Extent of Proton Transfer in the Transition States of the Reaction Catalyzed by the Δ^5-3-Ketosteroid Isomerase of *Comamonas* (*Pseudomonas*) *testosteroni*: Site-Specific Replacement of the Active Site Base, Aspartate 38, by the Weaker Base Alanine-3-sulfinate†

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ABSTRACT: Previous studies of the mechanism of the steroid isomerase of *Comamonas* (*Pseudomonas*) *testosteroni* have identified aspartate 38 as the proton porter which transfers the substrate's 4β proton to the 6β position of the product. Consequently, aspartate 38 functions as a base in the deprotonation of the substrate to form a dienol or dienolate intermediate, which then undergoes reprotonation from protonated aspartate 38 at C-6β to give the product. We have tried to characterize the transition states for the proton transfers by altering the pK_a' of aspartate 38 and then determining the effect of the alteration on the kinetics of the enzyme. Alteration of the pK_a' was accomplished by replacement of the carboxyl carbon of aspartate 38 by sulfur, a change which converts the carboxylate group to the much less basic sulfinate group. Employing Brønsted catalysis theory as applied to the individual steps of the isomerase mechanism, we find that in the enolization step of the reaction proton transfer to aspartate 38 is well advanced in the transition state. In the subsequent ketonization step, proton transfer from aspartate 38 has barely started when that transition state is reached. A series of mutant KSIs with alternative bases at position 38 have been constructed using a combination of site-directed mutagenesis and chemical modification: Asp-38 to Glu (D38E), His (D38H), and *S*-(carboxymethyl)cysteine (D38CMC). While the D38H and D38E mutants both retain significant isomerase activity, D38CMC is essentially inert. From the results of kinetic experiments it is possible to get a qualitative idea of the sensitivity of the enzyme's catalytic ability to the location of the base responsible for proton transfer.

Proton transfer from a substrate to a catalytic group of an enzyme is a ubiquitous event in mechanisms proposed for enzyme-catalyzed reactions. Enzymes which abstract or donate protons from or to carbon atoms of substrates or intermediates at some point in the mechanism include, but are not limited to, aldolases, racemases, enolases, transaminases, carboxylases, and isomerases (Walsh, 1979; Gerlt et al., 1991). Many isomerases use proton transfers to and from the enzyme to interconvert isomers. One particularly well studied isomerase is the Δ^5-3-ketosteroid isomerase (KSI)[1] from the soil bacterium *Pseudomonas testosteroni* (EC 5.3.3.1), which has been renamed *Comamonas testosteroni* (Tamaoka et al., 1987). This small homodimeric enzyme utilizes acid/base chemistry and/or hydrogen bonding to catalyze the reaction shown in Figure 1. Studies of the structure and mechanism of KSI have been reviewed (Schwab & Henderson, 1990; Pollack et al., 1989). Mutagenesis studies

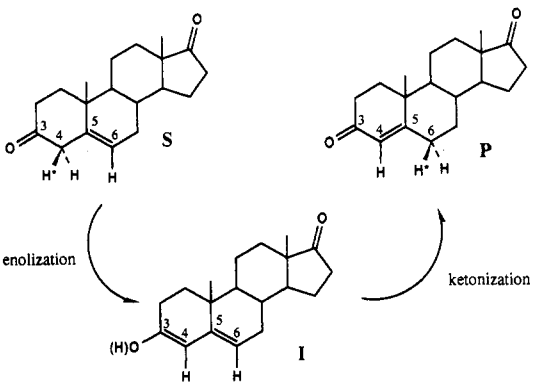

FIGURE 1: Isomerization of 5-AND by Δ^5-3-ketosteroid isomerase. The proton transferred from the 4β to the 6β position of the steroid is indicated by an asterisk (*). Substrate, intermediate, and product species are labeled S, I, and P. The uncertainty of the state of protonation of I is indicated by (H).

by Mildvan and Talalay (Xue et al., 1990, 1991a,b; Kuliopulos et al., 1989, 1990, 1991; Austin et al., 1992; Mildvan et al., 1992; Li et al., 1993) have identified residues D38 and Y14 as critical for enzyme function; the KSI mutants Y14F and D38N each possess a k_{cat} that is ca. 10^{-5} times that of the wild-type enzyme. The pH dependencies of k_{cat} and k_{cat}/K_m have been determined by Pollack and co-workers (Pollack et al., 1986). They found that a group with a pK_a' of 4.57 in the free enzyme and 4.74 in the enzyme–substrate complex was involved in catalysis. These pK_a's are close to that measured for D38 from the pH dependence of its alkylation by a steroidal affinity labeling reagent (Pollack et al., 1986). Recently, the pK_a' of Y14 has been directly measured in the KSI double mutant Y55F/Y88F and found to be 11.6 (Li et

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[1] Abbreviations: KSI, Δ^5-3-ketosteroid isomerase; ASI, alanine-3-sulfinic acid (cysteine sulfinic acid); ASO, alanine-3-sulfonic acid (cysteic acid); ASE, alanine-3-sulfenic acid (cysteine sulfenic acid); 5-AND, androst-5-ene-3,17-dione; 5,10-EST, estr-5(10)-ene-3,17-dione; DTT, dithiothreitol; TPCK, tosylphenylalanyl chloromethyl ketone; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; TFA, trifluoroacetic acid; IPTG, isopropyl thiogalactoside; CMC, *S*-(carboxymethyl)cysteine; CAMC, *S*-[(carboxamido)methyl]cysteine; IAA, iodoacetic acid; IAN, iodoacetamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); WT, wild type.

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al., 1993). Mechanisms for the isomerase have been proposed (Kuliopulos et al., 1989, 1990, 1991; Xue et al., 1990; Hawkinson et al., 1991a,b; Zeng et al., 1992; Li et al., 1993; Pollack et al., 1989) in which D38 functions as a base in the enolization steps and as an acid in the ketonization steps, while Y14 serves a role as an electrophile which polarizes the carbonyl group by protonation or hydrogen bonding in the enolization steps.

Although much is now known about the KSI mechanism, the nature of the transition states for enolization and ketonization is not yet clear. In particular, the question of how much breakage the C4–H bond has undergone in the transition state for enolization and the extent of bond formation to C6 in the ketonization transition state has not yet been answered. One approach to answering these questions is provided by Brønsted catalysis theory (Stewart, 1985; Maskill, 1985a). Brønsted theory has been used by physical organic chemists to determine the extents of bond formation and cleavage in the transition states of proton-transfer reactions involving catalytic acids and bases. In the case of general base catalysis, the extent of proton transfer from the substrate to the catalytic base in the transition state is approximated by the slope, β, of a plot of log k versus pK_a' for a series of bases of varying pK_a' used as catalysts for the reaction under investigation.

The use of Brønsted methods to elucidate enzyme reaction transition states has been extremely limited, due to the technical difficulty in modifying the pK_a' of enzyme bases without at the same time making large steric changes. Recently, the method of "chemical rescue" (Kirsch et al., 1990; Toney & Kirsch, 1989, 1992; Ehrig et al., 1991; Brooks & Benisek, 1992) has been utilized to allow variation of the catalytic base or acid by providing a series of low molecular weight side chain surrogates in free solution to an enzyme mutant from which the base or acid side chain has been deleted via mutation to alanine or glycine. However, the relevance of the results obtained by chemical rescue to the wild-type enzyme is difficult to judge, since the rescuing bases and acids may not faithfully mimic the spatial positioning adopted by the base or acid of the wild-type enzyme.

An alternative approach is to covalently modify the catalytic group in such a way as to alter its pK_a' with minimal perturbation of steric, electrostatic, and hydrophobic properties of the side chain. Such a modification is not usually achievable by standard *in vitro* mutagenesis methods; these are restricted to the side-chain repertoire provided by the 20 codon-specified amino acids. The use of group-specific chemical reagents is frequently plagued by the problems of multiple reaction sites and the introduction of increased bulk at the site of modification, which violates the criterion of isostericity.

Nevertheless, use of chemical reagents to achieve residue-specific structural alterations has been successful when the reactivity of a single residue toward a reagent is much greater than that of all other relevant residues in the protein. Cysteine is the most reactive codon-specified residue toward alkylating and oxidizing reagents. This property forms the basis for the strategy of "cysteine mutagenesis" (Falke & Koshland, 1987; Smith & Hartman, 1988; Traut et al., 1989; Planas & Kirsch, 1991). In this report we describe an application of cysteine mutagenesis to create a modified KSI in which the catalytic residue, D38, is replaced by the nearly isosteric, but much less basic, residue alanine-3-sulfinic acid (ASI). Comparison of the k_{cat}'s of the D38ASI "mutant" and the wild-type enzyme allows us to measure the influence of the basicity of residue 38 on k_{cat}. This influence, characterized by the Brønsted coefficient, β, provides a measure of the degree of proton transfer to and from the enzyme base in the enolization and ketonization steps of the isomerase reaction. Thus, the structures of the transition states in enolization and ketonization can be partially described.

EXPERIMENTAL PROCEDURES

Materials. Estr-5(10)-ene-3,17-dione (5,10-EST) was purchased from Steraloids and used without further purification. Androst-5-ene-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, and M13mp19-RF was from Bio-Rad. TPCK-treated trypsin was a product of Worthington Enzymes. IPTG, DTT, sodium deoxycholate, β-mercaptoethanol, sodium periodate, thiodiglycol, TFA, HEPES, CAPS, MES, MOPS, iodoacetic acid, iodoacetamide, and urea were obtained from Sigma. Other chemicals were of reagent grade or better. Oligonucleotides were synthesized by the UCD Protein Structure Laboratory using an Applied Biosystems Model 430A synthesizer. The deoxycholate–agarose affinity resin was synthesized as described previously (Linden & Benisek, 1986).

Construction of pKSItac.D38C and pKSItac.D38S. The wild-type KSI gene has been previously cloned and sequenced (Choi & Benisek, 1987, 1988; Kuliopulos et al., 1987) and ligated into the *Hin*dIII and *Eco*RI sites of plasmid pKK223-3 (Brooks & Benisek, 1992). The resulting expression vector, pKSI*tac*, carries the KSI gene under the control of the IPTG-inducible *tac* promoter. In order to construct similar expression vectors for the KSI mutants D38C, D38S, D38E, and D38H, the wild-type KSI gene was placed into M13mp19 for mutagenesis using the Bio-Rad mutagenesis kit, which is based on the method of Kunkel (1985). The 19-mer oligonucleotides 5′-CGGTGGAAUGCCCGTGGG-3′ and 5′-CGGTGGAATCCCCGTGGG-3′, corresponding to bases 104–122, were used to make the genes for D38C and D38S, respectively. The 19-mer oligonucleotides 5′-CGGTGGAAGAACCGTGGG-3′ and 5′-CGGTGGAACACCCGTGGG-3′, corresponding to bases 104–122, were used to make the genes for D38H and D38E, respectively. The changed bases which direct the mutations are underlined. Mutants were screened by single-track dideoxy sequencing using the TaqTrack sequencing system (Promega). The mutated DNA was isolated as the double-stranded replicative form from M13mp19, and the 391-bp fragment containing the mutant KSI gene was ligated into the *Eco*RI and *Hin*dIII sites of pKK223-3 to create pKSI*tac*.D38C, pKSI*tac*.D38S, pKSI*tac*.D38E, and pKSI*tac*.D38H. Accuracy of the mutations was verified by complete sequencing of both genes in these expression vectors.

Expression and Purification of Mutant and Wild-Type KSIs. Cells of the JM105 strain of *Escherichia coli* were transformed (Ausubel et al., 1989) with the plasmids pKSI*tac*, pKSI*tac*.D38C, pKSI*tac*.D38S, pKSI*tac*.D38E, and pKSI*tac*.D38H. For production of these KSIs, 1-L cultures inoculated with the transformed cells were grown in LB medium supplemented with 75–100 μg/mL ampicillin and 0.67 mM IPTG at 37 °C with shaking at 250 rpm for 18–24 h. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 8.5, and 5 mM DTT. The cells were disrupted using a Bronson Sonifier at 70 W for 45–90 s, and the resulting suspension was centrifuged to obtain a crude cell extract. Ninety-five percent ethanol was added to the cell extract until a final concentration of 50% ethanol was obtained.

The resulting solution was allowed to sit on ice for 30 min, after which it was centrifuged. The supernatant solution was then adjusted to 2 mM EDTA, 80% ethanol, 5 mM $MgCl_2$, and 5 mM DTT. This solution was allowed to stand overnight, during which time a precipitate containing the isomerase formed and settled out. Most of the supernatant solution was removed by careful decantation, and the precipitate was recovered by centrifugation of the turbid lower layer. The pellet obtained by centrifugation was extracted twice for 1 h each time with 0.4 M potassium phosphate, pH 7.0, and 5 mM DTT, and insoluble material was removed by centrifugation. The resulting extract was applied to a deoxycholate–agarose affinity column. The column was washed with at least 20 column volumes of 0.4 M potassium phosphate, pH 7.0, and 5 mM DTT before elution of the enzyme with either 1 mM potassium phosphate, pH 7.0, 25% EtOH, and 5 mM DTT or 1 mM potassium phosphate, pH 7.0, 3 mM sodium deoxycholate, and 5 mM DTT. Fractions containing KSI as determined by SDS–PAGE or by assaying for KSI activity were pooled and dialyzed against 50 mM potassium phosphate, pH 7.0, and 5 mM DTT. Contamination by traces of higher molecular weight material was removed either by gel filtration on a Sephacryl 100HR (Pharmacia) column or by preparative isoelectric focusing using a Bio-Rad Rotofor apparatus. Homogeneity of the final preparations was assessed by SDS–PAGE, analytical isoelectric focusing, and amino acid composition analysis.

Analytical Isoelectric Focusing. Ampholine PAGplates (LKB) having a pH range of 3.5–9.5 or 4.0–6.5 were used according to the manufacturer's instructions. Gels were run on a Desaga IEF apparatus at 10 °C. Low-p*I* calibration standards from Pharmacia were used to calibrate the pH gradient. At the beginning of electrophoresis the voltage was set at 100 V and then gradually increased to 1000–1200 V by the end of the run.

Preparative Isoelectric Focusing. Preparative IEF was carried out with a Bio-Rad Rotofor apparatus according to the manufacturer's instructions. Approximately 10 mg of mutant KSI was loaded into the apparatus in a total volume of 55 mL containing 18% glycerol, 0.15% β-mercaptoethanol, and 1.5% Pharmalyte (LKB), pH 4–6.5. IEF was conducted for 5–6 h at constant power. The fractions containing KSI were identified by SDS–PAGE and pooled, and their volume was increased to 55 mL in a solution containing 18% glycerol and 0.15% β-mercaptoethanol. This solution was subjected to a second stage of preparative IEF, as described above; the KSI-containing fractions containing the KSI of highest p*I* were identified by analytical IEF and pooled. Ampholytes were removed from the pooled fractions by making the solution 1 M in NaCl, dialyzing this solution vs 0.4 M potassium phosphate, pH 7.0, and 5 mM DTT, and loading the dialyzed solution onto a deoxycholate–agarose affinity resin column. Elution of the affinity column was performed as described above.

Oxidation of Mutant and Wild-Type KSI. Oxidation was carried out at 22 °C in 20 mM potassium phosphate, pH 7.0, after the removal of all low molecular weight thiols by desalting on a Bio-Rad Econo-Pak column or by dialysis under a nitrogen atmosphere. Typical concentrations of KSI were 50–100 μM of monomer. Sodium periodate or hydrogen peroxide was added to a final concentration of 0.05–30 or 30 mM, respectively. Aliquots were removed at various times for KSI activity measurement. Oxidation was quenched by the addition of thiodiglycol (final concentration, 0.4–0.75 M) and/or by desalting on a Bio-Rad Econo-Pak column.

Large-Scale Preparation of Periodate-Oxidized KSIs. D38C KSI in 20 mM potassium phosphate, pH 7, was treated with 10 mM sodium periodate for 1.5 or 60 min. Oxidations were quenched by adding thiodiglycol (0.74 M final concentration) and desalting on an Econo-Pak column. Under these conditions oxidation for 1.5 min results in maximal activation of D38C, while oxidation for 60 min results in a KSI which is less active than untreated D38C. The product obtained after 1.5 min of oxidation is designated D38Cox, while that obtained after 60 min of oxidation is designated D38Cox+. As controls, wild-type KSI was treated with periodate under the same conditions for 1.5 and 60 min.

Kinetic Methods and Data Analysis. Initial rates for the conversion of 5-AND and 5,10-EST to their Δ^4 isomers were determined at 25 °C from the change in absorbance at 248 nm, the absorption maximum of the Δ^4 products in water. Absorbance changes were monitored with a Gilford 240 spectrophotometer equipped with a Gilford A/D converter (digital absorbance meter, Model 410). Data acquisition and initial rate calculations were performed by using a modified version of the Applesoft BASIC program of Schriefer and Benisek (1984) written for an Apple IIe computer. Most reactions were conducted in 34 mM potassium phosphate, pH 7.0, 2.5 mM EDTA, and 1.7% methanol using varying concentrations of substrate. For wild-type KSI and D38Cox, kinetic constants were also determined in 330 mM potassium phosphate, pH 7.0, and 3.3% methanol. For both substrates, the increase in extinction coefficient due to product formation was assumed to be 1.63×10^4 M^{-1} cm^{-1} (Kawahara et al., 1962). The substrate concentrations used were 1–30 μM 5-AND (for D38C); 6–180 μM 5-AND and 5,10-EST (for D38Cox and wild-type KSI); 9–180 μM 5-AND (for D38S); 1.5–180 μM 5-AND and 9–144 μM 5,10-EST (for D38E); and 3–150 μM 5-AND and 4.5–180 μM 5,10-EST (for D38H). After the apparent background rate in the absence of enzyme was recorded, the reaction was initiated by the addition of 10 μL of the isomerase appropriately diluted with 10 mM EDTA and 1% bovine serum albumin, pH 7.0. Absorbances were recorded for 45 s. The enzyme concentration of stock solutions was determined by quantitative amino acid analysis, after total acid hydrolysis. For each mutant KSI, k_{cat} and k_{cat}/K_m were calculated by curve-fitting using the nonlinear regression data analysis program ENZFITTER (Elsevier-BIOSOFT).

The pH dependence of kinetic constants was determined in 20 mM sodium MES and 80 mM NaCl (pH 3.5–6.5) or 20 mM sodium MOPS and 80 mM NaCl (pH 6.5–8.75). At pH 6.5 rates measured with either buffer were the same, indicating a lack of specific buffer effects under the chosen conditions. In the case of the KSI mutant D38C, kinetic pK_a's could be determined. This was done by fitting the equation

$$k_{cat(obs)} = \frac{(k_{cat(upper)} - k_{cat(lower)})K_{es}}{K_{es} + [H^+]} + k_{cat(lower)}$$

to the data for k_{cat}(obs) and the equation

$$\left(\frac{k_{cat}}{K_m}\right)_{obs} = \frac{\left[\left(\frac{k_{cat}}{K_m}\right)_{upper} - \left(\frac{k_{cat}}{K_m}\right)_{lower}\right]K_e}{K_e + [H^+]} + \left(\frac{k_{cat}}{K_m}\right)_{lower}$$

to the data for k_{cat}/K_m(obs), using ENZFITTER. In these equations K_{es} is the acid dissociation constant for the enzyme–substrate complex and K_e is the acid dissociation constant for the unliganded enzyme.

The stabilities of wild-type KSI, D38C, D38Cox, D38E, and D38H at the extreme pH values were checked by adding the enzyme to the assay buffer, incubating the solution at 25 °C for 1 min, and then initiating the reaction by addition of substrate. The rate obtained was compared with that measured using the standard order of addition, substrate before enzyme.

Alkylation of D38C with Iodoacetic Acid. D38C was alkylated by exposure to 50 mM iodoacetamide (IAN) or 50 mM neutralized iodoacetic acid (IAA) in 200 mM HEPES and 8 M urea, pH 8, at 22 °C in the dark. After 10 min the reaction was quenched by the addition of excess β-mercaptoethanol. The enzyme was then renatured by removing urea and other low molecular weight molecules via gel filtration on an Econo-Pak column (Bio-Rad) preequilibrated with 10 mM potassium phosphate, pH 7, followed by dialysis against the same buffer. Kinetic constants of alkylated KSIs were determined at pH 6, 7, and 8 in 20 mM Na MOPS and 80 mM NaCl as described above under Kinetic Methods and Data Analysis.

Preparation of Proteins for Mass Spectroscopy. All proteins were dialyzed extensively vs 25% ethanol and 5 mM DTT and then concentrated using a Centricon-10 (Amicon) to a final concentration of 200–500 pmol/μL.

Preparation of Tryptic Peptides for Mass Spectroscopy. KSI in 5 mM potassium phosphate, pH 7.0, was heated at 95 °C for 20–25 min, cooled on ice, and made 1% in ammonium bicarbonate and 0.01% in thiodiglycol. TPCK-treated trypsin, 3–4% (w/w), was added, and digestion was allowed to proceed for 4 h at 37 °C with the addition of a second 3–4% (w/w) portion of trypsin after 2 h. Some samples were then treated with 50 mM DTT at room temperature for 4 h. Digestion was stopped by freezing the samples in liquid nitrogen, followed by lyophilization. The lyophilized digest samples were dissolved in 6 M guanidinium chloride, 0.1 M Tris-HCl, 1 mM EDTA, and 2 mM DTT, pH 8.2, and loaded onto a Waters C-18 μBondapack column, 0.39 × 30 cm, which had been preequilibrated with 5% acetonitrile and 0.1% TFA. The column was eluted at 1 mL/min with a linear gradient from 5 to 55% acetonitrile and 0.1%TFA over a period of 55 min. The eluent was monitored at 215 and 280 nm while 1-mL fractions were collected. The fractions containing the peptide consisting of residues 14–45 were identified by amino acid composition and pooled. The peptide solution was concentrated 8-fold on a Savant Speed-Vac, with periodic addition of acetonitrile/0.1% TFA, which was necessary to keep the peptide in solution. The final concentration of peptide was 75–125 pmol/μL.

Electrospray Mass Spectroscopy. Samples of protein or peptides, prepared as described above, were infused into an Ionspray nebulizer (Sciex), and the ions generated were analyzed in quadrupole 1 of a Sciex API III triple-quadrupole mass spectrometer. The quadrupole was scanned from 300 to 2000 Da using a step size of 0.1 or 0.5 Da and a 1.0-ms dwell time per step.

HCl Hydrolysis and Amino Acid Analysis. Samples were hydrolyzed *in vacuo* in 6 N HCl containing 0.1% phenol at 110 °C for 24 h, unless noted otherwise. Both liquid-phase and vapor-phase hydrolysis methods were employed. Amino acid analysis of the hydrolysates was performed on a Beckman Model 6300 amino acid analyzer equipped with a System Gold data system at the UCD Protein Structure Laboratory. Cysteine was determined as alanine-3-sulfonic acid (ASO) in performic acid-oxidized samples (Hirs, 1967). Serine and threonine values were corrected for decomposition losses by the use of 24–72-h hydrolyses and extrapolation of the values obtained to zero time. Valine and isoleucine values were those from 72-h hydrolysates.

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.

Thiol Determination. The thiol contents of D38C, its alkylated derivatives, and D38Cox were measured by the method of Ellman (Ellman, 1959; Means & Feeney, 1971a).

RESULTS

Mutagenesis and Expression of KSIs. The complete mutant genes were sequenced in the expression plasmids pKSI*tac*.D38C, pKSI*tac*.D38S, pKSI*tac*.D38E, and pKSI*tac*.D38H, and only the desired changes directed by the mutating oligonucleotides were found. The proteins were then overexpressed in JM105 and purified to homogeneity as described in Experimental Procedures. One liter of stationary-phase culture was found to yield approximately 100–150 mg of these KSIs, except for D38E, which was expressed at about 50 mg per liter of stationary-phase culture. In each case KSI was the predominant protein constituent of the crude cell extract as determined by SDS–PAGE. When the purified proteins were examined by SDS–PAGE, only one band was observed, even on an overloaded gel. However, when these apparently homogeneous preparations of KSIs were examined by pH 4–6.5 analytical gel isoelectric focusing, each was found to consist of several isoforms. This has been previously observed for wild-type KSI purified from *Comamonas testosteroni* cells (Ogez et al., 1977). In the present study wild-type KSI exhibited a major band at p*I* 5.0, a minor band at p*I* 4.8, and sometimes a very minor band at p*I* 4.75, depending on the particular preparation. The D38C preparation exhibited a major band at p*I* 5.3, with bands of decreasing intensity at p*I* 5.1, 4.9, and 4.85. The D38S preparation was similar to that of D38C, but with relatively less material at p*I* 5.1 and 4.9 and no band visible at p*I* 4.85.

Properties of KSI Mutants. Early in our studies of D38C it was found that exposure of this enzyme to air or chemical oxidants resulted in a substantial increase in its isomerase activity. Thus, in order to study the kinetic properties of this mutant, it was important to remove the small amount of oxidized D38C which was present in the preparations obtained by the standard purification procedure. This was accomplished by preparative isoelectric focusing to remove the oxidized material, which has a lower p*I* than unoxidized D38C. The separation of the p*I* isoforms on the Rotofor was not as clean as one would hope, but an isoelectrically homogeneous preparation could be obtained in poor yield by pooling those fractions containing only the p*I* 5.3 form. This procedure also served to remove the high molecular weight contaminants present after the affinity chromatography step, making the gel filtration step unnecessary. The p*I* 5.3 isomer of D38S was purified in the same manner. The amino acid compositions of D38C, D38S, D38E, and D38H are given in Table 1, verifying that these proteins contained the desired changes.

The pH dependencies of k_{cat} and k_{cat}/K_m for D38C isomerizing 5-AND are shown in Figure 2. The data could be fitted by theoretical curves for titration of a single acidic group having a pK_a' of 8.46 for the free enzyme (pK_e) and 8.27 for the enzyme–substrate complex (pK_{es}). Stability studies showed that the enzyme was stable over the pH range investigated for the time required to make the rate measure-

Table 1: Amino Acid Compositions of KSI and KSI Mutants

amino acid (AA)	AA composition of wild-type KSI from gene sequence	AA compositions of wild-type and mutant KSIs[e]				
		WT	D38C	D38S	D38E	D38H
Asp	12	11.9	11	10.9	10.9	11.2
Thr	7	7.3[a]	7.3[a]	7.2[a]	7.0[a]	7.4[a]
Ser	5	4.9[a]	4.9[a]	5.9[a]	5.0[a]	4.9[a]
Glu	12	11.6	12	12	13.3	12
Pro	5	5	5	5	4.8	4.9
Gly	9	9.1	9	9	9	9
Ala	21	20.8	20.9	20.4	20.0	20.7
Val	14	13.9[b]	13.6[b]	13.1[b]	13.1[b]	13.6[b]
Met	3	1.7[f]	2.9	2.7	2.7	2.0
Ile	4	3.8[b]	3.7[b]	3.6[b]	4.1[b]	3.8[b]
Leu	8	8.2	8.1	8.5	8.4	8.1
Tyr	3	2.9	2.9	3	2.9	2.8
Phe	8	8	7.9	7.8	7.8	7.9
His	3	2.9	3.2	2.8	2.8	3.9
Lys	4	3.9	4	4	4.4	4.1
Arg	7	6.8	6.8	6.7	6.5	6.8
Cys	0	0[c]	0.9[c]	*d*	*d*	*d*
Trp	0	*d*	*d*	*d*	*d*	*d*

[a] Corrected for decomposition during acid hydrolysis as described in Experimental Procedures. [b] From the 72-h hydrolysate. [c] Determined as ASO (cysteic acid) in a sample oxidized with performic acid before hydrolysis. [d] Not determined. [e] Determined from the 24-h hydrolysate, unless noted otherwise. [f] Methionine was low in this sample due to partial oxidation to methionine sulfone.

ments (<1 min). These pH dependencies are quite distinct from that of the wild-type enzyme (Pollack et al., 1986; Weintraub et al., 1970) and are consistent with the thiolate anion of C38 functioning as a catalytic base in place of D38. Table 2 lists the high-pH and low-pH limiting values for k_{cat} and k_{cat}/K_m used to generate the theoretical curves. The kinetic parameters for D38S, D38H, D38E, D38CMC, and D38CAMC at pH 7 are given in Table 2, also. It is seen that the low-pH limiting value of k_{cat} for D38C is similar to that of D38S, but the K_m of D38C is much lower than that of D38S, perhaps a reflection of the greater hydrophobicity of cysteine compared to serine. For comparison we include the kinetic parameters for D38N (Kuliopulos et al., 1989). As previously observed by these workers, replacement of the side chain of D38 with essentially nonbasic neutral side chains results in decreases in k_{cat} by large factors, 2.8×10^5 for D38C (un-ionized thiol group) and 1.6×10^6 for D38S.

Properties of D38CMC and D38CAMC. The D38C mutant was alkylated with iodoacetate (IAA) in order to convert Cys-38 to *S*-(carboxymethyl)cysteine (CMC), an analogue of aspartic acid which retains the carboxylic acid moiety but whose side chain is lengthened by one sulfur and one methylene group. As a control D38C was also alkylated with iodoacetamide (IAN), which converts cysteine to *S*-[(carboxamido)methyl]cysteine (CAMC). Alkylation of D38C with IAN could be accomplished with both native and denatured enzyme, while alkylation with IAA was only successful under denaturing conditions. In control experiments in which D38C, D38H, and WT KSI were denatured in the absence of alkylating reagent, it was found that 95–100% of the original activity could be recovered by removal of urea as described in Experimental Procedures. Treatment of WT and D38H with IAA and IAN under denaturing conditions resulted in no significant change in activity.

D38C treated under denaturing conditions with both alkylating reagents was renatured and characterized kinetically and structurally. The extent of alkylation, as judged by the loss of DTNB-titratable thiol groups, was at least 95%. The inability to activate D38CMC and D38CAMC by exposure

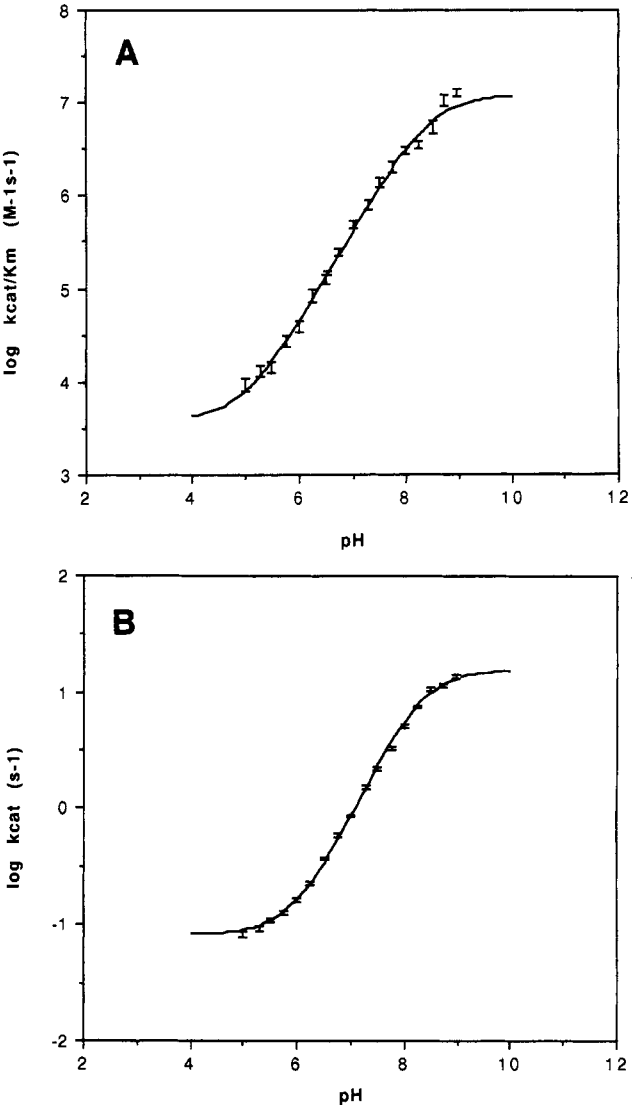

FIGURE 2: pH dependence of D38C. (A) pH dependence of k_{cat}/K_m. The solid curve is that calculated for $pK_e = 8.46$, k_{cat}/K_m(upper) = 1.2×10^7 M^{-1} s^{-1}, and k_{cat}/K_m(lower) = 3.8×10^3 M^{-1} s^{-1}. (B) pH dependence of k_{cat}. The solid curve is that calculated for $pK_{es} = 8.27$, k_{cat}(upper) = 16 s^{-1}, k_{cat}(lower) = 0.082 s^{-1}. Conditions used for the reactions are described in Experimental Procedures.

to sodium periodate (data not shown) provides additional evidence that the thiol has been modified, since exposure of untreated D38C to sodium periodate results in an 150-fold increase in specific activity (see below).

k_{cat} and K_m were determined for D38CMC and D38CAMC at pH 7 (see Table 2). The values of k_{cat} and K_m are virtually the same for both alkylated species. The fact that the K_m values determined for D38CMC and D38CAMC are indistinguishable from the K_m determined for D38ASI suggests that the observed activity is probably mostly due to the presence of contaminating D38ASI. This contamination could have occurred prior to and during alkylation, which is done in the absence of the DTT that is normally used to protect D38C from oxidation. About 0.1% contamination by D38ASI would account for the observed activity in D38CMC and D38CAMC. In any case, D38CMC is no more active than D38CAMC, which demonstrates that the carboxylic group of CMC is not contributing to catalysis. Therefore, the conclusion is made that D38CMC has negligible activity and that CMC is an extremely poor substitute for Asp-38.

Effects of Oxidation of D38C on Activity. Oxidation of D38C resulted in an increase in isomerase activity. Simply

Table 2: Kinetic Constants for KSI and Modified KSIs[a,b]

enzyme	substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
wild type	5-AND	3.0×10^4	123	2.4×10^8
wild type[c]	5-AND	2.3×10^4	106	2.2×10^8
$D38C_{low\ pH}$[d]	5-AND	8.2×10^{-2}	21	3.8×10^3
$D38C_{high\ pH}$[d]	5-AND	1.6×10^1	1.3	1.2×10^7
D38ASI	5-AND	4.4×10^2	42	1.0×10^7
D38ASI[c]	5-AND	3.6×10^2	40	9.0×10^6
D38S	5-AND	1.4×10^{-2}	88	1.6×10^2
D38N[e]	5-AND	1.3×10^{-1}	102	1.3×10^3
D38E[f]	5-AND	1.7×10^2	109	1.6×10^6
D38E	5-AND	1.7×10^2	73	2.4×10^6
D38H	5-AND	3.6×10^2	28	1.3×10^7
D38CMC	5-AND	5.3×10^{-1}	44	1.2×10^4
D38CAMC	5-AND	5.2×10^{-1}	48	1.2×10^4
wild type	5,10-EST	3.9×10^1	40	9.6×10^5
D38ASI	5,10-EST	7.6×10^{-1}	14	5.3×10^4
D38E	5,10-EST	2.1×10^{-1}	42	5.2×10^3
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, unless noted otherwise. [b] Standard errors are 5–10% in k_{cat} and K_m and 10–15% in k_{cat}/K_m. [c] Rate measurement made at 25 °C in 0.33 M potassium phosphate containing 3.3% methanol, pH 7.0. [d] Limiting values at high and low pH used to fit the data of Figure 2. [e] Data from Kuliopulos et al. (1989); 0.05 M Tris-HCl, 3.3% methanol, pH 7.5. [f] Data from Zawrotny et al. (1991); 0.034 M potassium phosphate, 3.3% methanol, pH 7.0.

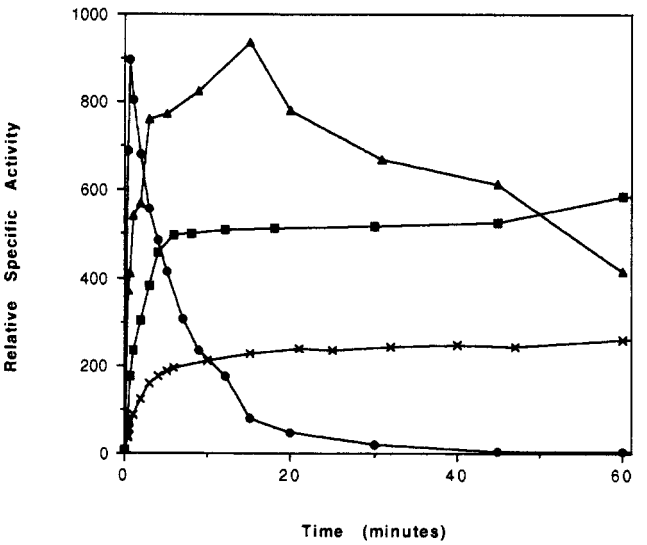

FIGURE 3: Effect of periodate on the specific activity of D38C. Time-dependent activity changes for four concentrations of periodate are shown: 0.1 (×), 0.2 (■), 1 (▲), and 15 mM (●).

exposing D38C to air in the absence of thiols resulted in a gradual increase in activity over the course of days. Treatment with 30 mM hydrogen peroxide at pH 7 resulted in a much more rapid activity increase, which was followed by a decrease in activity with prolonged exposure to this reagent. In contrast, wild-type KSI was not activated by 30 mM hydrogen peroxide, but, rather, was slowly inactivated by this treatment. Maximum activation of D38C was obtained with sodium periodate as the oxidant, and further studies were conducted using this reagent. In order to determine the optimal conditions for activation with periodate, a 0.09 mM solution of D38C in 20 mM potassium phosphate, pH 7.0, was treated with various concentrations of periodate (0.05–30 mM) and the time course for activity change was monitored. Figure 3 provides representative results. A rapid increase in activity was observed, followed by a slower decrease in activity, eventually to a level below that of unoxidized D38C. The rates of both activation and inactivation increased with increasing concentration of periodate. The maximum increase in specific activity obtained in this survey of reaction conditions was approximately 150-fold. At 0.05 and 0.1 mM periodate, the degree of activation was much less and no subsequent inactivation was observed, presumably because all of the periodate had reacted. Importantly, treatment of wild-type KSI or the mutants D38S and D38H with 15 mM periodate for up to 60 min resulted in no significant change in the activities of these enzymes. Thus, it seemed reasonable to conclude that both activation and subsequent inactivation of D38C by periodate involved oxidation of the thiol group. The optimized oxidation conditions (10 mM periodate for 1.5 min) were used to prepare oxidation-activated D38C, D38Cox, for further characterization and study. Upon removal of excess periodate by the addition of excess thiodiglycol and gel filtration the activity of D38Cox was stable for months when the protein was stored in the presence of 5 mM DTT.

Structure of D38Cox. (*a*) *Thiol Content.* Assays of D38C and D38Cox with Ellman's reagent (Means & Feeney, 1971a; Ellman, 1959) showed that the sulfhydryl group content had decreased from 0.85 ± 0.05/polypeptide for D38C to 0.05 ± 0.05/polypeptide for D38Cox, demonstrating that C38 was a site of periodate reaction.

(*b*) *Effects of Periodate Oxidation on the pI.* When D38C was treated with 20 mM periodate for 30 s or 17 min, the p*I* 5.3 species was almost entirely converted into species having p*I*'s of 5.1 and 4.9, with a faint band at p*I* 4.85. In contrast, treatment of D38S with periodate under these conditions resulted in little diminution of the p*I* 5.3 species and only slight formation of bands with p*I* 5.1 and 4.9. These results indicate that oxidation of the cysteine in D38C to give D38Cox produces an anionic oxidation product from the cysteine thiol, presumably the sulfinic acid and/or the sulfonic acid. The sulfenic acid is an unlikely species for the p*I* 5.1 and 4.9 products, since the pK_a's of sulfenic acids are thought to be above 6 (Bruice & Markiw, 1957). Also, a disulfide is not likely to be the oxidation product since the crystal structure of wild-type KSI (E. Westbrook, personal communication; J. Maxwell, W. F. Benisek, and E. Westbrook, unpublished studies) shows that D38 is situated deep in the pit-like steroid binding pocket, thus preventing the close approach of C38's from two KSI polypeptides which would be necessary for disulfide formation. Moreover, formation of a disulfide would not be expected to change the p*I*, since no charge change would have resulted from such a transformation. In addition, it was found that treatment of D38Cox with high concentrations of β-mercaptoethanol or DTT did not reverse the activation by periodate. Since these reducing agents readily reduce sulfenic acids and disulfides to thiols (Allison, 1976; Means & Feeney, 1971b), their lack of effect on the activity of D38Cox further substantiates the view that the high-activity form of D38Cox is neither a sulfenic acid nor a disulfide derivative of D38C.

(*c*) *Electrospray Mass Spectroscopy.* In order to more definitively characterize D38Cox, we turned to electrospray mass spectroscopy to measure the change in mass of the intact KSI polypeptide and of the tryptic peptide comprising residues 14–45 resulting from periodate treatment. In studies on intact KSI polypeptides, the measured masses of wild-type KSI and the D38C mutant were 13 398 and 13 386 Da, respectively, in close agreement with the masses calculated from the amino acid sequences (Choi & Benisek, 1988; Kuliopulos et al., 1987). Periodate oxidation of wild-type KSI and the D38C mutant resulted in mass increases for both proteins. In the case of wild-type KSI approximately 75% of the material showed a

mass increase of 16 Da, indicating the addition of one oxygen atom to the protein. This result agrees with earlier work (Benisek & Ogez, 1982) from this laboratory. In the case of D38C, periodate oxidation resulted in a mixture of polypeptides having masses corresponding to the addition of zero to five oxygens, with the predominant species having a mass of 13 434 Da, corresponding to the addition of three oxygens. After taking into account the incorporation of one oxygen by wild-type KSI, we calculate that 75% of the polypeptides in D38Cox have incorporated two more oxygens than wild type KSI, presumably at the Cys-38 sulfur.

This conclusion was confirmed by mass measurements of peptide 14–45 purified by HPLC from tryptic digests of wild-type KSI and D38C KSI which had been previously subjected to oxidation by 10 mM periodate for 0, 1.5, or 60 min. The mass spectra of the 14–45 peptide from each of the three wild-type samples showed that peptide 14–45 had a mass of 3261.6 Da, identical to the mass predicted by the gene sequence, indicating that this region of the KSI polypeptide contained no periodate-oxidizable residue. As predicted by its gene sequence, peptide 14–45 from unoxidized D38C KSI had a mass of 3249.4 Da. Peptide 14–45 from the 1.5-min-oxidized sample (D38Cox) was a mixture of peptides of masses 3249.4 Da (5%), 3281.6 Da (65%), and 3297.6 Da (30%), corresponding to the addition of zero, two, and three oxygen atoms. Peptide 14–45 from the 60-min-oxidized sample (D38Cox+) consisted almost entirely of the species of mass 3297.6 Da, corresponding to the addition of three oxygen atoms.

We conclude from the isoelectric focusing and mass spectrometric results that brief (i.e., 1.5 min) exposure of D38C to periodate results in an oxidation product which is mainly in the alanine-3-sulfinic acid form at residue 38, D38ASI. Since periodate treatment does not affect the activity of wild-type KSI, we conclude that oxygen incorporation due to periodate at sites other than C38 does not affect the activity of D38Cox. Furthermore, the 60-min oxidation producing D38Cox+ results in a low-activity form of KSI in which residue 38 is alanine-3-sulfonate, D38ASO.

Kinetic Properties of D38ASI. k_{cat} and K_m values for D38ASI were determined as described in Experimental Procedures. Since D38ASI is much more active than either D38C or D38ASO, the activity of a mixture containing these three forms is almost entirely due to its D38ASI content. Thus, the activity of D38ASI can be calculated from the activity of D38Cox by division by 0.75, the fraction of D38Cox which is in the form of D38ASI, as determined by the mass spectrometric measurements on the intact polypeptides. The values obtained are given in Table 2. It is seen that D38ASI is a much better catalyst than D38C, even when the latter is in its high-pH form. As expected, D38ASI is a much poorer isomerase than wild-type enzyme, presumably a reflection of the substantially weaker basicity of the sulfinate anion compared to the carboxylate anion. The pH dependencies of k_{cat} and k_{cat}/K_m for D38ASI were determined over the pH range 3.5–8.8, and these parameters were found to be independent of pH in this interval. This is in contrast to the behavior of the wild-type enzyme, which decreases in these parameters at pH values below 5.5 due to protonation of the D38 carboxyl (Pollack et al., 1986; Weintraub et al., 1970). Since the expected pK_a' of the sulfinic acid group is about 2, the observed pH independence of D38ASI is reasonable. Kinetic constants for D38ASI and wild-type enzyme in the isomerization of the $\Delta^{5,10}$ substrate, 5,10-EST, were determined at pH 7.0, and these values are also included in Table 2. The ratios of the k_{cat} or k_{cat}/K_m values for 5-AND and 5,10-EST are similar for D38ASI and wild-type isomerase.

DISCUSSION

In order to measure the extent of C–H bond breakage in the enolization transition state and provide information on the nature of the transition states leading to and from the intermediate, I, we have used Brønsted base catalysis theory (Maskill, 1985a) to determine the Brønsted coefficient, β, for this proton transfer. In a Brønsted analysis of a base-catalyzed reaction the rate constants for the reaction catalyzed by bases of varying pK_a' and similar structure are correlated with the pK_a's of the bases by the equation $\log k_{obs} = C + \beta pK_a'$. The slope of the plot of $\log k_{obs}$ versus pK_a' gives the β for the reaction. To a rough approximation β measures the extent of proton transfer to the base at the transition state. Values of β larger than 0.5 indicate that extensive proton transfer to the base has occurred at the transition state of the reaction, while values of β which are less than 0.5 indicate that proton transfer at the transition state is not well advanced. Thus, the value of β provides information on the structure of the transition state.

In order to apply Brønsted theory to enzyme-catalyzed reactions, it is necessary to either vary the pK_a' of the substrate or vary the pK_a' of the enzyme catalytic acid or base by means of suitable changes in structures. In the case of substrates which are carbon acids, such as 5-AND, alteration of the structure of the substrate leads not only to change in pK_a' but also to changes in resonance stabilization of the transition state which can lead to anomalous Brønsted coefficients not reflective of the extent of proton transfer at the transition state (Bernasconi, 1987, 1992). Thus, to measure the extent of proton transfer in the transition state for the KSI reaction requires an alteration in the catalytic base/acid. Alteration of the structure of D38 in such a way as to change its pK_a' with no change in steric properties, electric charge, nature of the proton-binding atom, or hydrogen bonding potential has not yet been achieved. Nevertheless, we reasoned that an approximation to this ideal could be achieved by replacing D38 with ASI, an amino acid which preserves the charge, hydrogen-bonding potential, proton-binding atom type, and side-chain volume of aspartic acid while reducing the pK_a' of the side chain by 2.75 log units. However, the shapes of sulfinates and carboxylates are different; the geometry of the carboxylate group is planar, while that of the sulfinate group is pyramidal. In the absence of other factors, the sulfinate oxygens of ASI38 would be located closer to the polypeptide backbone than would be the case for the carboxylate oxygens of D38. Crystal structures of methanesulfinic acid and sulfinate salts show that the S–O bond length is about 0.2 Å longer than the C–O bond length in carboxylates (Seff et al., 1969; Truter, 1962; Wagner et al., 1990; Langs & Hare, 1967) and that the C–S bond length in sulfinates is longer than the C–C bond in aliphatic carboxylates. These geometric differences between carboxylates and sulfinates work in opposite directions, the net result being that the distances from the β carbon to either the carboxylate or the sulfinate oxygens are nearly identical in these two classes of compounds. Manipulation of physical models reveals that if the Cβ atoms of D and ASI are superimposed, an oxygen of the sulfinate moiety can be brought into near congruency with an oxygen of the carboxylate moiety by small (<20°) rotations about the Cα–Cβ bond and the Cβ–S bond of ASI. Thus, the position occupied by the proton-abstracting oxygen of D38 in wild-type KSI in the ES complex can also be occupied by an oxygen

of ASI38 in D38ASI. Moreover, direct experimental evidence that the geometrical differences between carboxylates and sulfinates are not likely to be kinetically important comes from studies of acetone enolization catalyzed by carboxylates and phosphonates (Venimadhavan et al., 1989; Shelly et al., 1990). The Brønsted plots for alkyl carboxylates and alkyl phosphonates have similar values of β, 0.88 for carboxylates and 0.83 for phosphonates. From the data of these workers it can be calculated that, for carboxylates and phosphonates of the same pK_a', the rate constant for carboxylate catalysis is only 1.3-fold larger than that for phosphonate catalysis. As a result, we expect that steric effects are a minor contributor to the lowering of k_{cat} resulting from the carboxylate to sulfinate change.

This conclusion is supported by kinetic studies of the D38E and D38H mutant KSIs, proteins in which the catalytic base is much more severely perturbed sterically than in the case of D38ASI. The values of k_{cat} and k_{cat}/K_m for the D38E and D38H mutants given in Table 2 are relatively high compared to those of D38 mutants lacking a basic moiety, such as D38C at low pH, D38S, and D38N (Kuliopulos et al., 1989). Thus, both E38 and H38 are capable of functioning as fairly good substitutes for the D38 of WT. In D38E the carboxylate carbon of residue 38 is displaced at least 1.5 Å from its location in WT, possibly much more, and the orientation of the carboxylate oxygens has been changed relative to WT. The fact that D38E still retains significant activity implies that the steric requirements regarding the position of the carboxylic group are not stringent.

Even replacement of D38 by histidine results in only modest reductions in the kinetic parameters, although the side-chain volume of His is even larger than that of Glu. However, the δ1-N of His can be positioned so that it is nearly isosteric with the carboxylate oxygens of Asp, as close as ca. 0.15 Å. When one considers the possible close proximity of the His δ1-N to the position occupied by the Asp carboxylate oxygens, it is not surprising to find that H38 is a good substitute for D38 in terms of k_{cat}, somewhat better than E38. Studies of the pH dependence of catalysis by D38H (C. M. Holman and W. F. Benisek, manuscript in preparation) eliminate the possibility that the relatively high values of k_{cat} and k_{cat}/K_m for this mutant are due to a higher pK_a' of the catalytic base. In fact, the kinetic pK_a' of the catalytic base in this mutant is *lower* than that of wild-type or D38E KSI.

The conversion of D38C to D38CMC by alkylation with IAA results in a severe loss of KSI activity. Although the side-chain volume of CMC is about twice that of Asp, it is only about 7% larger than His, so the complete lack of activity in D38CMC probably cannot be explained as resulting simply from the bulk introduced into the active site by the CMC group. More likely it is a result of the 2.9-Å or greater displacement of the carboxylate carbon relative to its position in WT. This large alteration in active site geometry appears to have moved the CMC38 carboxylate completely out of position to act as the catalytic base. The progressive loss of activity seen as the side chain is lengthened in going from Asp to Glu to CMC gives a qualitative picture of the sensitivity of the catalytic ability of the enzyme to the location and orientation of the catalytic base. Since the steric perturbation in going from Asp to ASI is small compared to the perturbation in going from Asp to Glu or His, we expect that the former perturbation has little influence on the kinetic parameters and that the changes observed can be ascribed mainly to the change in pK_a' associated with the replacement of a carboxylate by a sulfinate.

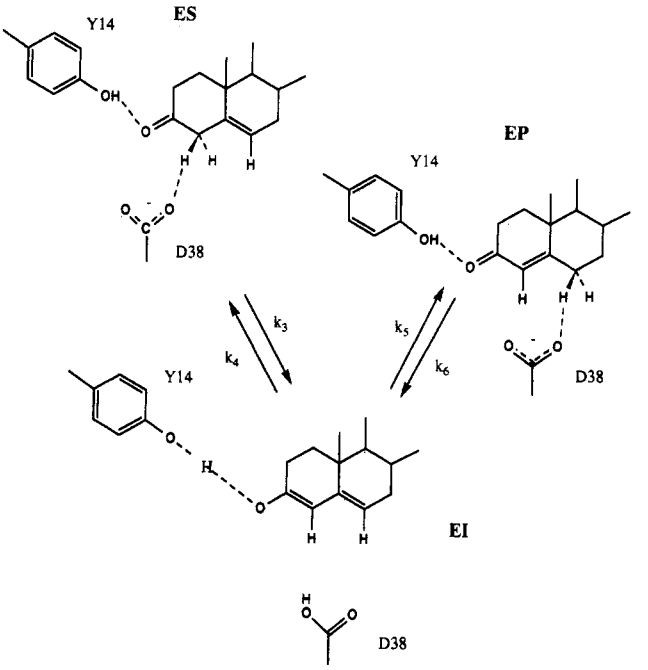

FIGURE 4: Mechanism of Δ^5-3-ketosteroid isomerase. Structures of the enzyme–substrate (ES), enzyme–intermediate (EI), and enzyme–product (EP) complexes are shown. The proton transfers in which D38 participates are shown.

Calculation of the Brønsted Coefficient, β. The kinetic mechanism of the KSI reaction is given by eq 1, in which S and P are the 5,6-enone and 4,5-enone, respectively, and I is the 3,5-dienol(ate) intermediate. The chemical mechanism for conversion of ES to EP is shown in Figure 4.

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

In the present study we attempt to measure β by determining the effect of the pK_a' change of the catalytic base (D → ASI) on the overall k_{cat} for the KSI reaction. It is not appropriate to include the D38H mutant in this analysis since the base of the histidine side chain is not of the same atom type as that of D and ASI and is neutral rather than anionic, as are D and ASI. Moreover, the side-chain volume of H is substantially larger than those of D and ASI.

Since the reaction proceeds via two consecutive chemical steps, enolization and ketonization, it is necessary to consider the consequences of the possibilities that one or both of these steps may be rate limiting. Clearly, as the pK_a' of the catalytic base is decreased, enolization will be slowed while ketonization will be accelerated, since D38 functions as a base in the enolization step and as an acid in the ketonization step, as shown in Figure 4. The effect of the pK_a' change on k_{cat} will depend on which step(s) is (are) rate limiting.

The results of secondary kinetic isotope effect measurements by Mildvan and co-workers have led them to the conclusion that ketonization is *not* rate limiting in the KSI-catalyzed isomerization of 5-AND (Xue et al., 1990); the similarity of the ratios of $k_{cat}^{5\text{-AND}}/k_{cat}^{5,10\text{-EST}}$ for wild-type and D38 mutant isomerases (Table 2) also suggest that ketonization might not be significantly rate limiting. In contrast, Pollack's group (Hawkinson et al., 1991b) has measured the partitioning of externally generated 3,5-dienol by the enzyme to partially determine the reaction profile for isomerization of wild-type KSI. These workers find that enolization, ketonization, and product dissociation are each partially rate limiting. They have argued that Xue et al.'s secondary kinetic isotope effect

experiments are probably not sensitive enough to distinguish between a mechanism in which enolization and ketonization are both partially rate determining and one in which enolization is solely rate limiting (Hawkinson et al., 1991b); however, the results of the kinetic partitioning experiments used by Hawkinson et al. to calculate the individual rate constants for the isomerization have also 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). The fact that replacement of tyrosine 14 by the more acidic electrophile 3-fluorotyrosine results in a *decrease* in k_{cat} of the double mutant KSI Y55, 88F [Brooks & Benisek, 1994 (accompanying paper)] suggests that ketonization is at least partially rate limiting for this high-activity mutant KSI.

If we assume that only enolization is significantly rate limiting, then the observed changes in k_{cat} due to the D38ASI change will faithfully reflect the changes in the rate constant for enolization of 5-AND, k_3. For this case a β value of 0.66 is obtained from eq 2 using the data in Table 2 and assuming

$$\beta = \frac{\log k_{cat}^{WT} - \log k_{cat}^{D38ASI}}{pK_a^{Asp} - pK_a^{ASI}} \quad (2)$$

that the difference in the pK_a's for D and ASI is the same as in model carboxylic and sulfinic acids ($\Delta pK_a' = 2.75$). If product dissociation is also partially rate limiting in the wild type catalyzed isomerization, then the β value for the enolization reaction will be greater than 0.66.

If the conclusion of Hawkinson et al. (1991b) that enolization, ketonization, and product dissociation are each partially rate limiting is correct, then a more complex analysis of our data needs to be made. The theoretical basis for this analysis is presented in the Appendix to the accompanying paper (Brooks & Benisek, 1994). Following the argument presented in the Appendix, it is found that

$$k_{cat}' = \frac{k_3 k_5 k_7}{(k_3 r^{\beta-1} + k_4 r^{\beta} + k_5 r^{\beta}) k_7 + k_3 k_5} \quad (3)$$

In eq 3 r is the ratio of the acid dissociation constants of ASI and D residues, while the rate constants on the right side have the significance given in eq 1. When the rate constants measured by Hawkinson et al. (1991b) are employed and r is taken to be 562 [$\Delta pK_a' = 2.75$ (Oae & Kunieda, 1977; Christensen et al., 1970)], we calculate from eq 3 that $\beta = 0.75$. The values of k_4 and k_5 which were used to obtain this result are the lower limits for these rate constants determined by Hawkinson et al. However, if k_4 and k_5 are increased while their ratio is maintained at 3, the value of β obtained from eq 3 is unchanged.

Thus, regardless of the outcome of the issue of which step is rate limiting, it appears that proton transfer from 5-AND to the catalytic base is well advanced in the enolization transition state of the KSI reaction. This result has implications for the nature of the transition state leading to EI: is it dienol-like or dienolate-like? The answer would depend on whether proton transfer from Y14 occurs in forming the transition state. Evidence that little proton transfer from Y14 occurs in the enolization transition state is presented in the accompanying paper (Brooks & Benisek, 1994).

The present measurement of β for KSI-promoted enolization may be compared with measurements of the Brønsted coefficients of similar reactions in nonenzymatic systems. General base catalysis of the isomerization of 5-AND by tertiary amines has been studied by Perera et al. (1980), who obtained a β of 0.68. Whalen et al. (1976) found that the base-catalyzed isomerizations of 3-cyclohexenone and 3-cyclopentenone by oxygen and nitrogen bases are characterized by β values of 0.5. Venimadhavan et al. (1989) have investigated catalysis of acetone enolization by various carboxylate anions, finding that β for this reaction is 0.89. This group has also investigated acetone enolization catalysis by aryl phosphonate dianions (Shelly et al., 1987) for which $\beta = 0.72$ and by alkyl phosphonate dianions (Shelly et al., 1990) for which $\beta = 0.83$. Thus the value for β which we have obtained for KSI enolization of 5-AND is similar to those of nonenzymatic systems studied so far. Since the nonenzymatic systems generally have $\beta > 0.5$, which is to be expected for endergonic reactions like enolization (Maskill, 1985b; Hammond, 1955), we must entertain the possibility that in the KSI reaction enolization is an endergonic process. At the lower limit value for k_4 (3×10^5 s^{-1}) measured by Hawkinson et al. (1991b), enolization is only slightly endergonic. If this were the true value for k_4, we would have expected a β close to 0.5. The fact that we find β to be substantially larger than this might suggest that k_4 and k_5 are significantly greater than the lower limits of Hawkinson et al., which would require that the energy of EI should be higher than that of ES. On the other hand, one might argue that our data are distorted by a steric effect arising from the somewhat different shapes of the carboxylate and sulfinate groups which decreases the k_{cat} via unfavorable interactions over and above the effect of r on k_{cat}. In such an instance the β due to r alone would be less than the measured value, 0.75, and an isoergonic enolization reaction could be accommodated. However, as discussed earlier, a steric contribution is not expected to be large. If the factor of 1.3 estimated from the comparison of carboxylate and phosphonate is assumed to be operative as a steric factor in D38ASI catalysis, then the effect on β will only be to lower it from 0.75 to 0.71.

The present analysis is based on kinetics measured for only two forms of the enzyme, one or both of which may be "anomalous" for reasons unknown to us at this time. In the field of physical organic chemistry measurements of Brønsted coefficients are based on larger numbers of catalysts in order to minimize the perturbing effects of anomalous members of the data set (Stewart, 1985). Thus, it is of great interest to investigate other derivatives of KSI with altered acid/base properties at residue 38 to see if they do or do not conform to the picture presented now.

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