Insights into the Catalytic Mechanism and Active-Site Environment of *Comamonas Testosteroni* Δ^5-3-Ketosteroid Isomerase as Revealed by Site-Directed Mutagenesis of the Catalytic Base Aspartate-38[†]

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ABSTRACT: Δ^5-3-Ketosteroid isomerase (KSI) of *Comamonas testosteroni* catalyzes the isomerization of a wide variety of $\Delta^{5(6)}$ and $\Delta^{5(10)}$ steroids through the formation of an enzyme bound dienol(ate) intermediate. Asp-38 has been strongly implicated in catalysis, apparently serving as a proton shuttle. In this paper the results of a detailed kinetic characterization of the KSI mutants D38E and D38H are presented. Both mutants retain significant activity, with k_{cat} and k_{cat}/K_m values 10^3-10^4 times greater than the D38N mutant. The results allow for a qualitative assessment of the sensitivity of the enzymes catalytic capability to the positioning and chemical nature of the catalytic base. The near identity of the ratios of $k_{cat}^{5\text{-AND}}/k_{cat}^{5,10\text{-EST}}$ is most easily explained by a mechanism in which the second chemical step, reketonization of the intermediate dienol(ate), is not significantly rate determining. The pH dependence of the rate constants for the D38E and D38H mutants is found to be consistent with earlier proposals that an as yet unidentified titrating functional group is present in the active site and indicates that the electrostatic environment of residue 38 is hydrophobic and positively charged.

A number of enzymes catalyze the abstraction of a proton from a carbon adjacent to a carbonyl/carboxylic acid group (the α-proton of a carbon acid), including β-hydroxydecanoyl thiol ester dehydrase (Bloch, 1971), mandelate racemase (Neidhart et al., 1991), enolase (Lebioda & Stec, 1991), glycolate oxidase (Lindqvist, 1989), ferricytochrome b_2 (Xia & Mathews, 1990), triosephosphate isomerase (Davenport et al., 1991), and citrate synthase (Karpusas et al., 1991). The α-proton is relatively acidic compared to most carbon-bound protons due to its proximity to the carbonyl/carboxyl group, which allows for resonance stabilization of the carbanion formed upon ionization. Still, the rate of enzyme-catalyzed proton abstraction is typically much greater than that predicted from the ΔpK_a between the substrate in solution and the active site base (Guthrie & Kluger, 1993; Gerlt & Gassman, 1992). Transiently stable intermediates are found in many of these reactions, suggesting that the pK_a values of the enzyme bound carbon acid and the proton abstracting base are similar (Gerlt et al., 1991). Electrophilic catalysis has often been invoked as an explanation for the ability of weak bases to abstract protons from weakly acidic substrates. The available X-ray structures of active sites which catalyze proton abstraction reveal that invariably electrophilic catalysts are positioned proximal to the carbonyl/carboxylic acid groups of the substrates (Gerlt et al., 1991).

An enzyme of this type which has been the focus of intensive study is the Δ^5-3-ketosteroid isomerase (KSI)[1] from the soil bacterium *Comamonas testosteroni*, formerly known as *Pseudomonas testosteroni* (EC 5.3.3.1) (Tamoaka et al., 1987). This enzyme, which is involved with both biodegradation (in bacteria) and biosynthesis (in mammals) of steroid hormones, catalyzes the isomerization of 3-oxo-$\Delta^{5,6}$- and 3-oxo-$\Delta^{5,10}$-steroids to their conjugated Δ^4-isomers by a predominantly conservative and stereoselective transfer of the 4β-proton of the substrate to the 6β- or 10β-position (Schwab & Henderson, 1990), as shown in Figure 1 for a $\Delta^{5,6}$ substrate. KSI is an extremely efficient catalyst, with a k_{cat}/K_m approaching the diffusion limit for Δ^5-androstene-3,17-dione (5-AND), the standard substrate used in kinetic studies (Hawkinson et al., 1991). The isomerization of $\Delta^{5,10}$ substrates proceeds at a rate ca. 1000-fold slower than that of $\Delta^{5,6}$ substrates (Pollack et al., 1989). The results of chemical modification and site-directed mutagenesis studies, as well as X-ray diffraction and NMR experiments, have strongly implicated Asp-38 and Tyr-14 as being critically involved in the enzyme mechanism (Schwab & Henderson, 1990; Kuliopulos et al., 1989). Asp-38 is believed to be the general base responsible for shuttling the substrate 4β-proton to the 6β-position, while Tyr-14 is thought to function as an electrophilic catalyst, either protonating or forming a low-barrier hydrogen bond with the 3-carbonyl oxygen of the steroid to form a dienolic intermediate. It is currently a

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[1] Abbreviations: KSI, Δ^5-3-ketosteroid isomerase; ASI, alanine-3-sulfinic acid (cysteinesulfinic acid); CMC, *S*-carboxymethylcysteine; 4-AND, androst-4-ene-3,17-dione; 5-AND, androst-5-ene-3,17-dione; 5,10-EST, estr-5(10)-ene-3,17-dione; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MES, 2-[*N*-morpholino]ethanesulfonic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WT, wild type.

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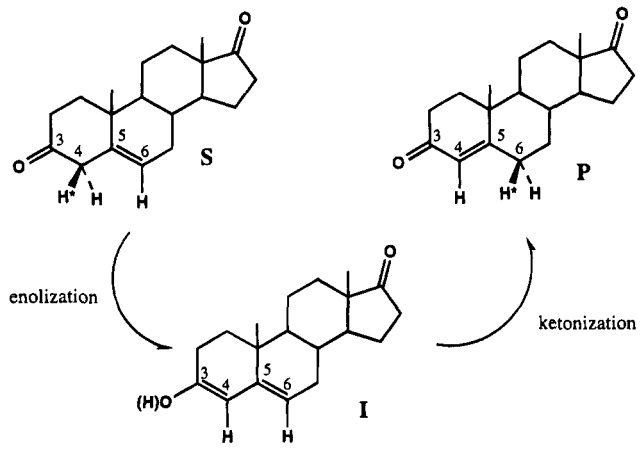

FIGURE 1: Isomerization of 5-AND by Δ^5-3-ketosteroid isomerase. The proton transferred from the 4β- to the 6β-position of the steroid is indicated by an asterisk. The numbering of relevant carbons is shown. Uncertainty with respect to the state of protonation of **I** is indicated by "(H)".

matter of some controversy as to whether the intermediate is a dienol or dienolate, with consensus appearing to favor the dienolate (Austin et al., 1992; Zeng et al., 1992; Guthrie & Kluger, 1993; Zhao et al., 1995). Gerlt and Gassman (1993) have recently proposed that the intermediate is neither one, but rather something in between. The pK_a of the protonated form of their proposed intermediate is matched to that of Tyr-14, the general acid catalyst, which could result in the formation a short, strong hydrogen bond.

Mildvan and co-workers have shown that the KSI mutants Y14F and D38N each retain ca. 10^{-5} the k_{cat} of the wild-type enzyme (Kuliopulos et al., 1989). The Y14F/D38N double mutant is completely inactive, although it does retain the ability to bind steroid substrate effectively (Kuliopulos et al., 1990). This has led them to propose that all of the catalytic power of the enzyme is derived from the Asp-38 and Tyr-14 residues. However, previous studies have suggested that another group with a pK_a of 9.5 might also be involved in catalysis. The catalytic activity of KSI has been found to decrease as the pH is raised above 9, with an apparent pK_a of ca. 9.5 (Weintraub et al., 1970). The same apparent pK_a is observed in fluorescence titration in the absence of substrate. However, the pK_a of Y14 has been directly measured in Y55F/Y88F and was found to be 11.6 (Li et al., 1993), suggesting that it is not the titration of Y14 that is responsible for the observed loss of activity and fluorescence quenching at high pH (Li et al., 1993). Austin et al. (1992) have also proposed the existence of a group that donates a hydrogen bond to Tyr-14, on the basis of the results UV resonance Raman spectroscopy studies. They suggested that the most likely candidate for this unidentified functionality would be a lysine residue; however, Mildvan and co-workers have recently singly mutated each of KSIs four lysines and found that all four mutants retain between 43% and 54% of wild-type activity (Li et al., 1993). Extending their earlier work (Austin et al., 1992), Austin et al. (1995) have found that Y14 and D38 operate semi-cooperatively to "polarize" the eneone system of bound 19-nortestosterone, and they propose strong hydrogen bonding of the Y14 hydroxyl to the bound steroid's C-3 oxygen to account for the spectral changes they observe.

We have previously determined the kinetic properties of D38ASI, a KSI mutant in which the catalytic base was replaced by the nearly isosteric but much less basic residue alanine-3-sulfinic acid (ASI) (Holman & Benisek, 1994). This mutant was used to determine that in the enolization step of the reaction proton transfer to Asp-38 is well advanced in the transition state. In the present paper we investigate the effect of lengthening the side chain of Asp-38 via the D38E and D38CMC (Asp-38 to carboxymethyl-cysteine) mutants and also the effect of replacing the carboxyl group with imidazole via D38H. The results allow for a qualitative assessment of the sensitivity of the enzyme's catalytic capability to the positioning and chemical nature of the catalytic base. We go on to compare the kinetic parameters for the wild-type (WT) and mutant KSIs toward the alternate substrates 5-AND and 5,10-EST. The near identity of the ratios of $k_{cat}^{5\text{-AND}}/k_{cat}^{5,10\text{-EST}}$ raises the possibility that the second chemical step in the proposed mechanism, the reketonization of the intermediate dienol(ate), is not significantly rate determining. The pH dependence of the rate constants for the D38E and D38H mutants is found to be consistent with earlier proposals that an as yet unidentified titrating functional group is present in the active site, and indicates that the electrostatic environment of residue 38 is hydrophobic and positively charged.

EXPERIMENTAL PROCEDURES

Materials. $\Delta^{5,10}$-Estrene-3,17-dione (5,10-EST) was purchased from Steraloids and used without further purification. Δ^5-Androstene-3,17-dione (5-AND) was prepared from Δ^5-androstene-3β-ol-17-one, obtained from Steraloids, by the procedure of Djerassi et al. (1956). pKK223-3 was provided by Pharmacia. All chemicals used were of reagent grade or better. Oligonucleotides were synthesized by the UCD Protein Structure Laboratory using an Applied Biosystems model 430A synthesizer.

Construction, Expression, and Purification of Mutant and Wild-Type KSIs. The wild-type KSI gene has been previously cloned, sequenced (Choi & Benisek, 1988; Kuliopulos et al., 1987; Choi & Benisek, 1987), and ligated into the *Hind*III and *Eco*RI sites of the expression plasmid pKK223-3 (Brooks & Benisek, 1992). The D38E and D38H mutants were constructed as previously described (Holman & Benisek, 1994). Accuracy of the mutations was verified by complete sequencing of both genes in these expression vectors. The mutant and wild-type expression plasmids were used to transform the JM105 strain of *Escherichia coli*, and the proteins were over-expressed and purified as described (Holman & Benisek, 1994). Homogeneity was assessed by SDS−PAGE and amino acid composition analysis (Holman & Benisek, 1994).

Kinetic Methods and Data Analysis. Initial rates for the conversion of 5-AND and 5,10-EST to their Δ^4-isomers were determined as previously described (Holman & Benisek, 1994). Kinetic constants as a function of pH at μ = 0.1 M were determined in either 20 mM NaMES, 80 mM NaCl (pH 3.5−6.5), or 20 mM NaMOPS, 80 mM NaCl (pH 6.5−9) (Holman & Benisek, 1994). Both buffers were used at pH 6.5 to rule out specific buffer effects. Kinetic constants as a function of pH at μ = 1.0 M were also determined in 20 mM sodium Tricine, 980 mM NaCl (pH 6.75−9.2). Kinetic pK_a values were determined for both D38E and D38H by fitting observed values of k_{cat} and k_{cat}/K_m to eq 1 and 2 using a nonlinear regression data analysis program,

$$k_{cat}^{obs} = \frac{k_{cat}}{1 + \frac{[H^+]}{K_{es1}} + \frac{K_{es2}}{[H^+]}} \quad (1)$$

$$\left(\frac{k_{cat}}{K_m}\right)^{obs} = \frac{\frac{(k_{cat})}{K_m}}{1 + \frac{[H^+]}{K_{e1}} + \frac{K_{e2}}{[H^+]}} \quad (2)$$

ENZFITTER (Elsevier-BIOSOFT). In these equations, K_{es1} and K_{es2} are the acid dissociation constants for the enzyme–substrate complex and K_{e1} and K_{e2} are the acid dissociation constants for the unliganded enzyme.

The stability of D38H and D38E at the extreme pH values was checked by adding the enzymes to the assay buffer, incubating the solution at 25 °C for 1 min, and then initiating the reaction by the addition of substrate. The rate obtained was compared with that measured using the standard order of addition, substrate before enzyme.

Protein Concentration. Amino acid analysis was used for quantifying the amount of protein in a stock solution used for kinetic studies. The concentration of hydrolyzed KSI in the sample analyzed was determined by dividing the sum of the concentrations of A, L, and K by the sum of the expected numbers of these amino acid residues in the KSI polypeptide.

UV-Monitored pH titration. Solutions containing ca. 45 μM D38H or WT in 5 mM NaMES, 195 mM NaCl, pH 6.2 (μ = 0.2), were titrated by the successive additions of small aliquots of 100 mM NaCAPS, 115 mM NaCl, pH 11.2 (μ = 0.2), at 28 ± 1 °C in a 3 mL quartz cuvette. Samples were passed through a 0.45 μm Millipore type HV filter as needed to remove the small amount of precipitate that sometimes formed after adjustment of pH. The reference cuvette was filled with 5 mM NaMES, 195 mM NaCl, pH 6.2 (μ = 0.2), and after each addition of titrant to the sample cuvette an identical amount was added to the reference cuvette. UV spectra were acquired on a Uvikon 941 spectrophotometer from 240–320 nm using a scan speed of 100 nm/min. ENZFITTER was used to fit the absorbance at 295 nm as a function of pH to the equation for the ideal titration of a single weak acid. Any deviation in protein concentration due to dilution or removal by filtration was corrected for by using absorbance at 277 nm, the isobestic point for the ionization of tyrosine, as a measure of protein concentration. At this wavelength the only significant absorbance should be from tyrosine, since the enzyme contains no tryptophan. Differences in concentration between different experiments were also corrected for using absorbance at 277 nm, assuming the same extinction coefficient for D38H and WT.

RESULTS

Mutagenesis and Expression of KSIs. Mutant wild-type KSIs were successfully over-expressed in the JM105 strain of *E. coli* and purified to homogeneity as described in Experimental Procedures. One liter of stationary phase culture was found to yield approximately 100–150 mg of D38H, while the yield of D38E was somewhat lower, about 50 mg KSI per liter of stationary phase culture. The purified enzymes were judged to be homogeneous by SDS–PAGE and amino acid composition analysis.

Table 1: Kinetic Constants for KSI and Mutated KSI's[a,b]

enzyme	substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
wild type[c]	5-AND	3.0×10^4	123	2.4×10^8
wild type[c]	5,10-EST	3.9×10^1	40	9.6×10^5
D38ASI[c]	5-AND	4.4×10^2	42	1.0×10^7
D38ASI[c]	5,10-EST	7.6×10^{-1}	14	5.3×10^4
D38E	5-AND	1.8×10^2	73	2.4×10^6
D38E	5,10-EST	2.1×10^{-1}	42	5.2×10^3
D38H	5-AND	3.6×10^2	28	1.3×10^7
D38H	5,10-EST	9.9×10^{-1}	36	2.8×10^4

[a] All rate measurements were made at 25 °C in 0.034 M potassium phosphate buffer containing 2.5 mM EDTA and 1.7% methanol, pH = 7.0. [b] Standard errors are 5–10% in k_{cat} and K_m, and 10–15% in k_{cat}/K_m. [c] Holman and Benisek (1994).

Table 2: Ratios of Kinetic Constants for the Alternate Substrates 5-AND and 5,10-EST

enzyme	$k_{cat}^{5\text{-AND}}/k_{cat}^{5,10\text{-EST}}$	$(k_{cat}/K_m)^{5\text{-AND}}/(k_{cat}/K_m)^{5,10\text{-EST}}$
WT	769	250
D38ASI	579	189
D38E	857	461
D38H	364	464

Comparison of Kinetic Parameters toward Alternate Substrates 5-AND and 5,10-EST. Kinetic parameters toward 5-AND and 5,10-EST were determined at 25.0 °C in pH 7 phosphate buffer for the D38E and D38H mutants and are presented in Table 1, along with kinetic parameters previously determined under the same conditions for wild type (WT) and D38ASI (a mutant of KSI in which D38 has been replaced by alanine-3-sulfinic acid, a structural analog of aspartate with a pK_a of ca. 2) (Holman & Benisek, 1994). The ratios of the kinetic constants for 5-AND and 5,10-EST are presented in Table 2.

pH–Rate Profiles for D38E and D38H. The pH dependencies of k_{cat}^{obs} and $(k_{cat}/K_m)^{obs}$ for D38E and D38H with 5-AND as the substrate are shown in Figures 2 and 3. The K_m of D38H increases dramatically as pH decreases, from 6 μM at pH 9 to 165 μM at pH 3.6. The maximum K_m for D38E is 86 μM at pH 7.25, dropping to 4.5 μM at pH 3.5 and to 48 μM at pH 8.75. Stability studies showed that both enzymes were stable over the pH range investigated for the time required to make the rate measurements (<1 min). Kinetic parameters determined for both mutants in pH 7 MOPS (μ = 0.1 M) did not vary significantly from those determined in the pH 7 phosphate buffer.

Since the limiting values of k_{cat}/K_m for D38E and D38H with 5-AND as the substrate are 10^2- to 10^3-fold lower than k_{cat}/K_m for WT (Holman & Benisek, 1994), it is reasonable to assume that 5-AND is a nonsticky substrate with respect to both mutant isomerases (Pollack et al., 1986). This assumption has been recently verified by Zawrotny and Pollack (1994), who have determined the free energy profile for the D38E KSI isomerization of 5-AND. For a nonsticky substrate, plots of $(k_{cat}/K_M)^{obs}$ vs pH should yield the pK_a values for the free enzyme and/or substrate, while plots of k_{cat}^{obs} vs pH should provide pK_a values of the enzyme–substrate complex whose decomposition to product is rate determining (Cleland, 1977; Knowles, 1976). This is not the case if the substrate is sticky, such as 5-AND in the reaction catalyzed by WT. The pK_e and pK_{es} values as well as limiting values of k_{cat} and k_{cat}/K_m were determined by fitting observed kinetic parameters to eqs 1 and 2 and are

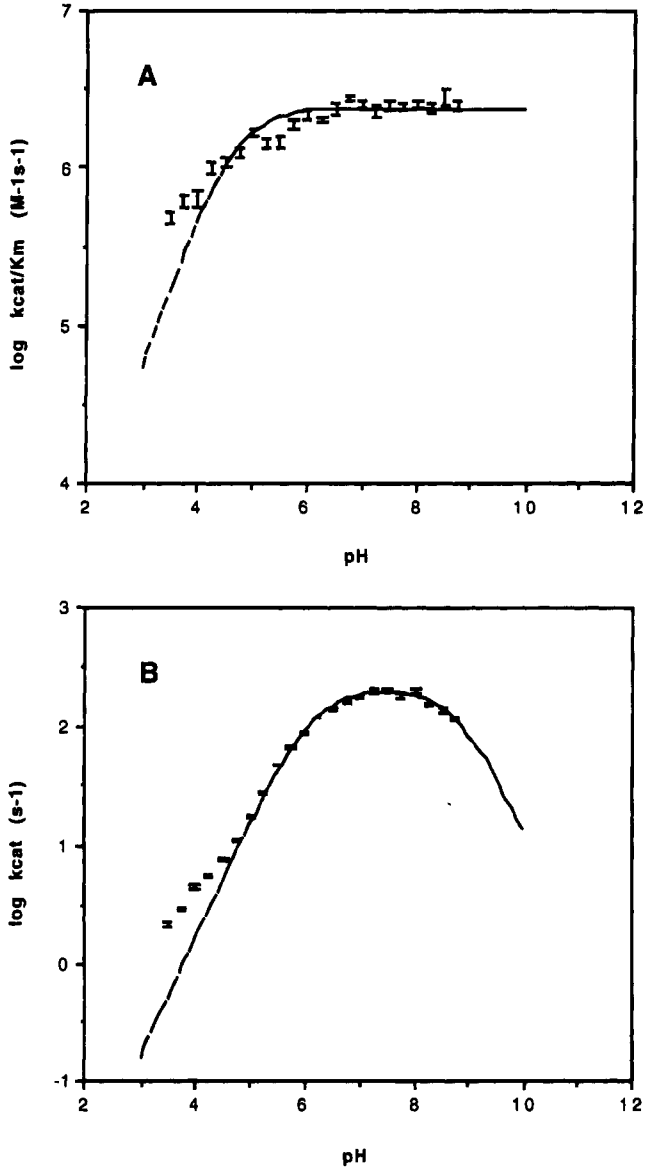

FIGURE 2: pH Dependence of D38E. *Panel A*, pH dependence of k_{cat}/K_m. The dashed line is the calculated curve for pK_{e1} = 4.65, k_{cat}/K_m (upper) = 2.4 × 10^6 M^{-1} s^{-1}. Panel B, pH dependence of k_{cat}. The dashed line is the calculated curve for pKes1 = 6.13, pK_{es2} = 8.83, k_{cat} (upper) = 210 s^{-1}. Conditions used for the reactions are described in Experimental Procedures.

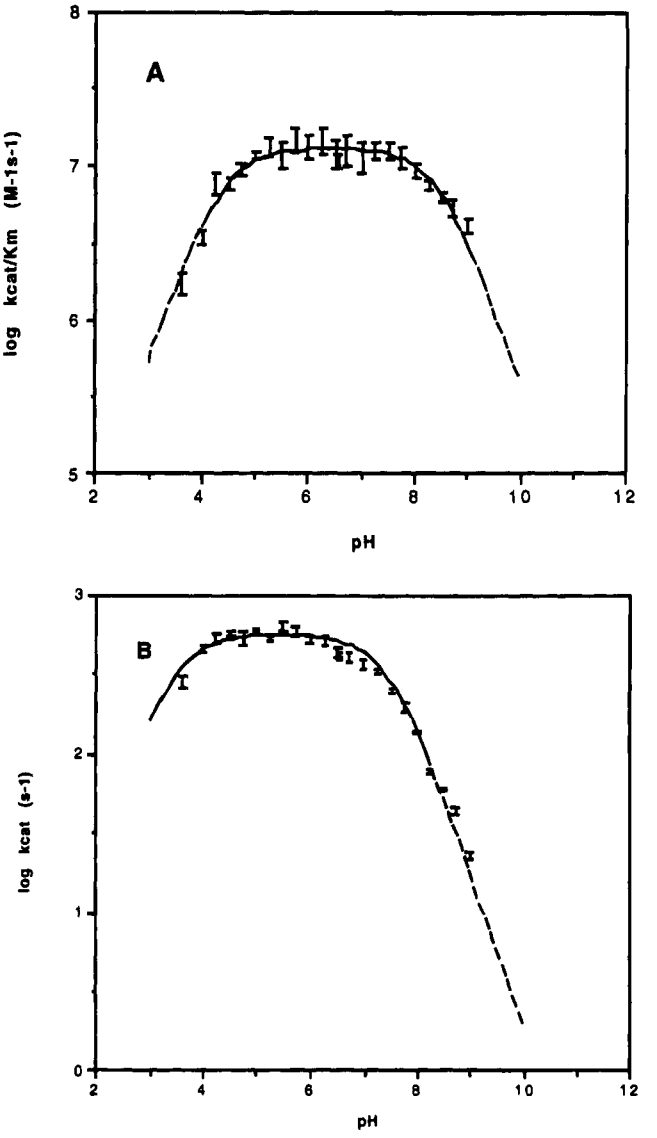

FIGURE 3: pH Dependence of D38H. Panel A, pH dependence of k_{cat}/K_m. The dashed line is the calculated curve for pK_{e1} = 4.37, pK_{e2} = 8.5, k_{cat}/K_m (upper) = 1.3 × 10^7 M^{-1} s^{-1}. Panel B, pH dependence of k_{cat}. The dashed line is the calculated curve for pK_{es1} = 3.41, pK_{es2} = 7.52, k_{cat} (upper) = 564 s^{-1}. Conditions used for the reactions are described in Experimental Procedures.

listed in Table 3. Included in the table are values previously determined for D38C, D38ASI (Holman & Benisek, 1994), and WT (Pollack et al., 1986). The kinetic pK_a's of WT were determined by these workers using the nonsticky substrate 5,10-EST.

Kinetic parameters of D38H(5-AND), D38E(5-AND), and WT(5,10-EST) were also determined from pH 6.75 to 9.2 in 20 mM sodium Tricine, 980 mM NaCl (μ = 1.0 M) in order to investigate the effect of elevated ionic strength on pK_{e2} and pK_{es2}. The enzymes' activities were found to be stable enough at pH up to 9.2 for the time required to assay (1 min) such that no corrections for loss of activity due to denaturation were required. Activity in 20 mM sodium Tricine, 980 mM NaCl, is identical to that found in 20 mM NaMOPS, 980 mM NaCl, and thus results can be compared directly to those obtained at μ = 0.1 M. The k_{cat} values for D38H, D38E, and WT are all very slightly altered by the increase in ionic strength, less than 20% at any given pH.

K_m, on the other hand, is significantly effected by ionic strength. For both D38E and WT, K_m is decreased at higher ionic strength for all pH values examined. The K_m values can be compared at the pH of optimal activity. At pH 7, WT K_m decreases from 40 to 24 μM in going from 0.1 to 1.0 M ionic strength, while K_m for D38E decreases from 75 to 45 μM. These results are consistent with the previous finding that the K_m of WT with 5-AND as the substrate decreases from 277 to 118 μM at pH 7 in going from 0.1 to 1.0 M ionic strength (Hawkinson et al., 1991). In contrast, the K_m of D38H increases dramatically as the ionic strength is increased, rising from 32 μM at μ = 0.1 M to 169 μM at μ = 1.0 M at pH 6.73.

Significantly, the pK_{es2} values of 8.8 for D38E and 7.5 for D38H were not affected by the increase in ionic strength. The pK_{e2} values for D38H were found to be 8.7 ± 0.1 at 0.1 M ionic strength and 8.5 ± 0.1 at 1.0 M ionic strength. No decrease in k_{cat}/K_m, and thus no pK_{e2}, could be detected for D38E up to a pH of 9.2.

Table 3: Kinetic pK_a Values and Limiting Kinetic Parameters at Optimal pH[a]

enzyme	pK_{e1}	pK_{e2}	pK_{es1}	pK_{es2}
wild type[b]	4.57	≥9.5	4.74	≥9.5
D38C[c]	8.46	≥9.5	8.27	≥9.5
D38ASI	<3	≥9.5	<3	≥9.5
D38E	4.65	≥9.5	6.13	8.83
D38H	4.37	8.5	3.41	7.52

enzyme	limiting k_{cat} (s^{-1})	limiting k_{cat}/K_m (M^{-1} s^{-1})	relative k_{cat} (s^{-1})	relative k_{cat}/K_m (M^{-1} s^{-1})
wild type	30 000	2.4×10^8	1	1
D38C	15.7	1.2×10^7	0.0005	0.050
D38ASI	439	1.0×10^7	0.015	0.0417
D38E	210	2.4×10^6	0.007	0.010
D38H	564	1.3×10^7	0.019	0.054

[a] All values determined for the isomerization of 5-androstene-3,17-dione unless otherwise noted. Conditions are described in Experimental Procedures. [b] pK_e and pK_{es} for wild-type were determined for the isomerization of 5,10-estrene-3,17-dione (Pollack et al., 1986). [c] Holman and Benisek (1994).

At low pH the data for the D38E pH−rate profiles deviate significantly from the calculated theoretical titration curves. A qualitatively similar deviation of k_{cat} and k_{cat}/K_m from theoretical titration curves was previously observed for WT using the substrates 5-AND, 5,10-EST, and 5-pregnen-3,20-dione (Pollack et al., 1986). This behavior could be the result of negative cooperativity in the titration of the catalytic base. In general, ideal titration curves are not to be expected in proteins due to the variation of the electrostatic potential of the molecule with pH (Parsons & Raftery, 1972). Additionally, some or all of the deviation could be explained if the enzyme retains measurable activity when residue 38 is protonated.[2]

In order to evaluate this apparent negative cooperativity in the titration of D38E, the pH−rate data for both D38E and D38H were fit to the Hill equation. Some negative cooperativity was seen in all of the titrations, with calculated n values in the range of 0.80−0.95. In most cases the pK_e and pK_{es} values obtained from the Hill equation were not significantly different from those obtained by fitting the data to the equation for an ideal acid−base titration, the largest difference being a pK_{e1} of 4.95 for D38E using the Hill equation as opposed to 4.65 when the data was fit to eq 2. The data was also fit to variations of eqs 1 and 2 in which k_{cat} and k_{cat}/K_m are not assumed to be zero at low pH. Again, the pK_e and pK_{es} values calculated were only slightly different than those obtained using eqs 1 and 2. Since these small differences would not affect any of the conclusions made from the data, only the values obtained using eqs 1 and 2 are reported.

UV-Monitored pH Titration of D38H and WT. Absorbance spectra between 240 and 320 nm were recorded from pH 6.2 to 10.4. The spectra at pH 7 for D38H and wild type were similar both to each other and to the spectra previously published by Kuliopulos et al. (1989) for wild-type and D38N. Increasing pH resulted in a general red-shift in absorbance between 240 and 320 nm, with maximum increases seen at 240 and 289 nm. The minimum increase in absorbance is seen at 277 nm, the isobestic point for protonated and unprotonated tyrosine. Absorbance at 295 nm was corrected for any changes in enzyme concentration by assuming that 277 nm is a measure of protein concentration, since absorbance change was minimal at this wavelength and contribution from ionization of tyrosine is expected to be negligible. Absorbance at 295 nm was chosen as an indicator of the ionization states of tyrosine since both phenylalanine and protonated tyrosine have negligible absorbance at this wavelength, while ionized tyrosine absorbs relatively strongly. There are no tryptophan residues in the protein; therefore at neutral pH the absorbance at 295 nm is very low. As the pH is increased from 6.5 to about 9, a gradual increase in absorbance at all wavelengths, including 295 nm, is seen, possibly reflecting slight changes in the enzymes structure and the environment of aromatic residues. Above pH 9 the absorbance is seen to rise dramatically. The changes in absorbance at 295 nm are virtually identical for wild type and D38H, as shown in Figure 4. The data was fit to the equation for the titration of a single ionizing group. "pK_a's" of 9.71 ± 0.08 and 9.95 ± 0.10 were calculated for D38H and wild type, respectively. This is not meant to imply that the observed increase in absorbance necessarily reflects the simple titration of a single tyrosine residue, but the apparent pK_as determined are a convenient way of quantitating the effect seen. In fact, it is likely that some denaturation of the enzyme is taking place at higher pH in the titration. However, it has been shown that the enzyme is stable at pH at least as high as 9, and if a tyrosine were titrating with a pK_a of 8.5, the kinetically determined pK_{es2} of D38H, the effects should have been readily apparent. To illustrate this, the expected effect of pH on the A_{295} of the

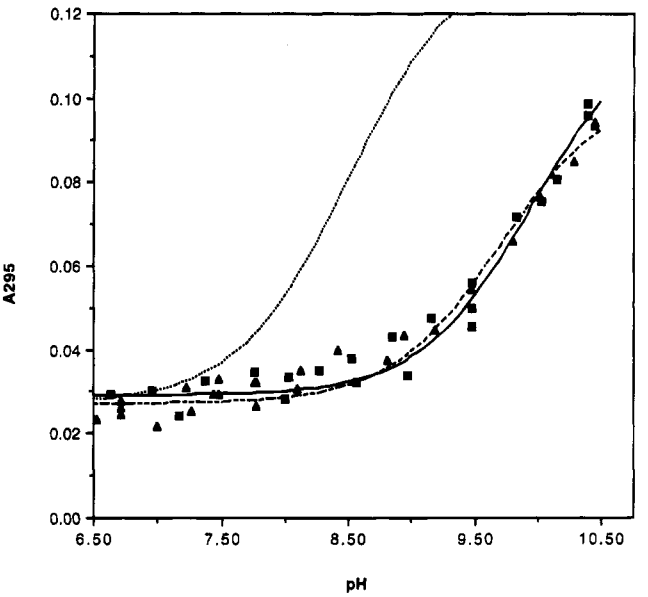

FIGURE 4: Absorbance of wild-type (square) and D38H (triangle) KSI at 295 nm as a function of pH. The dashed line (D38H) is the calculated curve for pK_a = 9.71, A_{295} (upper) = 0.103, A_{295} (lower) = 0.027, and the solid line (wild type) is the calculated curve for pK_a = 9.95, A_{295} (upper) = 0.119, A_{295} (lower) = 0.029. The dotted line is the calculated curve for pK_a = 8.5, A_{295} (upper) = 0.134, A_{295} (lower) = 0.027, the calculated ΔA_{295} for the ionization of one tyrosine.

[2] The data and theoretical curves shown in Figure 2A,B appear to show hollows between pH 5−6 and pH 4−5, respectively. The theoretical curves are based on eq 1 and 2 (Experimental Procedures), which assume that KSI fully protonated on D38 possesses no catalytic activity. However, if the data are fitted to more general equations which allow for nonzero activity of the protonated enzyme, then the apparent hollows vanish. That the protonated form of KSI has catalytic activity is consistent with the nonzero activity of the KSI mutant, D38N, (Kuliopulis et al., 1989; Hawkinson et al., 1994).

enzyme if one tyrosine were titrating with a pK_a of 8.5 while the other tyrosines remained fully protonated is plotted in Figure 4. Recently the pK_a of Tyr-14 in the Y55F/Y88F double mutant was determined to be 11.6 by UV-monitored titration (Li et al., 1993).

DISCUSSION

In this study we have characterized mutants of KSI wherein the catalytic base, Asp-38, has been mutated to the alternative bases Glu and His. The kinetics of the reactions catalyzed by D38E and D38H have been studied as a function of pH, and the activity of these mutants toward the alternate substrates 5-AND and 5,10-EST has been determined. In analyzing the results of mutagenesis experiments of this sort, the assumption must be made that no gross structural changes have occurred outside of the immediate vicinity of the mutation. Several lines of evidence support this assumption for these mutants. (1) The mutants are found to bind specifically to a deoxycholate affinity resin. (2) The K_m values obtained for the mutants toward the nonsticky substrate 5,10-EST are all very close to that of WT, while the mutant K_m values obtained toward the sticky substrate 5-AND are all close to the predicted K_s of WT (Hawkinson et al., 1991). (3) The close similarity in the ratio of $k_{cat}^{5\text{-AND}}/k_{cat}^{5,10\text{-EST}}$ for all the mutants to that of WT argues that the catalytic mechanism utilized by the mutants is very close to that of WT, implying a similar active site structure. (4) The C-2 protons of two out of the three wild-type histidines in D38H were identified using ^{1}H NMR (C. M. Holman and W. F. Benisek, unpublished results) and found to have pK_a's and limiting chemical shifts nearly identical to those reported for the wild-type enzyme (Benisek & Ogez, 1982; Kuliopulos et al., 1991). The C-2 protons of the third wild-type histidine and of His-38 could not be unambiguously assigned.

Effect of Alteration of Catalytic Base on the Ability of KSI to Isomerize Steroid Substrate. It has previously been demonstrated that the basic forms of cysteine- and alanine-sulfinic acid can substitute functionally for Asp-38, the D38C and D38ASI mutants of KSI having k_{cat} values of 16 and 440 s^{-1}, respectively (Holman & Benisek, 1994). Assuming that the backbone conformation of WT is conserved in the D38E mutant, the catalytic carboxylate of D38E is displaced at least 1.5 Å from its location in WT, and its orientation with respect to the substrate is changed. This displacement, however, could be attenuated by an alteration in the conformation of the protein backbone. The fact that the k_{cat} of D38E is over 1000-fold greater than that of D38N suggests that the precise location of the group is not critical for enzymatic function. The D38CMC mutant, however, in which the side chain is longer than that of D38E by one sulfur atom, is completely inactive (Holman & Benisek, 1994). This large displacement of the carboxylate (at least 2.9 Å, assuming no change in backbone conformation) evidently leaves it completely out of position to function in catalysis.

D38H is a surprisingly active mutant, with a k_{cat} nearly 3000-fold greater than D38N. Using molecular modeling it is possible to position the δ1-N of His so that it is nearly isosteric with the carboxylate oxygens of Asp, as close as about 0.15 Å. This might explain the success with which His can substitute for Asp as the catalytic base in KSI. Whalen and co-workers (1976) have examined the efficiency of various general bases in catalyzing the isomerization of 3-cyclopentenone. They found that neutral bases (tertiary amines) are about 100-fold more effective catalysts than negatively charged bases (hydroxide, phosphate, carbonate) of the same pK_a, although each charge type gives a good Brönsted plot with a β value of 0.5. This suggests that histidine might be an intrinsically better catalyst than aspartate if steric and pK_a differences could be eliminated. The introduction of an amine base more nearly isosteric with aspartate at position 38 would be an interesting unnatural mutation which might yield a form of KSI with high activity, possibly surpassing that of WT.

Analysis of the K_m values of D38H and D38E as a function of pH reveals that a charge on residue 38 is unfavorable for substrate binding. The K_m for D38E increases 20-fold as the pH is raised from 3.5 to 7.25, while it decreases almost 30-fold for D38H as the pH is raised from 3.5 to 9. Thus, for both mutants, K_m is much higher when residue 38 is charged. These findings are consistent with the hydrophobic nature of the steroid substrate.

Comparison of the Rates of Isomerization of the Alternate Substrate 5-AND and 5,10-EST. The ratios of $k_{cat}^{5\text{-AND}}/k_{cat}^{5,10\text{-EST}}$ and $(k_{cat}/K_m)^{5\text{-AND}}/(k_{cat}/K_m)^{5,10\text{-EST}}$ for all of the Asp-38 mutants of KSI investigated thus far are very close to those of WT. D38E and WT are nearly identical with respect to $k_{cat}^{5\text{-AND}}/k_{cat}^{5,10\text{-EST}}$, while D38ASI has a slightly lower value than WT and D38H has about half the value of WT. These results strongly suggest that all of the isomerases are catalyzing the reaction by the same enzymatic mechanism. Assuming that the A and B rings of the two steroid substrates are bound in approximately the same location in the active site (Carrell et al., 1978; Weintraub et al., 1977), it is likely that the C-4 protons of both bound substrates are positioned in about the same orientation with respect to residue 38; the distance between C-6 and C-10, on the other hand, is about 2.6 Å (Carrell et al., 1978). It is not difficult to envision an active-site geometry in which lengthening the side chain of residue 38 affects the rate of enolization for both substrates to the same extent. However, it seems less likely that a large displacement in the position of the catalytic base relative to the substrates would be felt equally at C-6 and C-10; therefore, one would predict that mutating residue 38 will effect the rates of ketonization for the two substrates in a quantitatively different manner. The similar value of $k_{cat}^{5\text{-AND}}/k_{cat}^{5,10\text{-EST}}$ for WT and all mutants, particularly the near identity of $k_{cat}^{5\text{-AND}}/k_{cat}^{5,10\text{-EST}}$ for WT and D38E, suggests that ketonization is not significantly rate limiting in the catalyzed reaction. The possibility does exist, of course, that because of fortuitous positioning of residue 38 steric changes introduced at this position are manifested equally at C-6 and C-10.

These findings are consistent with those of Xue et al. (1990), who have also proposed that ketonization is not rate limiting on the basis of the results of secondary kinetic isotope effect studies. Pollack's group, on the other hand, has proposed that both enolization and ketonization are partially rate limiting, on the basis of the results of kinetic partitioning experiments (Hawkinson et al., 1991). The validity of these kinetic partitioning experiments has been questioned due to the apparently improper ionization state of the initially formed enzyme−intermediate complex obtained when the 3,5-dienol binds to KSI (Xue et al., 1990).

Furthermore, the fact that replacement of Tyr-14 by the more acidic electrophile 3-fluorotyrosine results in a decrease in k_{cat} of the double mutant KSI Y55F/Y88F (Brooks & Benisek, 1994) suggests that ketonization is at least partially rate limiting for this high activity mutant of KSI.

pH Dependencies of the Enzymatic Rate Constants of WT, D38E, and D38H Are Used to Characterize the Electrostatic Environment of Residue 38. Proper analysis of the variation of k_{cat} and k_{cat}/K_m as a function of pH will yield kinetic pK_e and pK_{es} values, which often reflect the pK_a of an ionizable group in the free enzyme and in the enzyme−substrate complex, respectively (Cleland, 1977). The pH dependence of wild-type KSI has previously been determined, and the pK_{e1} and pK_{es1} have been shown to correspond to the titration of the catalytic base Asp-38 (Pollack et al., 1986). Pollack et al. (1986) saw no decrease in k_{cat} or k_{cat}/K_m as the pH was increased to a maximum of 8.8. Rapid denaturation of the enzyme at pH higher than this dissuaded these workers from studying the reaction at higher pH. However, in an earlier pH profile, a pK_{es2} of 9.5 was reported (Weintraub et al., 1977). No mention of the rapid denaturation seen at high pH was made by these authors. The only enzymatic functional group besides Asp-38 to be previously implicated in catalysis is Tyr-14 (Kuliopulos et al., 1989).

In the present paper the pH dependence of the D38H and D38E mutants of KSI is used to characterize the electrostatic environment of this critical catalytic base. Two factors are known to influence the pK_a values of active-site groups, the hydrophobicity of the environment, often referred to as the local dielectric constant, and the electric potential. The dielectric constant reflects the electronic and orientational polarizability of the relevant protein and/or solvent (Bashford & Karplus, 1990). The electric potential results from the overall charge on the protein, which itself changes with pH. A reduction in dielectric constant will lower the pK_a of cationic acids (i.e., His) and raise the pK_a of neutral acids (i.e., Asp, Glu). A positive electric potential, on the other hand, will lower the pK_a of either type of acid, with the converse being true for a negative electric potential. Thus, the observed pK_e and pK_{es} for the D38H and D38E mutants can be compared in order to assess the electrostatic environment of residue 38.

The pK_{e1} of D38E is very similar to that of WT, close to the predicted pK_a of an unperturbed glutamate. The pK_{es1} of D38E, however, is about 1.5 units higher than its pK_{e1}, while for both WT and D38C the differences between pK_{e1} and pK_{es1} are quite small, about 0.2 in each case. That the pK_{es} values are higher than the pK_e most likely reflects the increased hydrophobicity of the environment of residue 38 caused by the binding of the steroid substrate.

The pK_{es1} of D38H is *lower* than pK_{e1}, as one would expect upon increasing the hydrophobicity of the environment of a cationic acid. The pK_{e1} and pK_{es1} values for D38H are lower than those for D38E and WT, even though pK_a of an unperturbed histidine residue is about 2 units higher than those of Asp and Glu. This can be explained by an environment around residue 38 which is both hydrophobic and positively charged, the two effects complementing each other in the case of a cationic acid and opposing each other with respect to neutral acids. If one makes the assumption that the difference between the values of pK_{e1} and the known unperturbed pK_a values of His, Asp, and Glu is due solely to the electric potential and the dielectric constant at the site, and that the slightly different location of the titrating group in the different mutants is insignificant, one can use the pK_{e1} values along with the Tanford−Roxby equation ($\Delta pK_a = e\Delta\phi/2.303zKT$) (Tanford & Roxby, 1978) to estimate an electric potential of ca. +50 mV at residue 38.

A positive electric potential would help stabilize the negative charge that would develop with either a dienol or dienolate intermediate. Guthrie and Kluger (1993) have recently suggested that the only energetically plausible mechanism for a reaction such as this would have a dienolate intermediate, stabilized by electrostatic interactions with a positively charged group in a medium of low polarity. According to the mechanism proposed for KSI by Gerlt and Gassman (1993) the intermediate is neither a dienol or a dienolate, but rather a hybrid species which is stabilized by a negatively charged short, strong hydrogen bond with Tyr-14 (Zhao et al., 1995). This intermediate would also be stabilized by a positive electric potential in a hydrophobic environment. A short strong hydrogen bond requires that the pK_a of the protonated intermediate and the general acid catalyst be similar in magnitude. A positive electric potential might serve to fine tune the pK_a of Tyr-14 to optimize its potential to form a strong hydrogen bond with the reaction intermediate.

Lowered $pK_{e(s)2}$ of D38H and D38E Is Consistent with Existence of an As Yet Unidentified Titrating Residue that is Involved in Catalysis. The existence of an as yet unidentified functional group in the KSI active site with a pK_a of about 9.5 has been speculated upon for some time, on the basis of several lines of evidence. The pH dependence of the kinetic parameters has implicated a group titrating with a pK_a of about 9.5, which early on was believed to be Tyr-14. However, the pK_a of Tyr-14 was measured by UV titration in the KSI double mutant Y55F/Y88F and found to be 11.6 (Li et al., 1993). The kinetically observed pK_a of 9.5 is not likely to be explained by a change in rate-determining step for the reaction, since the same value of 9.5 is observed in fluorescence titrations in the absence of substrate. Austin et al. (1992,1995) have also proposed the existence of a hydrogen bond donor which interacts with the hydroxyl group of Tyr-14, on the basis of the results of UV resonance Raman experiments. They speculate that this group might participate in a proton relay with Tyr-14, stabilizing the negatively charged intermediate in the reaction. On the basis of energetic considerations, Guthrie and Kluger (1993) propose that electrophilic catalysis alone is inadequate to explain the rates observed for this type of reaction, and suggest that sufficient stabilization of the reaction intermediate could only be provided by electrostatic stabilization (i.e., a positive charge) in a medium of low polarity.

The pH dependence of the D38E, and particularly the D38H mutants of KSI has unexpectedly provided new evidence in support of the existence of this functional group. These mutants are found to have lowered pK_{e2} and pK_{es2} values compared to wild type. UV absorption at 295 nm is virtually identical for D38H and WT up to a pH of 10.4, whereas if Tyr-14 had a pK_a of 8.5−9.5 it would be readily apparent, as shown in Figure 4. Thus, the lowered values of $pK_{e(s)2}$ in the two mutants do not reflect a decrease in the pK_a of Tyr-14.

The pK_a of this unidentified group is lowered in the D38E mutant, probably due to the displacement of the carboxylate group of Asp-38 to a more distant location. A more dramatic

decrease in pK_a is seen in the D38H mutant as a result of completely removing the carboxylate and replacing it with a neutral imidazole. The titrating group responsible for $pK_{e(s)2}$ is probably a cationic acid, since substrate binding lowers its pK_a. In light of the fact that $pK_{e(s)1}$ reflects the effect of substrate binding on the pK_a of the cationic acid His-38, it is interesting to note that for D38H the difference between pK_{e2} and pK_{es2} is identical to the difference between pK_{e1} and pK_{es1}. This putative titrating group might be involved in a proton shuttle with Tyr-14, as proposed by Austin et al. (1992, 1995), and could provide the positive electrostatic potential which stabilizes the anionic intermediate.

pH−rate profiles for D38E, D38H, and WT were also obtained at high ionic strength (μ = 1.0M) in the pH range 6.75−9.2 in order to determine whether electrostatic shielding by high ionic strength leads to any alteration of $pK_{e(s)2}$. If $pK_{e(s)2}$ reflects the titration of a group whose pK_a is perturbed by the charge on residue 38, then elevated ionic strength might decrease this effect and lead to a change in $pK_{e(s)2}$. However, no significant shifts in either pK_{e2} or pK_{es2} were observed for either mutant or WT. The results imply that the space between residue 38 and the proposed titrating group is not freely accessible to ions in the medium. Fersht and co-workers have reported the insensitivity to high ionic strength of an electrostatically perturbed histidine pK_a in barnase (Sancho et al., 1992).

Increased ionic strength does, however, have an effect on K_m, increasing that of D38H and decreasing it for WT and D38E at neutral pH. Once again, this can be explained by noting that at neutral pH Asp and Glu are charged while His is neutral.

One can devise alternative explanations for the low values of pK_{e2} and pK_{es2} seen for D38E and D38H. A change in rate-determining step would seem unlikely, in light of the near identity of the ratio of $k_{cat}^{5\text{-AND}}/k_{cat}^{5,10\text{-EST}}$. Furthermore, if a step involving protonated Tyr-14 became less than completely rate limiting, the observed $pK_{e(s)2}$ would be *greater* than the actual pK_a of Tyr-14, not less. If the ratio of nonproductive to productive binding of substrate is pH dependent, this could perturb pK_{es} values, but no effect should be seen on pK_e (Fersht, 1985).The low pK_{e2} of D38H thus indicates that the effect is not solely due to nonproductive binding.

Probably the most plausible alternative explanation for the lowered values of $pK_{e(s)2}$ is that they reflect a conformational change in the enzyme. Mildvan and co-workers have proposed that kinetic pH dependence and the fluorescence titration of KSI, both which give an apparent pK_a of 9.5, reflect a conformational change in the enzyme (Li et al., 1993). However, it should be noted that pK_{es2} is significantly lower than pK_{e2} for both D38H and D38E. Thus, any conformational change that is resulting in a loss of activity is occurring at a lower pH for enzyme with substrate bound than for free enzyme. One would predict that substrate binding should encourage the enzyme to assume a conformation favorable for catalysis, not promote the loss of this conformation. If $pK_{e(s)2}$ actually *does reflect* a conformational change, then D38H might prove to be a valuable reagent for studying the phenomenon at a more readily accessible pH than WT.

The finding that all of the Lys residues in KSI can be mutated without any large loss of activity seems to rule out lysine as a candidate for the proposed positively charged catalytic group (Li et al., 1993). Arg is an unlikely but possible candidate. The pK_{e2} of 8.5 in D38H is very low for an Arg, but a pK_a shift of this magnitude is not unprecedented. The pK_a of a Lys in acetoacetate decarboxylase has an observed pK_a of 6.0, a perturbation of about 4.5 pH units from normal (Kokesh & Westheimer, 1971), while a His with a pK_a of about 15 has recently been reported in flavocytochrome b_2 (Lederer, 1992). The solution of the crystal structure of KSI is currently being attempted (E. Westbrook and J. E. Maxwell, personal communication), which could allow for the identification of this group.

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