Positioning of a Spin-Labeled Substrate Analogue into the Structure of Δ^5-3-Ketosteroid Isomerase by Combined Kinetic, Magnetic Resonance, and X-ray Diffraction Methods†

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ABSTRACT: We have shown by kinetic and magnetic resonance measurements that a spin-labeled substrate analogue, spiro[doxyl-2,3′-5′α-androstan]-17′β-ol, binds at the substrate site of crystalline Δ^5-3-ketosteroid isomerase (steroid Δ-isomerase; EC 5.3.3.1) of *Pseudomonas testosteroni*. The spin-labeled steroid is a linear competitive inhibitor with a K_i value (25 ± 5 μM) that is consistent with dissociation constants obtained by direct binding measurements based on changes in (a) the electron paramagnetic resonance spectrum of the nitroxide, (b) longitudinal relaxation rates of water protons, and (c) longitudinal and transverse relaxation rates of carbon-bound protons of the isomerase. These binding studies yield a stoichiometry for the nitroxide of 1 per subunit of the enzyme. Measurements of the longitudinal relaxation rates of water protons indicate that the 3-doxyl portion of the spin-label is highly immobilized yet is exposed to solvent. Paramagnetic effects of the nitroxide on T_1 defined distances to several previously assigned [Benisek, W. F., & Ogez, J. R. (1982) *Biochemistry 21*, 5816–5825] and newly assigned protons of the enzyme. These distances were then used to locate (with an accuracy of ±2 Å) the nitroxide moiety at a unique position in a partially refined 2.5-Å resolution X-ray structure of native isomerase. Three of five additional proton resonance peaks, attributed to ring-shielded methyl groups, could be assigned to specific residues on the basis of distances from the spin-label in the X-ray structure. The remaining portion of the spin-labeled steroid was then docked into the X-ray structure in a hydrophobic cavity of the enzyme. This position of the steroid is consistent with the steroid binding site previously proposed [Westbrook, E. M., Piro, O. E., & Sigler, P. B. (1984) *J. Biol. Chem. 259*, 9096–9103]. However, the rotational orientation of this steroid about its long axis could not be unambiguously established. If we assume that steroid substrates and the spin-labeled inhibitor bind to the same site, but with reversal of the 3- and 17-positions, then the phenolic hydroxyl of Tyr-55 is optimally positioned to function as the general acid that protonates the 3-keto group of the substrate, facilitated by the negative end of the dipole of a 10-residue α-helix, the only helix in the molecule. In this proposed binding mode, the 4- and 6-positions of the steroid substrate are near Asp-38 and Asn-57, suggesting that Asp-38 functions as the general base. Thus, the present results not only explain the reaction mechanism of steroid isomerase but are also consistent with the sites of covalent modification of the enzyme by affinity labels and mechanism-based inhibitors.

Δ^5-3-Ketosteroid isomerase (steroid Δ-isomerase; EC 5.3.3.1) (KSI)[1] catalyzes the isomerization of a variety of Δ^5-3-ketosteroids by a 4–6 cis, diaxial proton transfer via a presumed enolic intermediate (Batzold et al., 1976) (Figure 1). Analysis of the mechanism of this enzymatic isomerization has attracted considerable attention, and much effort has been invested into exploring the nature of the binding and catalytic sites. The enzyme is a small homodimer with a protomeric molecular weight of 13 394 and has two kinetically homogeneous steroid binding sites (Benson et al., 1975; Trivol et al., 1975; Weintraub et al., 1973). Each protomer consists of 125

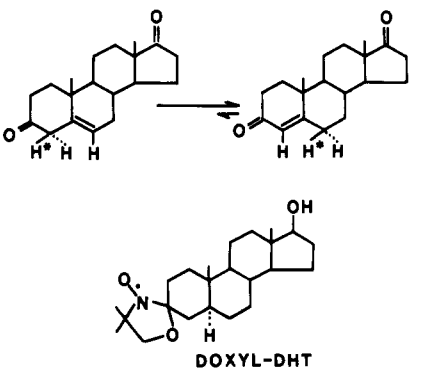

FIGURE 1: Reaction catalyzed by Δ^5-3-ketosteroid isomerase; conversion of 5-androstene-3,17-dione to 4-androstene-3,17-dione (above). Note the intramolecular stereospecific cis, diaxial C-4β-proton (asterisk) transfer to the C-6β-position. Comparison of the structure of the substrate with that of the spin-labeled steroid, spiro[doxyl-2,3′-5′α-androstan]-17′β-ol or doxyl-DHT (below).

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amino acids of known sequence (Benson et al., 1971). Interestingly, cysteine and tryptophan are both absent. The enzyme is highly hydrophobic, with 27% of its residues consisting of valine, leucine, isoleucine, and phenylalanine, an amino acid composition that is consistent with its low solubility of only 2 mg/mL in water and high solubility in ethanol.

Westbrook et al. (1984) have solved the 6-Å structure of KSI and have suggested a tentative location of the heavy atom of bound 17β-estradiol 4-mercurioacetate. The crystals were found to be catalytically active (Westbrook & Sigler, 1984). Recently, substantial progress toward the solution of the 2.5-Å X-ray structure has been made (E. M. Westbrook, P. B. Sigler, and R. Kahn, unpublished results). The α-carbon covalent chain from residue 3 to residue 110 has been interpreted and has been refined to a crystallographic R factor[2] of 38%. Extensive and intimate contact exists between the two subunits of the homodimer. The secondary structure of each monomer was found to have only 2.5 turns of α-helix involving 10 amino acids (residues 8–17) and 4 strands of antiparallel β-pleated sheet involving 29 amino acids.

Mechanistically, it may be anticipated that the enzyme provides a general acid to polarize the carbonyl group of the substrate, a general base to deprotonate the substrate, and hydrophobic residues to bind the substrate. Chemical and spectroscopic evidence suggests that one of the three tyrosine residues of this enzyme may function as the general acid. As detected by UV spectroscopy (Kawahara et al., 1962), one tyrosine residue titrates with a pK_a of 9.5 and the other two become titratable only after denaturation at high pH. Therefore, only one tyrosine residue in the native enzyme is accessible to H_2O. The fluorescence of KSI is typical of tyrosine and is partially quenched upon binding of 19-nortestosterone, a potent competitive inhibitor with a K_i of 7 μM (Wang et al., 1963). Nitration of one tyrosine residue of KSI with tetranitromethane changed its pK_a to 8.3 and resulted in 50% loss of enzymatic activity, suggesting that a protonated form of tyrosine is necessary for activity (Batzold et al., 1976).

In an early attempt to identify the general base participating in the catalytic mechanism, Weintraub et al. (1970) studied the effect of pH on the K_m and V_{max} values of the isomerase. These workers found that a group that titrated with a pK_a of 5.6 was essential for catalysis. This and other properties suggested that either a histidine residue or a carboxylate group was involved. A more recent study of the effect of pH on the rate of the reaction and of affinity labeling with oxiranes yielded pK_a values of 4.6–4.7 for the free enzyme and 4.7–5.5 for the EI and ES complexes (Pollack et al., 1986). Inactivation of the enzyme by diethyl pyrocarbonate and protection against such inactivation by the competitive inhibitor 19-nortestosterone also supported the role of histidine as the general base (Batzold et al., 1976). In addition, isomerase underwent a first-order photoinactivation in the presence of methylene blue with a pH dependence suggestive of histidine destruction. Again, protection from photoinactivation was provided by 19-nortestosterone (Colvin & Talalay, 1968; Batzold et al., 1976). Two other candidates for the general base have been suggested, namely, Asp-38[3] on the basis of specific chemical modification (Ogez et al., 1977) and affinity labeling (Martyr & Benisek, 1973, 1975; Benisek et al., 1980; Pollack et al., 1979, 1986; Kayser et al., 1983; Bevins et al., 1980, 1984) and Asn-57 on the basis of studies with an acetylenic secosteroid suicide inactivator (Penning & Talalay, 1981; Penning et al., 1981, 1982).

A proton NMR study of this enzyme by Benisek and Ogez (1982) assigned several resonances and observed the effects of binding of the competitive inhibitor 17β-estradiol. These workers argued against the role of a histidine in catalysis on the basis of negligible changes in the chemical shifts or of the pK_a* values of the histidines on binding of 17β-estradiol. Small upfield shifts of five aromatic protons of the enzyme were observed presumably due to the interaction with the aromatic A ring of the steroid. It is unclear, however, as the authors point out, whether the data reflect a direct or indirect interaction with aromatic residues of the enzyme.

We report here on magnetic resonance studies of the binding of a paramagnetically labeled steroid (doxyl-DHT; Figure 1) to the enzyme. Kinetics and binding studies using EPR and water proton relaxation rates show that the spin-labeled steroid binds at the active site with its nitroxide group exposed to the solvent. Distances from the bound nitroxide to selected protons of the enzyme, determined by the paramagnetic probe T_1 method (Mildvan & Gupta, 1978), allowed us to position the spin-labeled steroid into the X-ray structure of the enzyme. The location of the spin-labeled steroid overlaps with that of 17β-estradiol 4-mercurioacetate as determined by X-ray analysis at 6-Å resolution (Westbrook et al., 1984). Reports of this work have been published (Kuliopulos et al., 1984, 1987).

Experimental Procedures

Materials

Spiro[doxyl-2,3′-5′α-androstan]-17′β-ol (doxyl-DHT), mp 172–174 °C, was obtained from Aldrich Chemical Co. (Milwaukee, WI). Reagent grade methanol and ethanol were redistilled. Bovine serum albumin was a product of Armour Pharmaceutical Co. (Kankakee, IL). Potassium phosphate was purchased from J. T. Baker Chemical Co. (Philipsburg, NJ). Ultrapure Tris base was obtained from Mann Research Laboratory (Cambridge, MA). Tris-d_{11} base was obtained from Merck Inc. (Rahway, NJ). D_2O, NaOD, and CD_3OD were from Stohler Isotope Chemicals (Waltham, MA). All Tris solutions were treated with Chelex 100 to remove metal ions. Water was doubly distilled from glass and passed over mixed-bed ion-exchange resin. 5-Androstene-3,17-dione was synthesized from dehydroepiandrosterone (Kawahara, 1962). 19-Nortestosterone was a gift of Searle Chemical Co., Chicago, IL, and was purified via crystallization and high-vacuum sublimation. KSI was purified to homogeneity, crystallized as previously described (Kawahara et al., 1962; Talalay & Boyer, 1965; Benson et al., 1974; Jarabak et al., 1969), and stored as a suspension in 50% saturated $(NH_4)_2SO_4$ at 4 °C. The enzyme was found to retain almost full activity for at least 3 years. The specific activity of the preparation used for the

[1] Abbreviations: KSI, Δ^5-3-ketosteroid isomerase (steroid Δ-isomerase; EC 5.3.3.1); doxyl-DHT, spiro[doxyl-2,3′-5′α-androstan]-17′β-ol; pH*, pH measured in ^2H_2O; EPR, electron paramagnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; IU, international units.

[2] $R = \sum|F_{calcd} - F_{obsd}|/\sum F_{obsd}$. A total of 19 195 unique structure factor amplitudes (F_{obsd}) were measured. Refinement has been performed by the method of Hendrickson and Konnert (1980).

[3] Residue 38 is either asparagine as found by protein sequence analysis (Benson et al., 1971) or aspartate as indicated by affinity and photoaffinity labeling, by chemical modification (Martyr & Benisek, 1975; Ogez et al., 1977; Hearne & Benisek, 1985; Kayser et al., 1983; Bevins et al., 1984), and by sequence alignment with the homologous enzyme from *Pseudomonas putida* (Linden & Benisek, 1986). For purposes of discussion we assume residue 38 to be aspartate, which is also in agreement with preliminary information on the sequence of a DNA clone (A. Kuliopulos, unpublished observations).

magnetic resonance studies was 36 000 μmol of 5-androstene-3,17-dione isomerized/(min·mg of protein) when assayed as previously described (Kawahara & Talalay, 1960).

Methods

Kinetic Studies. KSI was assayed at 248 nm and at 25 °C in 3.0-mL systems containing 33 mM potassium phosphate buffer, pH 7.0, 0.37 ng of KSI, and concentrations of the substrate 5-androstene-3,17-dione of 14.5, 28.9, and 57.9 μM. Concentrations of the inhibitor, doxyl-DHT, were 0, 3.4, 8.3, and 11.7 μM. Both substrate and inhibitor were added in methanol, and the final methanol concentration was 1.7% by volume. The enzyme used in the kinetic experiments had a specific activity of 3.21×10^4 IU/mg of protein and was diluted into 1.0% bovine serum albumin, pH 7.0.

Sample Preparation. For the EPR studies and for measurement of the longitudinal relaxation rate ($1/T_1$) of water protons, the isomerase was desalted by passing it over a Sephadex G-25 column at 4 °C, equilibrated with 5 mM Tris-HCl and 100 mM NaCl at pH 7.5, and was concentrated by vacuum dialysis in cellulose acetate bags against 5 mM Tris-HCl, pH 7.0 at 25 °C. The isomerase used in the protein NMR experiments was concentrated with a Millipore immersible CX-10 ultrafiltration apparatus with Tris-HCl buffer replaced by Tris-HCl-d_{11} buffer, pH* 6.8 at 25 °C. Next, the exchangeable protons of the enzyme were deuteriated by lyophilization, redissolving in ^2H_2O, and heating the enzyme to 40 °C for 45 min in a shaker bath (Benisek & Ogez, 1982). This solution was again lyophilized, dissolved in 99.95% ^2H_2O, and centrifuged at 10 000 rpm for 10 min at 4 °C to remove insoluble matter. Final protein concentration was determined at 280 nm, assuming an absorption of 0.336 for a solution containing 1 mg/mL in a 1.0-cm light path cuvette (Benson et al., 1975). Enzyme, spin-label, and 19-nortestosterone were stored as dry powders at −20 °C and dissolved in deuteriated solvent just before use. Small (1–5-μL) aliquots of 5 mM spin-label in 99.5% CD_3OD were added as needed to yield final concentrations of 150–180 μM. Enzyme samples were typically 190–300 μM in subunits with 5–10 mM Tris-HCl-d_{11}, pH* 6.8. Volumes were generally 400–600 μL. The deuteriated enzyme samples were found to retain at least 86% of their original activity after prolonged (48–72-h) NMR experiments.

EPR Spectroscopy. EPR spectra of 40-μL aliquots of doxyl-DHT solutions were measured in quartz capillary tubes fitted with Teflon plugs in a Varian E-4 EPR spectrometer (Mildvan & Engle, 1972). The temperature was regulated at 24 °C by equilibration with N_2 gas. Spectra were centered on 3230 G with a scan range of 100 G, a scan time of 4–8 min, a time constant of 1–2 s, a receiver gain of 10^4, a microwave power of 5 mW, and a microwave frequency of 9.07 GHz. The modulation amplitude was usually sufficient at 2 G unless selective amplification of the broadened peaks of the enzyme-bound spin-label was necessary. In this case, the modulation amplitude was increased to 10 G. The binding of doxyl-DHT to KSI was studied as described by Weidman et al. (1973) by measuring the peak to peak amplitude of the low-field resonance which is proportional to the concentration of free doxyl-DHT. Controls contained equal concentrations of doxyl-DHT in buffer alone. These KSI–doxyl-DHT binding data were analyzed by a Scatchard plot to yield the number of doxyl-DHT binding sites (n) and the dissociation constant K_D of the KSI–doxyl-DHT complex.

$1/T_{1p}$ Measurements and Data Analysis. The paramagnetic contributions to the longitudinal relaxation rates ($1/T_{1p}$) of water protons were obtained at 24.3 MHz (Mildvan & Engle, 1972) by the pulsed method with an NMR Specialties PS 60-W NMR spectrometer. The observed molar relaxivity $(MR)_{obsd}$ is defined as $1/[(T_{1p})[\text{doxyl-DHT}]]$, where $1/T_{1p}$ is the paramagnetic contribution to the longitudinal relaxation rate. $(MR)_{obsd}$ is a weighted average of the molar relaxivity of the free $[(MR)_f]$ and enzyme-bound spin-label $[(MR)_b]$ (Mildvan & Weiner, 1969):

$$(MR)_{obsd} = ([\text{doxyl-DHT}]_b/[\text{doxyl-DHT}]_t)(MR)_b + ([\text{doxyl-DHT}]_f/[\text{doxyl-DHT}]_t)(MR)_f \quad (1)$$

The subscripts f, b, and t refer to the concentrations of free, bound, and total doxyl-DHT, respectively. Hence

$$[\text{doxyl-DHT}]_t = [\text{doxyl-DHT}]_f + [\text{doxyl-DHT}]_b \quad (2)$$

A plot of the $(MR)_{obsd}$ values against the reciprocal of the enzyme concentration was fitted by a calculated curve, based on eq 1–4, to obtain the values of the dissociation constant (K_D), the stoichiometry of the enzyme–doxyl-DHT complex, and the molar relaxivity of the bound spin-label.

$$K_D = [\text{doxyl-DHT}]_f[\text{sites}]_f/[\text{doxyl-DHT}]_b = [\text{doxyl-DHT}]_f([\text{sites}]_t - [\text{doxyl-DHT}]_b)/[\text{doxyl-DHT}]_b \quad (3)$$

$$[\text{sites}]_t = [\text{sites}]_f + [\text{sites}]_b \quad (4)$$

The latter value and the molar relaxivity of the free spin-label measured in the absence of enzyme permitted a calculation of the factor by which the enzyme enhances the paramagnetic effect of the nitroxide radical on the longitudinal proton relaxation rate of water.

$$\epsilon_b = (MR)_b/(MR)_f \quad (5)$$

NMR Studies of the Protein. ^1H NMR studies at 250 MHz and at 24 °C were carried out on a Bruker WM-250 NMR spectrometer with 16-bit A/D conversion and a sweep width of 2800 Hz. This frequency was used to provide easily measurable paramagnetic effects as well as the necessary resolution. Spectra were collected by using 90° pulses and quadrature phase detection. The instrument was calibrated with a sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) reference standard before each experiment. Selective proton irradiation was used to suppress the residual HDO peak. Spectra were obtained with 16K data points, 1024 transients, and an exponential multiplier applied in order to achieve line broadening of 1–1.5 Hz. $1/T_1$ values of protein resonances were obtained via a nonselective saturation–recovery sequence with use of a 90° pulse of 5-μs duration (Markley et al., 1971). Transverse relaxation rates ($1/T_2$) were calculated from the line width at half-height ($\Delta\nu$) by using the relationship $1/T_2 = \pi\Delta\nu$.

Paramagnetic effects of the nitroxide spin-label on the longitudinal relaxation rates of the proton resonances of the enzyme ($1/fT_{1p}$) were used to obtain nitroxide to proton distances by the theory described in detail elsewhere (Mildvan & Gupta, 1978; Mildvan et al., 1980). The normalization factor f is defined as [bound spin-label]/[enzyme subunits]. Correlation times (τ_c) for nitroxide–proton dipolar interaction were determined by two methods: the paramagnetic effect of enzyme-bound spin-label on the $1/T_1$ of H_2O protons at 24.3, 100, and 250 MHz and the T_{1p}/T_{2p} ratio of resolved proton resonances of the enzyme (Mildvan & Gupta, 1978; Mildvan et al., 1980).

Results and Discussion

Kinetic Analysis of the Binding of Doxyl-DHT to KSI. Dixon plots of the initial velocity of enzymatic catalysis, with

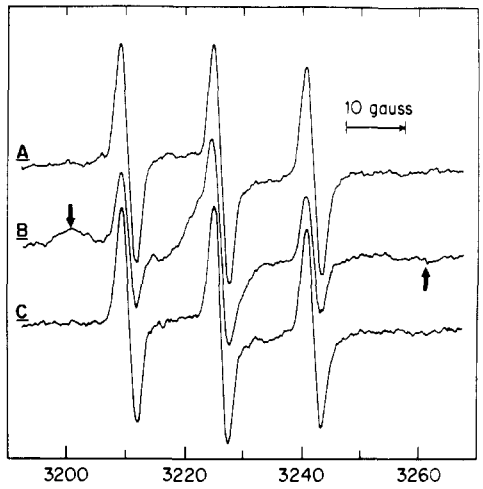

FIGURE 2: Electron paramagnetic resonance spectra of free doxyl-DHT and of the doxyl-DHT–KSI complex and displacement of doxyl-DHT by 19-nortestosterone. The upper curve (A) was obtained with 88.4 μM doxyl-DHT in 1.8% methanol (v/v), 49.1 mM Tris-HCl buffer, pH 7.5, in a final volume of 50.9 μL. Curve B was obtained with 98.0 μM doxyl-DHT, in 2.0% methanol (v/v), 97.25 μM KSI monomer, and 29.4 mM Tris-HCl buffer, pH 7.5. Sample C contained, in addition to all the components of sample B, 167 μM 19-nortestosterone in 1 μL of CH_3OH [final CH_3OH 3.85% (v/v)]. The instrument settings were as follows: scan range 100 G, time constant 1 s, modulation amplitude 2 G, microwave power 5 mW, receiver gain 1×10^4, field setting 3230 G, scan time 4 min, T = 24 °C, and microwave frequency 9.0 GHz. The enzyme used in this experiment had a specific activity of 3.21×10^4 IU/mg of protein. The arrows indicate the broadened and shifted signals of enzyme-bound doxyl-DHT.

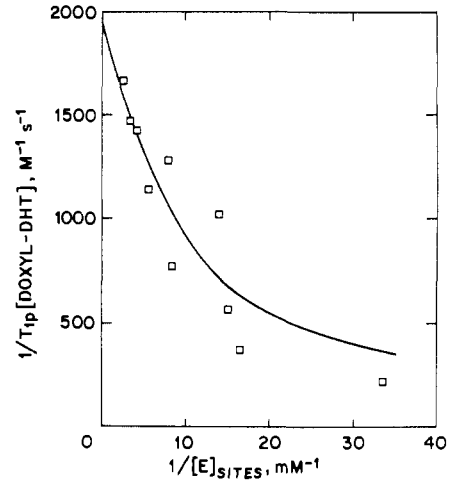

FIGURE 3: Titration measuring the binding of doxyl-DHT to KSI by EPR and $1/T_{1p}$ of water protons. Effect of KSI concentration on the molar relaxivity of doxyl-DHT on water protons. The theoretical titration curve shown was calculated by using n = 1 and K_D = 32 ± 13 μM according to eq 1 and 2 under Methods section. Solutions of 51.0-μL final volume contained doxyl-DHT at 98.0 μM in 2.0% methanol (v/v), an isomerase monomer concentration ranging from 48.0 to 230 μM, and a Tris-HCl buffer concentration ranging from 9.8 to 44.1 mM, pH 7.5, T = 24 °C.

respect to doxyl-DHT concentration, were typical of linear competitive inhibition. The K_i value, obtained from the intersection of the lines, was checked by a replot of the slopes of the Dixon plot with respect to 1/[S], according to the following equation, which defines the slope of the Dixon plot (Segel, 1975):

$$\text{slope} = (K_m/V_{max}K_i)(1/[S]) \quad (6)$$

Consistent with eq 6, the secondary plot gave a straight line through the origin with slope $K_m/V_{max}K_i$. A double-reciprocal plot, together with a linear replot of slope with respect to doxyl-DHT concentration, also indicated linear competitive inhibition and yielded the same K_i value. The limited solubility of the steroid (16 μM at 25 °C) precluded use of higher concentrations of doxyl-DHT. The K_i value determined from these data (25 ± 5 μM) is very similar to that of testosterone (K_i = 26 μM), which is a structural analogue of the spin-labeled compound.

Binding of Doxyl-DHT to KSI As Determined by EPR Spectroscopy. The nitroxide moiety of the unbound doxyl-DHT gave a typical hyperfine splitting pattern for a nitrogen nucleus where I = 1 (Figure 2). Since the nitroxide is moving rapidly and isotropically, the EPR signal consisted of three narrow signals of comparable height. This high degree of mobility is generally seen with small spin-labels that are tumbling rapidly in a nonviscous solution. Upon immobilization of the spin-label, nevertheless, a differential line broadening of the spectrum is observed. This is illustrated in Figure 2B where almost stoichiometric amounts of KSI (93 μM) were added to a solution of doxyl-DHT at 90 μM. Thus, as the correlation time for spin-label motion increased upon binding to KSI, a characteristic change was seen in the nitroxide spectra. The partially immobilized nitroxide yielded broadened resonances both upfield and downfield from the corresponding peaks of the residual unbound nitroxide. The central peak (I = 0) was also substantially broadened. Accordingly, as the free nitroxide was bound, the amplitudes of the three narrow lines of the paramagnetic resonance spectrum due to the free species decreased while the broad spectrum of the enzyme-bound spin-label increased. Addition of 19-nortestosterone, a competitive inhibitor with a K_i value of 7 μM (Wang et al., 1963), as in Figure 2C, completely abolished the line-broadening effect, reestablishing the peak amplitudes of the unbound nitroxide to their original values. Addition of saturating levels of the substrate Δ^5-androstene-3,17-dione (K_m = 320 μM) had a similar effect (not shown). A Scatchard plot based on the EPR data showed much scatter presumably because of the tendency of the unbound doxyl-DHT to aggregate, as also reflected in the variable amplitude ratios of the high- and low-field peaks (Figure 2). A least-squares analysis yielded an estimated binding stoichiometry of 0.94 ± 0.20 site per monomer with a K_D value of 31 ± 14 μM. Because of the large error in determining free doxyl-DHT, an independent study that measures bound doxyl-DHT was carried out.

$1/T_{1p}$ Studies of H_2O in the Presence of the Doxyl-DHT–KSI Complex. The effects of paramagnetic species, such as spin-labels and transition metals bound to macromolecules, on the relaxation rates of water protons can give valuable structural and thermodynamic information. The relaxation rate of the water protons may be enhanced because of the hindrance of free rotation of the spin-label and nearby water molecules by the macromolecule, which increases the correlation time for dipolar interactions between the unpaired electron and water protons. The molar relaxivity of the free doxyl-DHT was found to be 140 M^{-1} s^{-1}, which is about half that found for more hydrophilic nitroxides such as 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl (282 ± 3 M^{-1} s^{-1}) (Mildvan & Weiner, 1969). A plot of the observed molar relaxivity of H_2O protons with respect to reciprocal enzyme concentration (Figure 3) could be fit by assuming 1 binding site per subunit for doxyl-DHT with a K_D = 32 ± 13 μM and a molar relaxivity of 1960 ± 300 M^{-1} s^{-1} for the bound spin-label. The dissociation constants obtained by kinetic measurement, EPR, and $1/T_{1p}$ (Table I) are in reasonable agreement, yielding an average K_D value of 29 ± 11 μM. The

Table I: Comparison of Parameters from Kinetic and Magnetic Resonance Studies of the Doxyl-DHT–Ketosteroid Isomerase Complex

method	K_i or K_D (μM)	stoichiometry (binding sites/subunit)	molar relaxivity (M^{-1} s^{-1})	enhancement factor[a] (ϵ_b)
kinetics	25 ± 5[b]			
EPR	31 ± 14[c]	0.94 ± 0.2		
$1/T_{1p}$ (H_2O)	32 ± 13[c]	1.0 ± 0.2	1960 ± 300	14 ± 8

[a] ϵ_b = (molar relaxivity of bound doxyl-DHT)/(molar relaxivity of free doxyl-DHT). [b] Conditions as described under Methods. [c] Conditions as described in Figure 2.

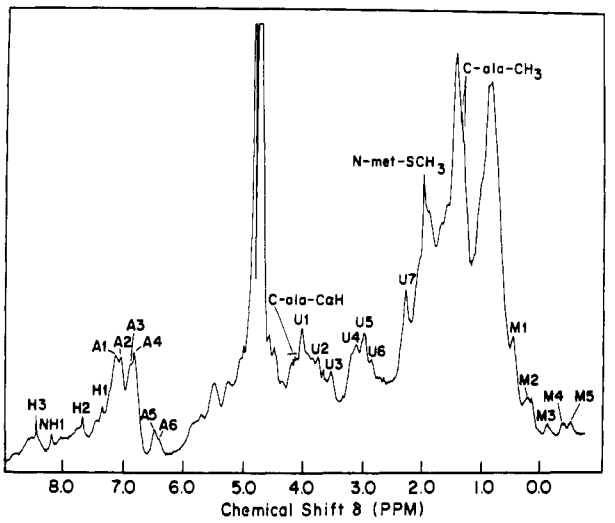

FIGURE 4: Proton NMR spectrum of KSI. The 400-μL sample contained 290 μM KSI monomer in 5 mM Tris-HCl-d_{11}, pH* 6.8. The spectrum was obtained at 250 MHz by using a 90° pulse with quadrature phase detection, 16-bit A/D conversion, 1024 transients with 16K data points, a spectral width of 2800 Hz, an aquisition time of 2.93 s, a relaxation delay of 6.0 s, a continuously applied decoupler pulse 20 dB below 0.2 W centered on the residual HDO peak, and T = 24 °C. A 1.5-Hz line broadening was applied to the spectrum. Chemical shifts are indicated with reference to external DSS. Peaks H3, H2, and H1 were assigned to histidines, and C-Ala-CαH, C-Ala-CH_3, and N-Met-SCH_3 were assigned by Benisek and Ogez (1982). Other labeled resonances were assigned as described in Tables II and III and in the text.

binding of the spin-label to the enzyme enhances its effect on $1/T_{1p}$ of water protons by a factor of 14 ± 8. As in the EPR experiments, 19-nortestosterone was used to displace the paramagnetic spin-label from the enzyme. Thus, the addition of 167 μM 19-nortestosterone to a solution containing 97.3 μM enzyme sites and 98.0 μM spin-label caused a 66 ± 9% reduction in the molar relaxivity presumably by displacement of bound doxyl-DHT. From the dissociation constants of 19-nortestosterone and of doxyl-DHT, a 74 ± 5% decrease was predicted under these conditions. Addition of 761.4 μM substrate (5-androstene-3,17-dione) to a solution containing 95.4 μM enzyme sites and 192.3 μM spin-label caused a 22 ± 7% reduction in the molar relaxivity. Under these conditions a 29 ± 5% decrease was predicted from the K_m, K_D, and K_i values.

Protein NMR Spectra. The proton NMR spectrum of KSI at 250 MHz is shown in Figure 4. Possible assignments of individual resonances were made on the basis of chemical shifts (Wüthrich, 1976). With respect to the aromatic region of the spectrum, Benisek and Ogez (1982) have already assigned the C-2 protons of the three histidines of KSI on the basis of the effect of pH on their chemical shifts. The pK_a values of 7.71, 5.94, and 4.68 were found for the resonances labeled H3, H2, and H1, respectively. In addition, another resonance (NH1) attributed to a very slowly exchanging amide NH is located at 8.18 ppm. Typically, after 48–72 h in D_2O at 24 °C, the intensity of the NH1 peak and of other unresolved amide NH resonances diminished as they became more deuteriated. Most of these NH protons, including resonance NH1, could be removed by heating the enzyme solution in D_2O and lyophilizing more than once.

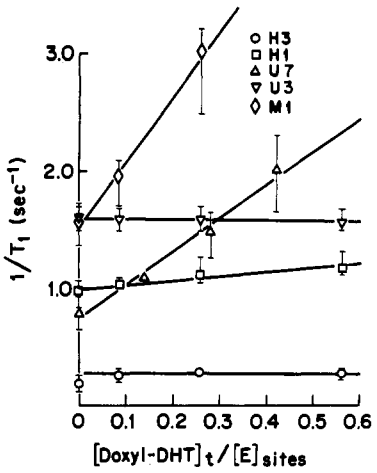

FIGURE 5: Paramagnetic effects of doxyl-DHT on the longitudinal relaxation rates ($1/T_1$) of selected proton resonances of KSI. Peaks are assigned according to Tables II and III. NMR spectra at 250 MHz were obtained on a 400-μL sample containing 290 μM KSI monomer (except for U4 where KSI monomer was 174 μM) in 5 mM Tris-HCl-d_{11} buffer, pH* 6.8, which was titrated with doxyl-DHT in CD_3OD up to a concentration of 157 μM for the spin-label. Final CD_3OD was 3.1% (v/v). The solid line is a least-squares fit of the $1/T_1$ data. The measurements of T_1 by the saturation–recovery method required a variable delay ranging from 0.1 to 6.0 s, as described under Methods. NMR conditions were otherwise as described in Figure 4.

Resonances A1 through A4 are located on top of the more broadened resonances comprising the aromatic envelope and are assigned either to the ring protons of the eight phenylalanines or to the C-2 and C-4 protons of the three tyrosine residues found in KSI, since isomerase contains no tryptophan. Further upfield, between 6.65 and 6.3 ppm, are peaks A5 and A6. At the beginning of the experiment, these two peaks integrated to 6.8 protons, and about 48–72 h later they integrated to 5.8 protons. The slow loss of one proton signal over the course of the experiment was most likely due to the deuteriation of a single amide NH in this region. The remaining aromatic resonances are very likely to arise from the six C-3 and C-5 protons of the three tyrosine residues, which, in most cases, are the most upfield of all aromatic protons (Wüthrich, 1976).

In the aliphatic region, an assigned resonance (Benisek & Ogez, 1982) is a quartet at 4.16 ppm arising from the α-proton of the C-terminal alanine residue of KSI which is coupled to a methyl doublet at 1.35 ppm. The only other assigned resonance, that at 1.99 ppm, arises from the methyl protons of the N-terminal methionine. These assignments and those of other resolvable peaks of KSI are listed in Table II. Some of these assignments are based on the position of doxyl-DHT in the X-ray structure (see below).

Interaction of Doxyl-DHT with KSI. KSI was titrated with doxyl-DHT in order to probe the active site of the enzyme. This was accomplished by measuring the paramagnetic effects on the longitudinal relaxation rates ($1/T_{1p}$) of all the resolved resonances. $1/T_1$ values were determined by the saturation–recovery method and were linear with [doxyl-DHT]. Typical data are shown in Figure 5. The values of the normalized

Table II: Chemical Shifts and Probable Assignments for Selected Proton Resonances of KSI. Paramagnetic Relaxation Rate Effects and Calculated Distances from Enzyme-Bound Doxyl-DHT

designation of resonance peak[a]	chemical shift (ppm)	no. of protons	probable assignment	NMR: av normalized relaxation rates $(1/fT_{1p})_b$ (s^{-1})	NMR: av normalized relaxation rates $(1/fT_{2p})_b$ (s^{-1})	NMR: distance from doxyl-DHT calcd from $(1/fT_{1p})_b$ (Å)	X-ray, distance from doxyl-DHT[d] (Å)
H3	8.44	1	His-6[b] C2H	≤0.28		≥19.5	34.2
NH1	8.18	1	amide NH	0.46		18.0 ± 2.2	
H2	7.67	1	His-122 C2H[b]	0.525		17.9 ± 2.2	
H1	7.34	1	His-100 C2H[b]	0.40		18.4 ± 2.6	20.3
A1	7.125	1	Phe-207,[b] -86, -30 ring protons[c] > Tyr-14, -55, -88 C2H, C4H	0.82		16.3 ± 1.1	
A2	7.05	1		0.78		16.7 ± 1.8	
A3	6.87	1		0.96		16.0 ± 2.5	
A4	6.82	1		1.24		15.3 ± 4.3	
A5	6.47	4	Tyr-14 C3H, C5H	2.42		14.1 ± 2.5	13.6
			Tyr-55 C3H, C5H	2.42		14.1 ± 2.5	14.7
A6	6.38	2	Tyr-88 C3H, C5H	2.29		14.7 ± 3.2	16.8
C-Ala-CαH	4.16	1	Ala-125 CαH[b]	≤1.16		≥15.5	
U1	4.02	2	Gly-105, -107, -21 $C\alpha H_2$ > Ser-85 $C\beta H_2$[b]	1.01		15.8 ± 1.4	
U2	3.76	2	Ser-85 $C\beta H_2$ > Gly-105, -107, -21 $C\alpha H_2$[b]	0.93		16.0 ± 1.4	
U3	3.54	2	Pro-4, -97 δCH_2; Arg-52, -91, -72, -45 δCH_2; His-6 CH_2[b]	0.00		≥20.0	
U4	3.12	2	Phe-103, -101 CH_2 > Arg-197 δCH_2 > Lys-108 ϵCH_2 > Asn-19, -57 βCH_2	3.40	102.2	12.9 ± 1.1	
U5	2.99	2	Lys-92 ϵCH_2 > Asn-2 CH_2 > His-6 CH_2; Arg-52, -91, -72, -45 δCH_2 > Met-1, -6 γCH_2	0.00		≥20.0	
U6	2.86	2	Asn-19, -57, -158 βCH_2 > Lys-108 ϵCH_2; Tyr-14 CH_2 > Phe-103, -101 CH_2 > Arg-72 δCH_2[c]	5.81	88.7	12.25 ± 2.0	
U7	2.29	4–5	Pro-39 $\beta CH_2 + \gamma CH_2$	5.50	94.0	11.19 ± 1.3	11.4; 10.9
N-Met-SCH_3	1.99	3	Met-1 δCH_3[b]	0.325		≥17.1	
C-Ala-CH_3	1.35	3	Ala-125 CH_3[b]	1.26		19.1 ± 4.7	

[a] See Figure 4. [b] Benisek and Ogez (1982) made assignments to residue types but not to their specific positions in the sequence (except for C-terminal alanine and N-terminal methionine). Residues 1–125 and 126–250 refer to the two subunits, respectively. [c] The carboxyl-terminal 16-residue peptide, which has not been clearly identified in the X-ray structure, contains the sequence: Ser^{111}-Met-Arg-Ala-Leu-Phe-Gly-Glu-Lys-Asn-Ile-His^{122}. These residues provide alternative assignments where the chemical shifts are appropriate. [d] Only values for unambiguous assignments are given.

relaxation rates $(1/fT_{1p})_{obsd}$ were calculated from the slopes of linear regression fits to the data (Figure 5) according to

$$(1/T_{1p})_{obsd} = \frac{[\text{doxyl-DHT}]_t}{[\text{subunit}]}(1/fT_{1p})_{obsd} + (1/T_1)_0 \quad (7)$$

The $(1/fT_{1p})_{obsd}$ data were then used to calculate $(1/fT_{1p})_b$, the relaxation rate of the resonance in the presence of the bound spin-label, determined from the dissociation constant of doxyl-DHT and

$$(1/fT_{1p})_b = (1/fT_{1p})_{obsd}\frac{[\text{doxyl-DHT}]_t}{[\text{doxyl-DHT}]_b} \quad (8)$$

The values of $(1/fT_{2p})_b$ were calculated in an analogous manner.

Displacement of Spin-Label from KSI with 19-Nortestosterone. At the end of each titration, doxyl-DHT was displaced from the enzyme by adding 19-nortestosterone. Displacement of paramagnetic doxyl-DHT with diamagnetic 19-nortestosterone resulted in a decrease by 55 ± 13% of the $1/T_{1p}$ values, which agreed well with the calculated reduction of $1/T_{1p}$, 57 ± 4%, based on the affinity of the enzyme for doxyl-DHT and for 19-nortestosterone. Similarly, in a separate experiment, reductions in $1/T_{2p}$ for six different resonances averaged to 63 ± 21%, when the calculated decrease in $1/T_{2p}$ should have been 75 ± 5%.

Calculation of Distances from Nitroxide to Selected Protons of KSI. When $(1/fT_{1p})_b$ of a proton resonance of an enzyme is smaller than the rate of exchange of the spin-label out of the enzyme, and the outer-sphere contribution to $(1/fT_{1p})_b$ is small, then $(1/fT_{1p})_b$ may be used to calculate distances from the nitroxide to the proton (Mildvan et al., 1980; Mildvan & Gupta, 1978). In the present case, fast exchange is established by the observation that the largest value of $(1/fT_{2p})_b$ sets a lower limit to the exchange rate of 102 s^{-1}, which exceeds all of the $(1/fT_{1p})_b$ values by more than 1 order of magnitude (Table II). The absence of residual paramagnetic effects on $1/T_1$ of the protein resonances, after displacement of the spin-label by 19-nortestosterone, indicates negligible outer-sphere contributions to the $(1/fT_{1p})_b$ values. The different $(1/fT_{1p})_b$ values for the various resonances (Figure 5, Table II) argue against a significant effect of spin diffusion on the relaxation rates (Kalk & Berendsen, 1976; Andree, 1978).

The following equation relates nitroxide–proton distances (r) to the relaxation time of the bound nitroxide (Mildvan et al., 1980; Mildvan & Gupta, 1978):

$$r = C\left[qfT_{1p}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right)\right]^{1/6} \quad (9)$$

In eq 9, C is a constant equal to 539 Å $s^{-1/3}$ for nitroxide–proton interactions, q is the binding stoichiometry of nitroxide to KSI, ω_I and ω_S are the nuclear and electron precession frequencies, and τ_c is the correlation time. The value of q was found to be 1 per subunit as discussed above. The τ_c was determined by measuring $(1/T_{1p})_b$ values of water protons in solutions of enzyme (109 and 255 μM sites) and doxyl-DHT (70 μM) at three frequencies. A value of 8.74×10^{-10} s was obtained by a least-squares-fitting computer program that allowed the frequency dependence of τ_c to vary. An independent estimate of τ_c was obtained from the T_{1p}/T_{2p} ratio for two well-resolved resonances of KSI and was found to be 4.73×10^{-10} s. The average of these values $(6.30 \pm 1.26) \times 10^{-10}$ and the $(1/fT_{1p})_b$ data calculated for each resonance were used to calculate distances from the nitroxide to each enzyme resonance as given in Table II.[4] These distances were used

to position the spin-labeled steroid into the X-ray structure of the enzyme.

Active Site Binding of Doxyl-DHT. In order to use the information obtained by NMR studies with doxyl-DHT to locate the substrate binding site of KSI, it is essential that the spin-labeled steroid occupy this site. Evidence obtained by several independent approaches has been presented (Figures 2 and 3, Table I) that doxyl-DHT binds at the active site of KSI. First, a kinetic analysis demonstrated that doxyl-DHT was a linear competitive inhibitor with a K_i value of 25 ± 5 μM. This value agrees closely with the K_i of testosterone, 26 μM, another competitive inhibitor which is structurally similar to doxyl-DHT. Next, EPR and proton relaxation rate (PRR) studies of the interaction of the spin-label with the enzyme yielded K_D values of 31 ± 14 μM and 32 ± 13 μM, respectively. In both of these studies, displacement of the spin-label by the substrate 5-androstene-3,17-dione or by the competitive inhibitor, diamagnetic 19-nortestosterone, was detected by the complete disappearance of the EPR line-broadening effect of the enzyme and, in the case of the $1/T_{1p}$ study of water, by a reduction in the molar relaxivity of enzyme-bound doxyl-DHT. The Scatchard analysis of the EPR data gave a stoichiometry of 0.94 ± 0.2 binding site per monomer, which was confirmed by the $1/T_{1p}$ data since the latter could be fit only by assuming 1 binding site for each subunit. Finally, binding of the spin-label in competition with 19-nortestosterone was shown by the reversal of the paramagnetic effects on the longitudinal and transverse relaxation rates of specific proton resonances of the enzyme.

Environment of Enzyme-Bound Doxyl-DHT. The $1/T_{1p}$ measurements of water protons also yielded information that positioned the enzyme-bound nitroxide with respect to solvent. Specifically, the molar relaxivity of the bound doxyl-DHT was enhanced by a factor of 14 ± 8 over that of the free spin-label, indicating that the nitroxide moiety of the steroid is highly immobilized yet is accessible to water protons (Mildvan & Weiner, 1969). The mode of hydration of the nitroxide of free doxyl-DHT may be determined by eq 9, using the molar relaxivity (140 ± 60 M^{-1} s^{-1}) and the rotational correlation time of the free spin-label estimated from Stokes' law (8.4×10^{-11} s), as consisting of either one hydrogen-bonded water proton at 2.71 Å from the unpaired electron or two hydrogen-bonded water protons at 3.05 Å from the unpaired electron. These distances are reasonable for one or two protons that are hydrogen-bonded to the nitroxide oxygen at a tetrahedral N—O---H angle, making the approximation that the unpaired spin density is entirely on the nitrogen, as suggested by the large isotropic hyperfine coupling of 15.2 G (Figure 2; Hubbell & McConnell, 1971). For hydrogen bond lengths of 2.00 and 2.37 Å to the oxygen, proton to spin distances of 2.72 and 3.05 Å, respectively, are calculated. When doxyl-DHT bound to KSI, the increase in the rotational correlation time (τ_c) closely paralleled the increase in the relaxation rate. This fact leads us to conclude that the solvation of the bound nitroxide proton of doxyl-DHT is very similar to that of the free nitroxide. Thus, the paramagnetic end of the enzyme-bound spin-label is exposed to solvent.

NMR Docking of Doxyl-DHT into the X-ray Structure of KSI. Distances from the nitroxide group to certain protons of the enzyme were used to locate more precisely the steroid binding site. Since the crystals used in the X-ray study were shown to be catalytically active with a diffusion-limited rate (Westbrook & Sigler, 1984), it is reasonable to assume that the crystalline enzyme structure is identical with or very similar to that existing in solution. The relative positions of steroid and enzyme were manipulated by computer graphics methods on an Evans and Sutherland PS 300 display unit. By use of the MOGLI and FRODO programs (Jones, 1982), the steroid was moved relative to the enzyme, while distances from the nitroxide to specific protons were monitored. Because the 15 carboxyl-terminal residues (111–125) have not yet been located in the X-ray structure, the distances of the spin-label group to His-122 and to the carboxyl-terminal alanine residue could not be used in the positioning maneuvers.

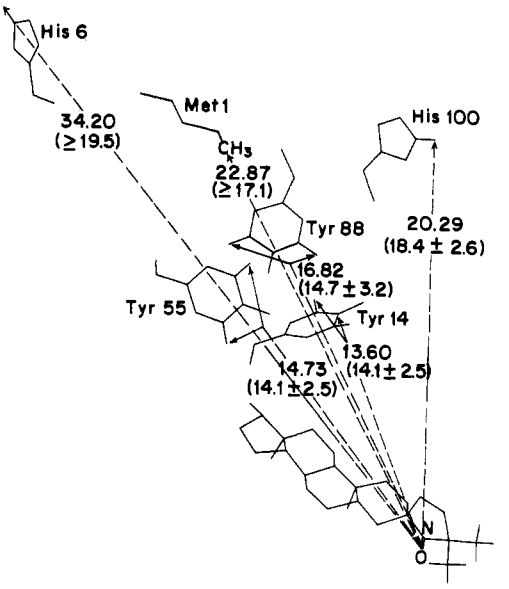

FIGURE 6: Computer graphics representation showing the location of bound doxyl-DHT relative to the protons of assigned resonances of KSI used to position the spin-label in the X-ray structure of the enzyme. Indicated root-mean-sixth average distances in angstroms are those measured by NMR (in parentheses) together with distances derived by positioning the spin-label into the X-ray structure. The errors in the latter distances are ±2 Å.

The nitroxide portion of the steroid was first positioned by using distances to the C-2 imidazole protons of two of the three histidines, the ortho protons of the three tyrosine residues, and the N-terminal methionine CH_3S protons (Table II, Figure 6). Since the measured distances from the nitroxide to the H-2 protons of two of the three histidine residues were almost equivalent (18.4 ± 2.6 and 17.9 ± 2.2 Å), it was unnecessary to obtain an unambiguous assignment of either of them. One of these was assigned to His-100. The histidine H-2 proton, which gave rise to resonance H3, determined to be at least 19.5 Å from the nitroxide group, was assigned to His-6. It was not sterically possible to place both His-6 and His-100 at a position 18 Å from the nitroxide. Similarly, the C-3 and C-5 protons of the three tyrosine residues of KSI were almost equidistant from the nitroxide (Table II). These data could easily be fit by assigning the two nearer tyrosine resonances as Tyr-14 and Tyr-55 and the slightly farther one as Tyr-88 (Figure 6). The nitroxide was placed at least 17.1 Å from the methyl group of the N-terminal methionine. One the basis of these findings, the nitroxide could be positioned within a sphere of 2-Å diameter relative to the X-ray coordinates (Figure 6). As required by the enhanced $1/T_{1p}$ values of water protons, access of the nitroxide group to the solvent was possible through a

[4] Estimates of τ_c, made by Stokes' law, assuming a spherical protein, or from the T_2 of the unpaired electron as measured by the EPR line width, are 1 order of magnitude greater than those used. As discussed elsewhere, these methods of estimating τ_c are less satisfactory than those obtained by direct measurement (Mildvan & Gupta, 1978; Mildvan et al., 1980).

Table III: Probable Assignments of Ring-Shifted Methyl Groups Based on NMR and X-ray Data

designation of resonance peak[a]	no. of protons	probable assignment	NMR: obsd δ (ppm)	NMR: obsd $\Delta\delta$[b] (ppm)	NMR: av $(1/fT_{1p})_b$ (s^{-1})	NMR: distance from nitroxide (Å)	X-ray: calcd $\Delta\delta$[c] (ppm)	X-ray: distance from nitroxide (Å)
M1	6	Val-84 $(CH_3)_2$[d]	0.47	0.43	6.99	11.4 ± 1.4	0.1 ± 0.4	12.3
M2	6	Val-110, -65, -109, -74, -29, -196 $(CH_3)_2$; Leu-23, -192 $(CH_3)_2$[e,f]	0.222	0.68	1.08	15.7 ± 2.6		
M3	3	Val $(CH_3)_2$; Leu $(CH_3)_2$; Ile γCH_3 + δCH_3	−0.12	1.02	0.63	≥15.4		
M4	3	Val-11 γ_2CH_3[g]	−0.39	1.29	0.51	17.7 ± 1.8	0.6 ± 0.7	17.8
M5	3	Val-11 γ_1CH_3[g]	−0.52	1.42	0.70	17.3 ± 2.9	1.4 ± 0.7	18.7

[a] See Figure 4. [b] Difference in chemical shift from random coil value (Wüthrich, 1976). [c] From X-ray structure by use of the Johnson–Bovey ring current equation. [d] The $\gamma_{1,2}CH_3$ groups of Val-84 are near the aromatic rings of Phe-86 and Phe-101. [e] The carboxyl-terminal 15-residue peptide, which has not yet been interpreted in the X-ray structure, contains Leu-115 and Phe-116. These residues could provide alternative assignments if the appropriate relative orientations and distances are satisfied. [f] These are the only residues that contain methyl groups located within the distance constraints imposed by the NMR data. [g] The γ_1CH_3 and γ_2CH_3 groups of Val-11 are near the aromatic rings of Tyr-88 and -55 and Phe-54.

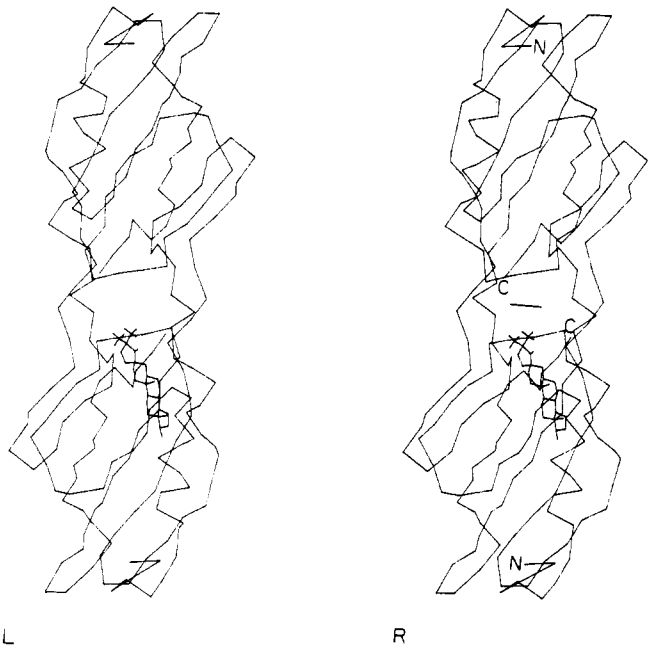

FIGURE 7: Stereo drawing showing the position of doxyl-DHT in one of the two subunits of KSI, viewed along the 2-fold axis of symmetry. The amino (N) and carboxyl (C) termini are identified. The doxyl moiety of the steroid protrudes into the cleft which communicates with the bulk solvent. The 10-residue α-helix is to the right of and below the steroid.

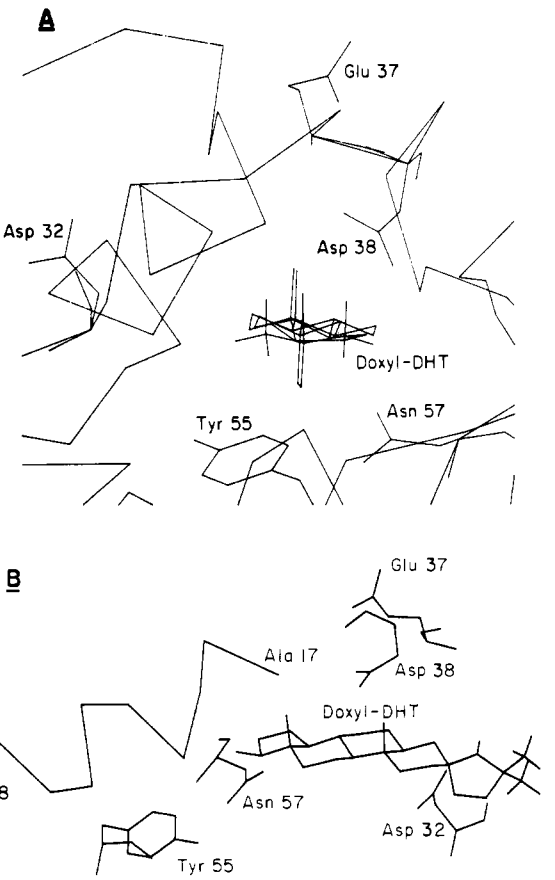

FIGURE 8: Details of the doxyl-DHT binding site on KSI. (A) View along the long axis of doxyl-DHT about which the steroid can be rotated by 180° to provide an alternative fit. (B) View perpendicular to the long axis of the steroid showing neighboring residues and the only α-helix in the molecule.

wide cleft near the internal 2-fold symmetry axis of the enzyme dimer (Figure 7).

The position of the nitroxide group having been established, the position of the remainder of the steroid ring system of doxyl-DHT was obtained by rotating the steroid about the nitroxide, minimizing the van der Waals overlap with the neighboring atoms of KSI. By this approach, a unique location for the spin-labeled steroid was found (Figures 7 and 8), which overlaps that determined for a heavy metal derivative of 17β-estradiol by X-ray analysis at 6-Å resolution (Westbrook et al., 1984). While the position and the longitudinal orientation of the spin-labeled steroid are uniquely determined, an alternative fit to the NMR data is obtained by an approximately 180° rotation of the steroid about its long axis, i.e., by reversing the α and β faces of the steroid with respect to the cleft in the protein (Figure 8).

Assignment of Other Proton Resonances of KSI. The spin-label having been positioned by using distances to protons arising from resonances previously assigned to amino acid types (Benisek & Ogez, 1982), the assignment of other resonances then became possible. The first of the resonances to be addressed were the upfield methyl resonances of the spectrum, namely M1 through M5 (Figure 4 and Table III). These resonances arise from the protons of ring-current-shifted methyl groups, and their distances from the nitroxide group were determined. An additional advantage of these resonances is that they could be independently analyzed in terms of their expected chemical shifts from the protein X-ray coordinates in conjunction with the Johnson–Bovey ring current equations (Johnson & Bovey, 1958) and their variations (Pople, 1956; Haigh & Maillion, 1971; Perkins & Wüthrich, 1979; Perkins & Dwek, 1980). In most cases such analyses can predict chemical shifts from X-ray structures with an error of less than

0.3 ppm, although larger errors as great as 0.6 ppm can occur when uncertainties in atomic coordinates are 0.5–1.0 Å. In the present case, assignments of the five resonances M1 through M5 were based on two considerations: (a) a comparison of calculated and observed chemical shift values and (b) a comparison between the distance to the nitroxide as measured by NMR and as positioned in the X-ray structure of the enzyme (Table III). The resonances M1, M4, and M5 were explicitly assigned to the methyl groups of Val-84 and the γ-1 and the γ-2 methyl groups of Val-11, respectively.

The chemical shifts of resonances U1–U7 are consistent with their origin from methylene protons of the main or side chains of some amino acids. Efforts have been made to correlate the distances of these protons to the spin-label by calculating changes in the relaxation rates of these protons induced by the nitroxide group. In this way, a number of candidate protons consistent with these measurements have been assigned (see Table II).

Active Site Residues of KSI. The combined X-ray and NMR data permit the steroidal portion of doxyl-DHT to be positioned in a hydrophobic cavity of KSI, surrounded by such apolar amino acid residues as Phe-82, Phe-86, and Phe-100, as well as Val-74 from the second subunit. The X-ray and NMR data also allow us to draw some conclusions regarding the candidates for the general acid and general base involved in the catalytic mechanism of KSI. If we assume that the substrate 5-androstene-3,17-dione binds in a manner similar to that of the spin-labeled steroid, with its 3-keto group pointing outward toward the external environment, the difficulty arises that, with the possible exception of Thr-35, no proton donor is positioned nearby to polarize this carbonyl group. Since threonine is an even weaker general acid than water, it is unlikely to function as the general acid involved in the catalytic mechanism. Hence, the opposite orientation of the substrate with its 3-keto group pointing inward and its D ring pointing outward must be considered.

Two modes of steroid binding have been previously invoked to explain (a) the alkylation of Asp-38 of KSI by both 3β- and 17β-oxiranylsteroids (Bevins et al., 1984), (b) the spectral differences between the reversibly and irreversibly bound forms of an aromatic 17β-oxiranylsteroid (Bevins et al., 1986), and (c) the dual activity of 3α,20β-hydroxysteroid dehydrogenase on both 3α- and 20β-hydroxysteroids (Strickler et al., 1980). In this alternative orientation, the A and B rings of the substrate are deeply located in the enzyme in a hydrophobic environment, near several potential catalytic residues (Figure 8). The negative (carboxyl) end of the dipole (Hol et al., 1978; Wada & Nakamura, 1981; Hol, 1985) arising from the sole α-helix in the molecule (Thr-8 to Ala-17) approaches, to within 5 Å, the A ring of the substrate (Figures 7 and 8B). The 3-keto group of the substrate can be oriented toward and closely approach the phenolic hydroxyl group of Tyr-55, which could donate a proton or a hydrogen bond to the 3-carbonyl oxygen of steroid (Figure 8B). Tyr-14, also in the pocket, is slightly farther away, and Tyr-88 is farthest from the 3-keto group of the substrate (Figure 6). The proximity of the A ring of the substrate to two tyrosine residues is consistent with the quenching of 70% of the tyrosine fluorescence of the enzyme by 19-nortestosterone, a potent competitive inhibitor of the enzyme (Wang et al., 1963). Binding of this inhibitor also induces an upfield shift of about five aromatic protons (Ogez & Benisek, 1982). The participation of tyrosine in the catalytic mechanism has been suggested also on the basis of the effect of pH on the kinetic parameters of the isomerase, which reveal a $pK_a \geq 9$ in the enzyme–substrate complex (Wang et al., 1963; Weintraub et al., 1970). Furthermore, nitration of two tyrosine residues results in substantial loss of enzyme activity (Batzold et al., 1976). Similarly, iodination of the enzyme, which removes tyrosine fluorescence, greatly decreases activity, and protection against both effects is afforded by 19-nortestosterone.[5] Model studies show the ability of phenols to function as general acids in the nonenzymatic isomerization of 5-androstene-3,17-dione (Jones & Wigfield, 1969).

With the 3-keto group of the steroid oriented inward and the D ring oriented outward, the only polar amino acid residues that can approach both the 4- and 6-positions of the steroid are Asp-38[3] and Asn-57 (Figure 8). With considerable rearrangements of the conformation of their side chains both Asp-32 and Glu-37 can approach the A and B rings of the bound steroid from opposite faces. At the current state of the crystallographic analysis Asp-38 is, therefore, the best candidate for the general base although the participation of Glu-37 and Asp-32 cannot be ruled out.

The presence of Asp-38 at the steroid binding site was previously suggested by the observation that photoinactivation of the enzyme in the presence of 3-keto-4-estren-17β-yl acetate resulted in the conversion of this residue to alanine. Furthermore, when the enzyme was inactivated with carbodiimide, Asp-38 was converted to an amidate (Ogez et al., 1977; Benisek et al., 1980). Protection was afforded by the competitive inhibitor 19-nortestosterone against both of these inactivation procedures. The functioning of two carboxyl groups is also consistent with the observation that a carboxylate residue between position 14 and 45 is alkylated on the α face by a 3,2′-oxirane and that Asp-38 is alkylated by 17β-oxiranes (Pollack et al., 1986) presumably after protonation of the oxirane by another residue on the opposite face of the steroid. Depending on the detailed rotation about the long axis of the steroid, either Asp-32 or Asn-57 could approach the same region of the steroid from the face opposite to Asp-38 (Figure 8). The proximity of both Asn-57 and the general base to the 4- and 5-positions of the steroid would explain the mechanism-based inactivation of KSI by the acetylenic secosteroid, 5,10-secoestr-5-yne-3,10,17-trione (Penning et al., 1981, 1982; Penning & Talalay, 1981). Thus, isomerization of the secosteroid by the enzyme, with Tyr-55 functioning as the general acid and Asp-38, Glu-37, or Asp-32 functioning as the base, would generate a reactive allene between C-4 and C-6. Because of its location near the electrophilic C-5 of the allene, Asn-57 could be alkylated.

Reaction Mechanism. With the detailed information now available on the environment and residues at the active site of ketosteroid isomerase a qualitative (Figure 9) and quantitative consideration of the reaction mechanism is feasible. The k_{cat} of this enzyme is very high (8.7×10^4 s^{-1}), exceeding the spontaneous rate of steroid isomerization in water at pH 7.0 by a factor of $10^{9.5}$. The additional catalytic effect of the enzyme is due in part to a 6 kcal/mol lowering of the enthalpy barrier of the rate-limiting step (Jones & Wigfield, 1969), namely, the deprotonation of C-4 of the substrate (Malhotra & Ringold, 1965). The entropic barrier ($-T\Delta S^{\ddagger}$) is also lowered by 7 kcal/mol.[6]

The $10^{9.5}$-fold rate acceleration of substrate isomerization by the enzyme may result from the product of several factors.

[5] J. L. Daubek and P. Talalay, unpublished observations (1966).

[6] The suggestion that the enzyme lowers only the enthalpic barrier to catalysis (Jones & Wigfield, 1969) arose from an underestimate of the catalytic enhancement effect of the enzyme, resulting from a comparison of the second-order rate constant for base-catalyzed steroid isomerization with k_{cat}, which is a first-order rate constant.

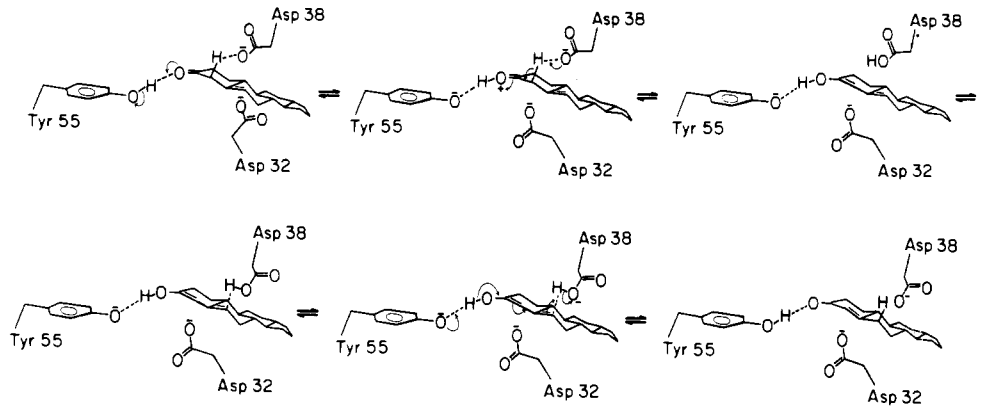

FIGURE 9: Mechanism of KSI consistent with the NMR and X-ray data. As discussed in the text, Asp-38 is better positioned than Glu-37 and Asp-32 to function as the general base, although Glu-37 and Asp-32 are not ruled out. The facilitating effect of the negative end of the α-helix dipole on the protonation of the carbonyl group of the substrate is not shown.

The tighter binding of the transition state (in which ring A approaches a half-chair conformation) than the substrate (in which ring A is a chair; Figure 9) may contribute a factor of approximately 10, based on the greater affinity of the enzyme for steroids planar in the A ring (Batzold et al., 1976; Carrell et al., 1978). The partially hydrophobic environment at the active site would facilitate the loss of charge that occurs in association with the removal of the 4β-H. Following the partial or complete protonation of the 3-keto group by Tyr-55, the carbonium–oxonium ion at C-3 would be stabilized by the negative end of the helix dipole and the carboxylate of Asp-38, Glu-37, or Asp-32 on the α face. As the carboxylate group deprotonates the substrate at C-4 in the rate-limiting step, its charge and that of the carbonium–oxonium ion are lost (Figure 9). A rate enhancement of $\geq$10 due to hydrophobicity is estimated from the effects of increasing methanol concentration on the rate constant for the base-catalyzed isomerization of steroids (Jones & Wigfield, 1968). The stabilization of the carbonium–oxonium ion by the helix dipole and the additional carboxylate groups, while thermodynamically facilitating the protonation of the 3-keto group of the substrate by Tyr-55, would not be expected to contribute greatly to k_{cat} since the protonation step is only partially rate limiting in the KSI reaction (Malhotra & Ringold, 1965).

Interestingly, the negative end of the α-helix (residues 25–36) of hen egg white lysozyme is oriented toward the E and F rings of the bound substrate (Blake et al., 1967). It is noteworthy that the proton-donating residue of lysozyme, Glu-35, located at the negative end of this helix has an unusually high pK_a (Parsons & Raftery, 1972), possibly due in part to the helix dipole. As in the KSI reaction, lysozyme converts a neutral substrate to a cationic intermediate. The rarity of such uses of the negative ends of helix dipoles in catalysis probably results from the fact that most substrates are anions.

The remaining and major factor of $10^{7.5}$ by which KSI accelerates steroid isomerization very likely results from general-acid catalysis by Tyr-55, from general-base catalysis most likely by Asp-38, and from the lowering of the entropic barrier to the reaction by the simultaneous presence of these catalytic groups near the substrate (Swain & Brown, 1952a,b; Jencks, 1975). While the individual contributions to catalysis of each of these effects have not yet been evaluated, they are clearly large ($\geq 10^2$ each) as judged by the profound loss of enzymatic activity resulting from the chemical modification of tyrosine residues (Batzold et al., 1976) and of Asp-38 (Martyr & Benisek, 1973, 1975). A more precise evaluation of these factors may be possible by the selective mutation of these residues.

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