2O	0	86.7	13.3	0.867				21
	3	34	D_2O	99.5	0.5	0	0.05				21
D38E	**1**	34	D_2O	14.0	43.1	42.9	1.29	60	81	1.41	20
	1	800	D_2O	12.6	41.4	46.0	1.33				20
	1d	34	D_2O	0	26.6	73.4	1.73	80	81	1.61	20
	1d	800	D_2O	0	22.8	77.2	1.77				20
	1d	34	H_2O	42.3	57.7	0	0.577	64	6[g]	0.71	20
	1d	800	H_2O	40.6	59.4	0	0.594				20

[a] Reproducibility of the mass spectral determinations was excellent. Each entry is the average of three or more mass spectral determinations of the isotopic composition of the compound. Agreement within runs was generally within ±0.5%. When multiple experiments on different days are compared, agreement is within ±2%. [b] Percent product with no deuteriums by mass spectrometry. [c] Percent product with one deuterium by mass spectrometry. [d] Percent product with two deuteriums by mass spectrometry. [e] Mole fraction of deuterium by mass spectrometry. [f] The accuracy of NMR determinations is judged to be ±10% for positions which have significant exchange. [g] This number is the difference between two relatively large numbers and, thus, has a significant error associated with it and may not be different from 0.

Scheme 2



at C-6β was determined from the peak at 2.5 ppm after the 30 min exchange period (supporting information).

Isomerization of **1** to **3** by WT in D_2O (34 mM phosphate, 20 °C) and mass spectral analysis of the product show that 5.7 at. % deuterium is incorporated during the reaction (Table 1). Control experiments demonstrate that this exchange is due to the isomerization catalyzed by KSI. Prior incubation of substrate for up to 20 h in MeOD gives no increase in deuterium incorporation. Addition of substrate to phosphate buffer (D_2O) prior to addition of enzyme, however, results in a considerable increase in deuterium content in the product. A 5 min delay before addition of enzyme gives 18% incorporation of D, and a 20 min delay gives 44% incorporation. Therefore, all experiments were done with substrate added last to eliminate nonenzymatic proton exchange of the reactant prior to enzymatic isomerization. Furthermore, experiments in both 34 and 800 mM phosphate gave similar results, showing that there is no nonenzymatic exchange prior to reaction with KSI. Incubation of **3** in D_2O without enzyme results in the incorporation of less than 1% deuterium from solvent, demonstrating that product is stable to exchange under these conditions.

Mass spectral analysis of **3** from the isomerization of **1d** by WT in H_2O at 20 °C and 34 mM phosphate reveals that 65% of the deuterium is retained in the product. NMR shows that approximately 65% of this deuterium is transferred to the 6β position and 35% is retained at C-4 (42 and 23% of the original deuterium, Scheme 2). When **1d** is isomerized in D_2O, a second deuterium is found in 13% of the product, but localization of this deuterium by NMR was not possible. These results clearly demonstrate that abstraction of the 4α proton is competitive with abstraction of the 4β proton with **1d** as the substrate. Only minor amounts (ca. 5%) of deuterium are incorporated from D_2O with **1** as a substrate.

When D38E is used as a catalyst, significantly more incorporation of solvent hydrogens is observed. In deuterium oxide (34 mM phosphate), isomerization of **1** results in 1.29 at. % deuterium in the product. NMR analysis shows that 43% of the product has deuterium at both C-4 and C-6β, 12% at C-4 alone, and 31% solely at C-6β. Similarly, when **1d** is isomerized by D38E in D_2O (34 mM phosphate), ca. 73% of the molecules pick up a second deuterium to give deuteration at both C-4 and C-6β, with deuterium in singly deuterated product almost evenly distributed between C-4 and C-6β. Reaction of **1d** in H_2O results in 58% retention of deuterium as analyzed by mass spectroscopy. NMR analysis shows that virtually all of that deuterium is at C-4, although a small amount (ca. 10%) at C-6β cannot be ruled out by these measurements. A summary of these experiments is given in Table 1 and Scheme 2.

DISCUSSION

Stereochemistry of Proton Transfer with Wild Type. In early work with WT and **1d** in H_2O, Malhotra and Ringold (1965) reported that 67% of the deuterium was transferred from C-4β to C-6β, with the rest of the label lost to solvent. They concluded that proton transfer is stereospecific from C-4β to C-6β. This conclusion, however, was later questioned by Viger and co-workers (Viger & Marquet, 1977; Viger et al., 1981), who observed that 25% of the deuterium at C-4β was retained at C-4 after enzymatic isomerization of **1d** in H_2O, suggesting some labilization of the 4α proton as well as the 4β proton. The determinations of Malhotra and Ringold (1965) relied on the localization of the deuterium on the basis of infrared stretching frequencies. Viger and co-workers (Viger & Marquet, 1977; Viger et al., 1981) determined the position of the isotope by mass spectrometry, following catalytic reduction and selective exchange of deuterium at C-4 with sodium hydroxide.

Because of the discrepancy between the previous reports, we reinvestigated the stereochemistry of the reaction of KSI with **1d** in both H_2O and D_2O, as well as with **1** in D_2O. Mass spectrometry was used to determine the amount of deuterium in the product, and high-field ^1H NMR was used to determine the position of the deuterium. There is good agreement of the total amount of deuterium present for reactions for which both mass spectrometry and NMR analyses were performed (Table 1). In our hands, catalysis of the isomerization of **1d** in H_2O by wild type KSI occurs with retention of 65% of the deuterium as determined by mass spectroscopy and 75% as determined by 500 MHz ^1H

NMR; the remaining label is lost to solvent. Localization of the deuterium by ^{1}H NMR spectroscopy (and using the mass spectroscopy results for total deuterium) shows that 42% of the product has a deuterium at C-6β and 23% has a deuterium at C-4 (Scheme 2). These findings confirm the results of Viger et al. (Viger & Marquet, 1977; Viger et al., 1981) and are consistent with some labilization of the 4α proton, in addition to the 4β proton. Thus, although WT KSI preferentially abstracts the 4β deuterium, the reaction is not completely stereospecific. Isomerization of **1d** by WT KSI in D_2O results in 13% incorporation of a second deuterium (as determined by mass spectroscopy), further strengthening this conclusion.

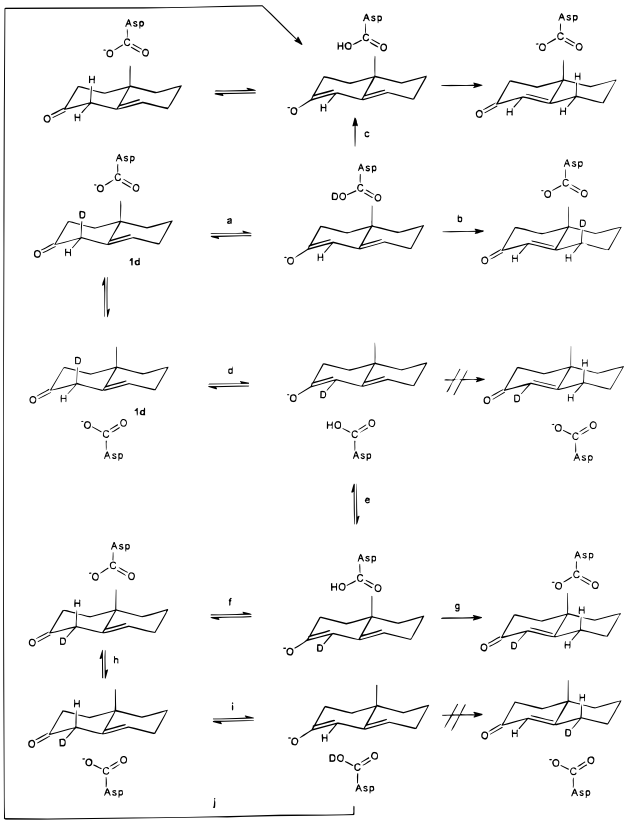
A lack of stereospecificity of proton abstraction has also been observed with crotonase. In the dehydration of 3(*S*)-hydroxybutyryl-CoA, the *pro-R* hydrogen is abstracted, with no detectable labilization of the *pro-S* hydrogen. However, the stereospecific exchange of the *pro*-2*S* proton of butyryl-CoA is catalyzed by crotonase. Furthermore, exchange of both α protons is observed with several more acidic derivatives (D'Ordine et al., 1994). In all of these cases, the postulated base is the same glutamic acid.

An important corollary of the ability of KSI to labilize both protons of **1** is that the interpretation of kinetic isotope effects with deuterated **1** and KSI becomes quite complicated. Due to primary kinetic isotope effects, the relative amounts of 4α and 4β proton abstraction vary depending on the isotopic state of the hydrogens at the α and β positions. Thus, observed effects cannot be interpreted as simple primary or secondary effects, and reliable conclusions cannot be drawn from interpretations of isotope effects with 4α-deuterated **1** as simple secondary effects [see, for example, Xue et al. (1990)].

Mechanism of Proton Exchange. Viger et al. (1981) interpreted the labilization of the 4α proton in terms of a two-base mechanism. In order to account for the competitive abstraction of the 4α and 4β protons, they proposed that the 4α proton is abstracted either by a second enzyme base or by a solvent molecule. However, no evidence has been presented as yet for the intervention of any amino acid residues other than Asp-38 or Tyr-14. In addition, the rate of formation of the intermediate dienolate from **1** with the D38N mutant (Asp $\rightarrow$ Asn) is almost 10^4-fold too slow to account for the rate of isomerization catalyzed by the D38E mutant (Xue et al., 1991; Zawrotny & Pollack, 1994). If a second base were important, then the intermediate should be formed from abstraction of a proton at C-4 by this second base at about the same rate with both the D38N and D38E mutants. Thus, the simplest explanation is that the entire catalytic reaction with both the WT and D38E is due to residue 38 (Asp or Glu) of the enzyme acting as the sole catalytic base. It is, of course, possible that the action of a second base (e.g. a water molecule) requires the presence of Asp-38, but there is no evidence yet to support this possibility.

We propose a modification of the Viger scheme, in which there is only one base present at the active site, and this base (Asp-38) is capable of abstracting both the 4α and the 4β protons. The rationale for this hypothesis is that (1) Asp-38 is clearly the base that is involved in the isomerization reaction (Benisek et al., 1980; Bounds & Pollack, 1987; Kuliopulos et al., 1989), so it must have access to the β face of the steroid, and (2) Asp-38 has been shown to have access to the α face of the steroid by inactivation studies with 3β-oxiranyl steroids (Bounds & Pollack, 1987). Thus, the steroid may bind in either of two orientations, with Asp-38 at the α face of the steroid or at the β face, and it is reasonable to assume that this amino acid residue can abstract both the 4α and 4β protons. Competitive abstraction of both the 4α and 4β protons is also consistent with the finding that, in the nonenzymatic isomerization of **1** catalyzed by acetate ion, the rates of abstraction of the 4α and 4β protons are comparable (Zeng & Pollack, 1991). Furthermore, we postulate that protonation at C-6α does not occur to any significant extent. Viger et al. (1981) found no indication of transfer of deuterium from **1** deuterated at C-4α to C-6, but since reaction of this compound occurs almost exclusively by abstraction of the 4β hydrogen, this evidence is not strong. With acetate as catalyst, protonation at C-6α is about 100-fold slower than at C-6β (Pollack et al., 1989b), presumably due to better orbital overlap for C-4β protonation. It is likely that the same effect is operating with the enzyme.

Scheme 3



Scheme 3 illustrates the proposed mechanism, using **1d** as an example. Substrate (**1d**) can bind to KSI in either of two orientations, with Asp-38 at the α side of the steroid or at the β side. These complexes are interconvertible, either by rotation of the steroid, by movement of Asp-38 within the complex, or by dissociation/reassociation of the steroid from/to the active site. Proton abstraction and donation can, in principle, occur from either complex. Reversible abstraction of the 4β deuterium by Asp-38 (path a) is followed either by deuteration at C-6β to form **3** deuterated at C-6 (path b) or by exchange of the deuterium with a proton from solvent to form a complex with loss of deuterium (path c). This complex then leads to formation of undeuterated **3**. Abstraction of the 4α proton (path d) leads to an intermediate that we assume cannot form product. Instead, this intermediate can interconvert with a complex having Asp-38 on the β face of the steroid (path e). From here, protonation can occur

at C-4β to give epimerized substrate (path f) or at C-6β to give product with deuterium at C-4α (path g). Finally, C-4α-deuterated substrate can bind with Asp-38 at the α face of the steroid (path h), which, upon abstraction of the α deuterium and washout with solvent, gives undeuterated product (paths i and j).

Stereochemistry of Proton Transfer with D38E. In contrast to the reaction of WT with **1** in D_2O, which results in incorporation of only 5 at. % deuterium, reaction of D38E with **1** in D_2O results in the incorporation of ca. 130 at. % deuterium. NMR analysis reveals that both C-4 and C-6β are heavily deuterated (Table 1). Similarly, reaction of **1d** in D_2O (34 mM phosphate) results in incorporation of an additional 73% deuterium, to a total of 173 at. %, which is evenly distributed between C-4 and C-6β. With D38E in H_2O, **1d** retains 58% of the deuterium, but surprisingly, virtually all of the remaining deuterium is at C-4.

These results can be rationalized by a scheme similar to that for **1d** with WT. The large increase in the amount of deuterium incorporation into **1** and **1d** in D_2O can be accounted for on the basis of the much lower rate constant for protonation of the intermediate dienolate by D38E (ca. 300 s^{-1}; Zawrotny & Pollack, 1994) compared to that with WT (ca. 10^5 s^{-1}; Hawkinson et al., 1991). Thus, proton exchange in the enzyme−dienol complex can compete more effectively with protonation to product with D38E than with WT. The occurrence of dideuterated products with both enzymes is clear evidence that the α hydrogen may be abstracted at some time during the reaction. Although a process can be envisaged that involves abstraction of the C-4β deuterium and reprotonation at C-4α with concomitant epimerization of **1d**, microscopic reversibility would require that deprotonation at C-4α also be possible.

D38E also differs from WT in that the predominant atom lost from **1d** at C-4 with D38E is the α hydrogen (ca. 62%), rather than the β deuterium (ca. 38%). With WT, it is predominantly the β deuterium that is lost (ca. 75%). With the assumption of a kinetic isotope effect of 6 on this process (Xue et al., 1990), it can be calculated that in the undeuterated compound proton abstraction is ca. 20% α and 80% β with D38E. In contrast, with undeuterated **1** and WT, a similar calculation shows that proton abstraction is about 5% α and 95% β. In both systems, the more reactive hydrogen is the β one, but abstraction of the α hydrogen is competitive (5% for WT, 20% for D38E).

SUPPORTING INFORMATION AVAILABLE

A figure showing the partial 500 MHz ^{1}H NMR spectrum of **3** before and after the exchange of labile protons with NaOD (4 pages). Ordering information is given on any current masthead page.

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