Proton Nuclear Magnetic Resonance Studies of *Pseudomonas testosteroni* 3-Oxo-Δ^5-steroid Isomerase and Its Interaction with 17β-Estradiol†

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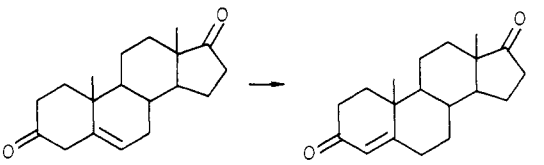
ABSTRACT: 3-Oxo-Δ^5-steroid isomerase (EC 5.3.3.1) and its complex with the competitive inhibitor 17β-estradiol have been studied by ^{1}H NMR at 360 MHz. The histidine C-2 protons of the enzyme give rise to distinct peaks in the spectral region below 7.3 ppm. The pK_a' values for the histidine residues in the presence and absence of 17β-estradiol have been determined by titrations monitored by NMR. In the absence of 17β-estradiol the pK_a' values obtained were 4.68, 5.94, and 7.71, while in the presence of 17β-estradiol the pK_a' values of the histidines were 4.90, 5.83, and 7.61. Binding of 17β-estradiol results in substantial changes in the aromatic region of the spectrum. The resonances of 4.8–7.8 protein aromatic protons undergo an upfield shift as a result of 17β-estradiol binding; however, the structural basis for this perturbation is not yet known. The spectrum of the enzyme contains several resonances which are unusually narrow ($\Delta\nu_{1/2}$ = 1–2 Hz) for a molecule of molecular weight 27 000. Several types of evidence indicate that these resonances arise from groups of the protein rather than from contaminating small molecules. These resonances are not removed by prolonged dialysis or by preparative ultracentrifugation of the enzyme. They are absent from buffer species, water, and deuterium oxide used for preparation of the enzyme for spectroscopic study. The longitudinal relaxation times for these resonances have been measured and are found to be substantially shorter than those for protons on free amino acids determined under the same conditions. In addition, the narrow resonances broaden significantly when the enzyme concentration is increased to a level at which aggregation of the enzyme occurs. Spin decoupling experiments, pH titration studies, and chemical modification of the enzyme with sodium periodate have provided data which suggest that the narrow resonances arise from the methyl groups of N-terminal methionine and its sulfoxides and from the methyl and α hydrogen of the C-terminal alanine. The narrowness of these resonances provides qualitative evidence that these residues have a greater mobility than the rest of the protein globule.

Certain members of the bacterial genus *Pseudomonas* have the ability to utilize steroids as their sole carbon source (Stanier et al., 1966). When presented with steroids in the growth medium, *Pseudomonas testosteroni* (ATCC 11996) synthesizes a number of steroid-metabolizing enzymes, among which is 3-oxo-Δ^5-steroid isomerase (EC 5.3.3.1). This enzyme catalyzes the conversion of 3-oxo-Δ^5-steroids to their Δ^4 isomers, as shown in Scheme I.

Scheme I



Since its discovery by Talalay and his co-workers (Talalay & Wang, 1955), this enzyme has been under continuous study by several laboratories from both structural and functional standpoints. The properties of the enzyme have been reviewed by Batzold et al. (1976), and some more recent chemical modification studies have been summarized by Benisek et al. (1982). The isomerase is a dimer of similar or identical subunits. The subunit molecular weight is 13 400 (Benson et al., 1975; Tivol et al., 1975; Weintraub et al., 1973). The primary structure has been determined by Benson et al. (1971), and the crystal structure is under investigation by Sigler and co-workers (Westbrook, 1976; Westbrook et al., 1976).

The nature of the active site has been explored by various chemical modification studies and by spectroscopic techniques. Histidine may be an important residue of the active site since dye-sensitized photooxidation (Batzold et al., 1976; Colvin & Talalay, 1968) and the histidine-selective reagent ethoxyformic anhydride (Batzold et al., 1976) inactivate the enzyme. Steroids which are competitive inhibitors reduce the rates of these inactivating reactions. Residue 38, reported to be aspartic acid by Ogez et al. (1977) and asparagine by Benson et al. (1971), has been implicated as a critical residue by its photochemical conversion to alanine (Ogez et al., 1977) and by carbodiimide-mediated amidation (Benisek et al., 1980). Both of these modifications inactivate the enzyme, and protection is afforded by steroidal competitive inhibitors. The presence of tyrosine at the active site is suggested by the marked quenching of fluorescence which is produced by the binding of steroids at the catalytic site (Wang et al., 1963). Recently, the site of covalent attachment of the suicide reagent 5,10-secoestr-5-yne-3,10,17-trione has been reported to be on asparagine-57 by Penning & Talalay (1981).

It seemed reasonable that ^{1}H NMR[1] spectroscopy would be a useful tool for learning more about the functional groups present in the active site of isomerase. The carbon-bound protons of the aromatic rings of histidine, tyrosine, and phenylalanine (isomerase has no tryptophan) possess chemical shifts which are well downfield of the highly complex aliphatic proton envelope. Each enzyme subunit contains three histidine, three tyrosine, and eight phenylalanine residues (Benson et

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[1] Abbreviations: NMR, nuclear magnetic resonance; ^{1}H NMR, proton nuclear magnetic resonance; Me_4Si, tetramethylsilane; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate.

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al., 1971). In view of the earlier studies, the environment of one or more of these residues might be perturbed by the binding of steroids. Such perturbations might be reflected by changes in the aromatic and histidine regions of the ^1H NMR spectrum.

The present report describes ^1H NMR studies of the enzyme in D_2O as a function of pD in the presence and absence of the competitive inhibitor 17β-estradiol [1,3,5(10)-estratriene-3,17β-diol]. We find that 17β-estradiol induces large changes in the aromatic region of the spectrum. In contrast, 17β-estradiol binding results in relatively minor changes in the spectral parameters and pK_a' values for the three histidine residues. The isomerase spectrum is found to contain several narrow resonances ($\Delta\nu_{1/2}$ = ca. 2 Hz). Some of these may arise from mobile parts of the enzyme molecule.

Experimental Procedures

3-Oxo-Δ^5-steroid isomerase was purified from progesterone-induced *Pseudomonas testosteroni* following the procedure of Jarabak et al. (1969) as modified by Benson et al. (1974) except that the affinity chromatography step was performed by using a deoxycholate–ethylenediamine–agarose resin. The species of the enzyme having an isoelectric point of 4.75 was prepared from the mixture of isozymes following the procedure of Ogez et al. (1977). All studies were made with this species. The enzyme was prepared for NMR spectroscopy by extensive dialysis against dilute aqueous potassium phosphate buffer, pH 7.0, followed by thorough lyophilization. The residue was twice redissolved in 99.7% D_2O, equilibrated at 40 °C for 1 h, and lyophilized again. It was then taken up in a volume of 100% D_2O such that the final concentrations of phosphate and enzyme subunit were 0.03–0.1 M and ca. 0.2 mM, respectively. Solutions of enzyme at higher concentrations exhibited NMR spectra of noticeably reduced resolution, due to the tendency of the enzyme to aggregate above 0.2 mM (Benson et al., 1975; Tivol et al., 1975).

17β-Estradiol was obtained from Steraloids, Inc.; 99.7% D_2O was obtained from Bio-Rad Laboratories, and 100% D_2O was from Bio-Rad or from Aldrich. Deuteriomethanol (99.5 atom % D) was obtained from Merck Sharp & Dohme, Canada. DCl (20%) in D_2O (99+ atom % D) was a product of Aldrich. Sodium deuteroxide (30%) in D_2O (99+ atom % D) was acquired from Sigma. Tetramethylsilane was purchased from Aldrich. Water was deionized and then distilled in an all-Pyrex apparatus. Sodium metaperiodate was a reagent grade product of Mallinckrodt. L-Methionine, L-threonine, L-alanine, and L-methionine *d,l*-sulfoxide were products of Schwarz/Mann. Dialysis tubing was Spectrapore 3, a product of Spectrum Medical Industries. NMR tubes, 5-mm o.d., Model 528 PP, were obtained from Wilmad Glass Co.

^1H NMR spectra in D_2O solution were obtained at 360 MHz on a Nicolet NT-360 narrow bore spectrometer employing a Nicolet 293′ pulse programmer. In most experiments, 400–2000 transients were collected into 16 384 data points covering a spectral width of 4504 Hz, with the spectrometer operating in the quadrature mode of detection. Suppression of the residual HDO signal was achieved by using a long weak pulse at the observed frequency, according to the procedure of Cutnell et al. (1980). The pulse repetition time was 3.84 s. Prior to Fourier transformation, the free induction decays were apodized with a single exponential function to give a line broadening of 1 Hz, or 2 Hz if the signal to noise ratio was poor. Longitudinal relaxation times, T_1, were determined in argon-purged solutions at 21 °C by using the 180–τ–90 pulse sequence with altenating phases (Cutnell et al., 1976). The relaxation data were fitted by a three-parameter exponential function (Levy & Peat, 1975). Chemical shift values, δ, are reported on a scale set such that internal CHD_2OD has a proton chemical shift of 3.30 ppm and external Me_4Si in CCl_4 has a chemical shift of 0.16 ppm relative to internal DSS at 0.00 ppm.

^1H NMR was used to monitor the titration of the enzyme over the pH range 2.5–10.4 at 21 °C in 0.1 M potassium phosphate buffer containing 2.0% (v/v) tetradeuteriomethanol in the presence and absence of 0.2 mM 17β-estradiol. The concentration of enzyme subunits was 0.2 mM. The pH of each sample was measured with a Corning Model 130 pH meter equipped with a Broadley-James Model 9100-C combination glass electrode standardized against aqueous buffers. The pH was found to vary less than 0.02 pH unit before and after acquisition of the spectrum. The standardization of the pH meter was checked at frequent intervals during the titrations and was found to not require adjustment. The pH values are reported as pH*, the meter readings uncorrected for the deuterium isotope effect. Adjustment of the pH* was made by addition of small amounts of dilute NaOD or DCl by using a microsyringe. The observed chemical shifts of selected resonances at various pH* values were fitted by a nonlinear least-squares program to the Hill equation having as adjustable parameters the pK_a', Hill coefficient, n, the low pH limiting chemical shift, δ_{H^+}, and the high pH limiting chemical shift, δ_{H^0}, unless indicated otherwise.

Sedimentation of the enzyme from dilute potassium phosphate buffer, pH 7.0, was achieved by preparative ultracentrifugation using a Beckman Model L8-70 ultracentrifuge and a Ti 75 rotor at 70 000 rpm for 30 h at 4 °C. Upon completion of the sedimentation run, the supernatant liquid was removed, and the pellet of enzyme was redissolved in aqueous 0.05 M potassium phosphate buffer, pH 7.0. Sixty percent of the initial enzyme activity was recovered in this solution; 36% remained in the supernatant solution.

Periodate oxidation of enzyme methionine was performed in D_2O solution containing 0.05 M sodium deuteriophosphate, pH* 6.8, following the procedure of Yamasaki et al. (1982). A 0.15 mM solution of isomerase was made 0.15 mM in $NaIO_4$ and incubated in an NMR tube at room temperature for 17 h, at which time the ^1H NMR spectrum was obtained. Enzyme activity was not affected by this treatment. For determination of the changes in the amino acid composition of the enzyme produced by periodate treatment, a separate reaction was conducted under the same conditions, but in H_2O solution. A control sample in which the addition of $NaIO_4$ was omitted was prepared, also. After reaction, the excess reagents and buffer species were removed by dialysis against H_2O. Aliquots of the dialyzed protein samples were taken for acid hydrolysis (5.7 N HCl, 110 °C, 24 h, in vacuo) and for alkaline hydrolysis (4.0 N NaOH, 110 °C, 24 h, in vacuo). The analyses of the acid hydrolysates were conducted by using a standard "hydrolysate" elution program on a Durrum D-500 amino acid analyzer. The analyses of the alkaline hydrolysates were conducted by using a modified elution program in which the first part of the elution with pH 3.25 sodium citrate buffer at 53 °C was replaced by an elution with pH 2.90 sodium citrate buffer at 49 °C. This change served to resolve the methionine sulfoxides from aspartic acid.

Partial exchange of the isomerase histidine C-2 protons with solvent deuterium was achieved by incubating a 0.2 mM solution of enzyme in 0.05 M sodium deuteriophosphate, pH* 6.8, containing 0.01% (w/w) NaN_3 for 144 h at 40 °C. The areas of the His C-2 proton peaks from pre- and postincubation

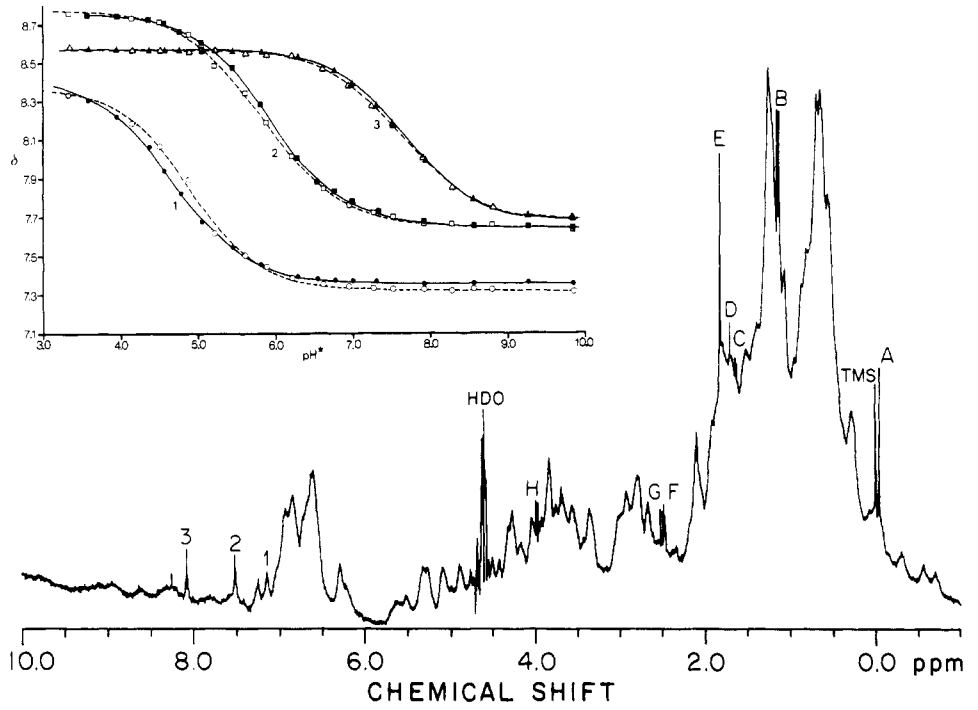


FIGURE 1: ^{1}H NMR spectrum of 3-oxo-Δ^5-steroid isomerase. The sample contained 0.2 mM isomerase subunits in 0.03 M potassium phosphate, pH 7.0. 2100 pulses were accumulated. Narrow resonances, A–H, are indicated in the spectrum. Resonances attributable to histidine C-2 protons are labeled 1–3. Resonances due to external Me_4Si (TMS) and residual HDO are as indicated. Resonance F is actually a closely spaced pair of singlets, F_1 and F_2 (see Results). (Inset) Effect of pH* on the chemical shift of resonances 1–3. The conditions of the titration are described under Experimental Procedures. The reversibility of the titrations was confirmed by back-titration of enzyme samples from pH* 3.3 or 10.0 to neutrality. Samples brought to pH* values below 3.3 or higher than 10.0 precipitated out of solution. (—) Titration in the absence of 17β-estradiol; (---) titration in the presence of 17β-estradiol. Symbols used are (●) resonance 1, (■) resonance 2, and (▲) resonance 3. The data points are the experimental observations while the curves are the computer-calculated nonlinear least-squares fits to the data.

spectra were used to assess the extent of deuterium exchange at these positions.

Results

^{1}H NMR Spectrum. The ^{1}H NMR spectrum of 3-oxo-Δ^5-steroid isomerase between −1 and +10 ppm is shown in Figure 1. The spectrum is characterized by the usual poorly resolved envelope expected for proteins of modest molecular weight resulting from the overlapping broad resonances from hundreds of protons. However, some special spectral features are noteworthy. First, four peaks are observed downfield from the aromatic region. Three of these, labeled 1–3, have chemical shifts at pH* 7.0 relative to internal DSS of 7.35, 7.76, and 8.38 and can be assigned to the three C-2 protons of the three histidines present in each enzyme subunit (see below). Second, some resonances are located in the high field region above 0 ppm. Three distinct peaks are observed in this region, and presumably, they arise from ring-current-shifted methyl protons. Third, several resonances which are unusually narrow for a protein of molecular weight 27 000 are observed in some or all of the various independent samples of enzyme used in these studies. These resonances are designated A–H in the spectrum of Figure 1. Their chemical shifts at pH* 7.0 in parts per million relative to an internal standard of DSS are the following: A, 0.12; B, 1.34; C, 1.83; D, 1.91; E, 2.03; F_1, 2.69; F_2, 2.67; G, 2.71; H, 4.17.

Effect of Long-Term Incubation in D_2O on Resonances 1–3. Comparison of the areas under resonances 1–3 before and after 144 h of incubation in 0.05 M sodium deuteriophosphate buffer showed that peaks 2 and 3 were reduced in area by 75% and 55%, respectively. It was not possible to determine the effects of the incubation on resonance 1 due to a concomitant shift of resonances in the aromatic region into the region around 7.4 ppm, which had the effect of obscuring resonance 1.

Effect of pH on Resonances 1–3.* The chemical shifts of resonances 1–3 were found to depend on pH*. The inset of Figure 1 shows the variation of chemical shifts with pH* in the absence and presence of an amount of 17β-estradiol equimolar with the amount of enzyme subunits. The curves obtained are typical of those observed for histidine C-2 protons in other proteins (Markley, 1975). The chemical shift of the resonance located between 7.4 and 7.5 ppm was altered less than 0.1 ppm over the pH* range of the titration. The titration parameters obtained from the four-parameter fits to the data are summarized in Table I. The three resonances titrate with pK_a values and chemical shifts spanning the range for histidines reported by Markley (1975). The chemical shift difference between the protonated and unprotonated states is 0.9–1.1 ppm, the expected pH* shifts for histidine C-2 protons. We conclude that resonances 1–3 arise from the C-2 protons of the three histidines of each enzyme subunit. The deuterium exchange results (see above) confirm this assignment for resonances 2 and 3.

The effects of the isomerase competitive inhibitor 17β-estradiol (Weintraub et al., 1977) on the histidine titration parameters are not large. The pK_a values of histidines-2 and -3 are shifted down by about 0.1 unit. The limiting values for their chemical shifts are virtually unaffected by the steroid. Somewhat larger perturbations of the His-1 resonance are observed. Its pK_a' is raised by 0.2 unit while both limiting chemical shifts are decreased by approximately 0.04 ppm.

Effect of 17β-Estradiol on the Aromatic Resonances. The presence of 17β-estradiol was found to have a major effect on the appearance of the aromatic resonance envelope. In the presence of an equivalent amount of estradiol, the aromatic peaks at 7.03 and 7.13 ppm were diminished in area whereas the peak at 6.82 ppm was increased in magnitude. The peaks near 6.47 and 6.40 ppm move upfield by approximately 0.05

Table I: Titration Parameters[a] of Resonances 1, 2, 3, B, E, F_1, F_2, and H

resonance	±17β-estradiol	pK_a	n	δ_{H^+}	δ_{H^0}
1	−	4.68 ± 0.02	0.93 ± 0.03	8.406 ± 0.014	7.356 ± 0.003
1	+	4.90 ± 0.03	0.97 ± 0.06	8.363 ± 0.024	7.320 ± 0.007
2	−	5.94 ± 0.01	0.90 ± 0.02	8.755 ± 0.005	7.649 ± 0.004
2	+	5.83 ± 0.02	0.85 ± 0.03	8.773 ± 0.010	7.649 ± 0.005
3	−	7.71 ± 0.02	0.87 ± 0.03	8.569 ± 0.004	7.665 ± 0.01
3	+	7.61 ± 0.02	0.91 ± 0.02	8.564 ± 0.004	7.675 ± 0.006
B	−	3.71 ± 0.07	1[b]	1.406 ± 0.005	1.343[b]
B	+	3.53 ± 0.12	1[b]	1.412 ± 0.008	1.341 ± 0.0005
E	−	7.45 ± 0.02	0.83 ± 0.02	2.094 ± 0.0007	1.926 ± 0.001
E	+	7.34 ± 0.02	0.86 ± 0.03	2.094 ± 0.0009	1.927 ± 0.001
F_1	−	7.00 ± 0.04	0.94 ± 0.08	2.735 ± 0.001	2.643 ± 0.002
F_1	+	6.83 ± 0.02	0.96 ± 0.04	2.736 ± 0.001	2.644 ± 0.001
F_2	−	6.91 ± 0.01	0.88 ± 0.03	2.725 ± 0.0005	2.630 ± 0.0006
F_2	+	6.75 ± 0.03	0.85 ± 0.05	2.725 ± 0.001	2.630 ± 0.001
H	−	3.67 ± 0.07	1[b]	4.313 ± 0.013	4.157 ± 0.0006
H	+	3.82 ± 0.11	1[b]	4.258 ± 0.010	4.152 ± 0.001

[a] The conditions of the titrations are described under Experimental Procedures. [b] For resonance B the Hill coefficient was fixed at 1. A unique fit could not be obtained if the Hill coefficient was permitted to float. Because δ_{H^0} was very constant at pH* values above 6.29, the calculated value of δ_{H^0} was fixed at 1.343, the measured value.

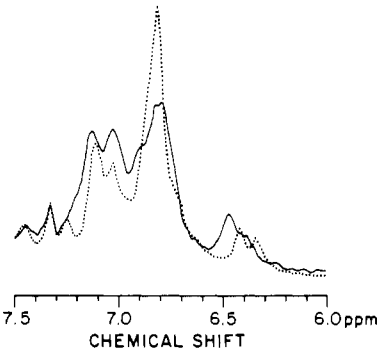


FIGURE 2: Effect of 17β-estradiol on the aromatic ^1H resonances. The spectrum of 0.2 mM steroid isomerase (—) is compared with that of 0.2 mM steroid isomerase plus 0.2 mM 17β-estradiol (···). Both solutions were 0.05 M potassium deuteriophosphate, pH 7.26. The sample containing 17β-estradiol was also 2.0% in CD_3OD. In a separate experiment CD_3OD at this concentration was without effect on the spectrum except for the introduction of a peak at 3.30 ppm due to residual CD_2HOD.

ppm. These changes are shown in Figure 2, in which the spectra of a sample of enzyme before and after the addition of 17β-estradiol are compared. These changes in the aromatic region can be crudely analyzed by the following considerations. Note that an isosbestic point is present at a chemical shift of 6.9 ppm. Downfield from this chemical shift the total area of the aromatic resonances is decreased by 17β-estradiol while upfield from 6.9 ppm the area of the aromatic proton resonances is increased by the presence of 17β-estradiol. Isomerase subunits contain three tyrosine, eight phenylalanine, but no tryptophan residues. Thus, the aromatic resonances between 6.7 and 7.3 ppm represent the contribution of 52 protons in the free enzyme and 55 protons in the enzyme–estradiol complex. Integration of the areas between 7.3 and 6.9 ppm and between 6.9 and 6.7 ppm shows that the low field region from 7.3 to 6.9 ppm was equivalent in area to 30.3 protons in the free enzyme, while in the enzyme–estradiol complex, the corresponding region was equivalent to 25.5 protons. In contrast, the high field aromatic protons between 6.9 and 6.7 ppm increased from 21.6 to 29.3 upon addition of 1 equiv of estradiol. Therefore, estradiol binding results in a loss of 4.8 protons from the 7.3–6.9-ppm region and a gain of 7.7 protons to the 6.9–6.7-ppm region. Since the steroid bears three aromatic protons, itself, these must affect the gain and loss figures.

Narrow Resonances of the Isomerase ^1H NMR Spectrum: Dialysis and Ultracentrifugation Studies. Spectra of various isomerase samples from several separate preparations of the enzyme contained the narrow resonances A–H. Resonances B, E, F, and H (Figure 1) were observed in all enzyme samples at essentially constant peak height relative to the envelope spectrum whereas resonances A, C, D, and G were of variable occurrence and were even absent from some samples, suggesting that the latter group of resonances were due to low molecular weight species in some enzyme solutions whereas the former group, B, E, F, and H, might be due to protons of the isomerase itself. Support for this assignment of these resonances was provided by dialysis and ultracentrifugation experiments.

Dialysis of enzyme samples vs. 0.01–0.1 M potassium phosphate buffer was capable of largely removing the species responsible for resonance D; however, the other narrow peaks remained. The possibility existed, however, that the substance(s) giving rise to the other narrow peaks were low molecular weight species which were inadvertently introduced during dialysis or during subsequent sample preparation steps such as lyophilization and equilibration with D_2O. ^1H NMR "spectra" of D_2O, deuteriophosphate buffer, and blank eluates of the affinity column and Sephadex gel filtration columns used in the later steps of the enzyme purification were devoid of peaks A–H. Peaks A–H could not have arisen from ampholytes used in the isoelectric focusing since a sample of enzyme which had not been subject to isoelectric focusing had a spectrum which was very similar to that of Figure 1.

Evidence for contaminants as the source of peaks A, C, and G was provided by a carefully controlled dialysis experiment in which a sample of isomerase in 0.05 M potassium phosphate buffer containing 10% ethanol and a sample of the buffer only were dialyzed in the same container vs. five changes of dilute potassium phosphate buffer. The retenates from each bag were lyophilized simultaneously on the same lyophilizer and the residues equilibrated with D_2O by the usual procedure described under Experimental Procedures. The ^1H NMR spectra of each sample was obtained by using identical spectrometer parameters. The enzyme sample contained all narrow resonances except C and D. The blank sample contained peak G in an amount very similar to the amount of peak G in the enzyme sample. The blank spectrum also showed a peak at the chemical shift of resonance A, but its height was only about 25% of the height of the A resonance in the enzyme sample.

Table II: Longitudinal Relaxation Times of Isomerase and Amino Acid Protons[a]

resonance	T_1 (s)	resonance	T_1 (s)
aliphatic envelope	0.6[b]	G	2.3
aromatic envelope	0.9[b]	H	1.5
His 2	1.7	Thr CH_3	1.16
His 3	1.7	Thr α-H	5.7
A	3.0	Met CH_3	3.5
B	0.55	Met α-H	5.0
D	5.8	Ala CH_3	2.1
E	1.35	Ala α-H	7.7
F	0.7		

[a] Sample preparation and the method of T_1 estimation are described under Experimental Procedures. The enzyme sample used did not contain resonance C in its spectrum. [b] Estimated by the "null method" (Martin et al., 1980).

Peaks B, E, F, and H as well as the broad protein resonances were absent from the "blank" spectrum.

The question of the origin of the narrow resonances was also addressed by an ultracentrifugation experiment. As described under Experimental Procedures, the enzyme was pelleted by centrifugation, and the pellet was redissolved in buffer and exchanged into D_2O by equilibration and lyophilization. The spectrum of the pelleted and redissolved enzyme exhibited greatly reduced amounts of peak D, and an absence of peak G, while the other narrow resonances, A, B, C, E, F, and H, were clearly observed. The supernatant solution from the ultracentrifuge run contained about one-third of the total amount of enzyme. It was lyophilized and exchanged into D_2O. The spectrum of this supernatant fraction exhibited enhanced amounts of peaks A, C, D, and G relative to the broad protein resonance envelope.

T_1 of Narrow Resonances. T_1 values for protons of low molecular weight species are longer than T_1 values for protons of similar functional groups in macromolecules (Wuthrich, 1976). The T_1 values for the narrow resonances as well as those of selected "authentic" protein resonances were measured as described under Experimental Procedures in order to obtain a completely independent approach to the question of the origin of the narrow resonances. In addition, T_1 values for certain protons of the amino acids alanine, threonine, and methionine were measured under the same conditions as were those of the enzyme solution. The measured T_1 values are summarized in Table II. Resonances A, D, and G all have much longer relaxation times than the composite relaxation time exhibited by the protein aliphatic envelope. Resonances B and F, on the other hand, possessed relaxation times which were very similar to that of the aliphatic envelope. Resonances E and H represented an intermediate case. For the purpose of comparison, the T_1 values for protons of threonine, methionine, and alanine were measured under the same conditions as pertained to the protein T_1 measurements. With the exception of the methyl protons of threonine, all of the free amino acid T_1 values are much longer than those of resonances B, E, F, and H and are similar in magnitude to those of resonances A, D, and G. In summary, the T_1 values of resonances B, E, F, and H are "proteinlike" whereas those of resonances A, D, and G are "free amino acid like".

Effect of Isomerase Concentration on Resonance Line Widths. Steroid isomerase undergoes aggregation to a high molecular weight form at concentrations above 0.22 mM (3 mg/mL) (Benson et al., 1975; Tivol et al., 1975). Therefore, resonances which arise from isomerase protons should increase in line width, whereas the line widths of resonances from small molecule contaminants should be unaffected by a modest increase in isomerase concentration into the aggregation range.

Table III: Effect of Isomerase Concentration on Line Widths of Resonances A–H

resonance	line width[a] (Hz) 0.2 mM	0.6 mM	change (%)
A	0.6	0.5	−17
B	1.6	2.2	+38
C	0.8	*b*	*b*
D	0.5	<0.5	<0
E	1.8	2.6	+44
F	1.1	1.4	+27
G	0.4	*c*	*c*
H	0.9	1.3	+44
2	4.8	8.1	+69
3	2.5	5.3	+120

[a] Line widths at peak half-height were determined by fitting theoretical Lorentzian peaks to the resonances of 1-Hz line-broadened spectra. The measured line widths were corrected for the effects of line broadening by subtraction of 1 Hz. [b] Resonance C was absent from this sample's spectrum. [c] Peak G was partially obscured by peak F_1.

The line widths of the narrow resonances of a 0.20 mM isomerase sample and a 0.59 mM isomerase sample were compared in an independent attempt to distinguish isomerase resonances from nonisomerase resonances. The line-width measurements are summarized in Table III. The expected line-broadening effect was observed for the C-2 proton resonances of His-2 and His-3 and also for resonances B, E, F, and H but not for resonances A and D. Unfortunately, the line width of resonance G could not be measured at the higher enzyme concentration due to the broadening of the nearby F resonances. This experiment has provided further evidence that resonances A and D do not arise from protons of the isomerase and that resonances B, E, F, and H are due to functional groups of the isomerase.

Effect of pH on Resonances B, E, F_1, F_2, and H.* The chemical shifts of resonances B, E, F_1, F_2, and H were found to vary significantly with pH*, though to a much smaller extent than those of the histidine C-2 protons. The chemical shifts were readily fitted by the simple titration curves which are illustrated in Figure 3. From these curves, values for pK_a, n (Hill coefficient), δ_{H^+}, and δ_{H^0} were derived. These titration parameters are presented in Table I. Inclusion of 1 equiv of 17β-estradiol in the titration solution resulted in no more than slight changes in the estimated values of the titration constants. In the absence of 17β-estradiol resonance B titrated with an apparent pK_a of 3.7; however, only about 60% of the theoretical titration curve was covered by data, due to denaturation of the enzyme by low pH. Thus, the accuracy of this pK_a is not great, and the arbitrary assignment of the pH*-dependent chemical shift changes of resonances B to the titration of a single group with a Hill coefficient of 1 is open to question. Similar reservations regarding the titration of resonance H must be held. On the other hand, resonances E and F could be titrated over their complete range of pH* dependency, resulting in data which could be fitted very well by simple titration curves. Resonance E titrated with a pK_a of 7.45 and a total change in chemical shift of 0.168 ppm. Resonance F, a closely spaced pair of resonances, F_1 and F_2, titrated with pK_as of 7.00 and 6.91 and total changes in chemical shift of 0.092 and 0.095, respectively.

Effect of Periodate Treatment on Resonances E and F and on Amino Acid Composition. The value of 2.03 for the chemical shift of resonance E at neutral pH and its singlet spin–spin splitting character suggested that resonance E might be due to the protons of a methionine methyl group. The thioether sulfur of methionine residues is easily oxidized to

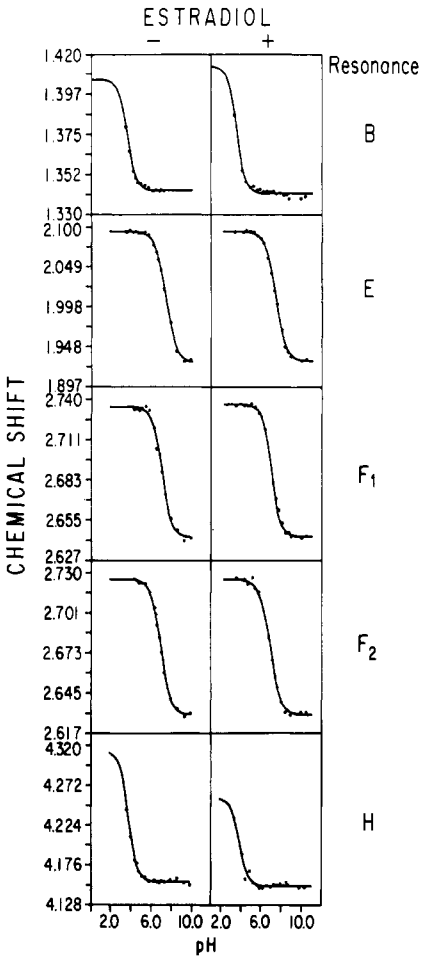


FIGURE 3: Effect of pH* on the chemical shifts of resonances B, E, F_1, F_2, and H. The conditions of the titrations are the same as those of the histidine titrations of Figure 1. (−) Titration in the absence of 17β-estradiol; (+) titration in the presence of 17β-estradiol. The data points (●) are the experimental observations while the curves are the computer-calculated nonlinear least-squares fits to the data.

a mixture of the (*R*)- and (*S*)-sulfoxides by a variety of oxidizing agents, including periodate (Yamasaki et al., 1982). In the free amino acids, this modification results in a substantial downfield shift in the chemical shift of the *S*-methyl protons from 1.915 to 2.533 ppm. The line width of resonance E was only 1.8 Hz, a value suggesting that the group responsible for this resonance had a greater rotational mobility than is typical of protons of globular proteins. Crystallographic studies of many globular proteins have demonstrated that surface residues are usually more highly disordered than internal residues. Consequently, the group responsible for resonance E might be susceptible to chemical modification under nondenaturing conditions by the polar species, periodate. When isomerase was treated with 1 equiv of periodate under the conditions described under Experimental Procedures the ^{1}H NMR spectrum was altered significantly. Figure 4 shows a portion of the spectrum of periodate-treated isomerase. Comparison of this spectrum with that of native isomerase (Figure 1) shows that resonance E is eliminated by periodate treatment while resonances F_1 and F_2 are greatly augmented. Other changes in the NMR spectrum resulting from the treatment with periodate were not apparent. Amino acid analysis of alkaline hydrolysates, the periodate-treated enzyme, and a periodate-free control showed that the control enzyme contained trace amounts (<0.1 residue) of methionine sulfoxides while the treated sample contained 0.85 residue of methionine sulfoxides per enzyme subunit. Periodate treatment resulted in a decrease in methionine content from 2.71 to 1.88 residues per enzyme subunit.

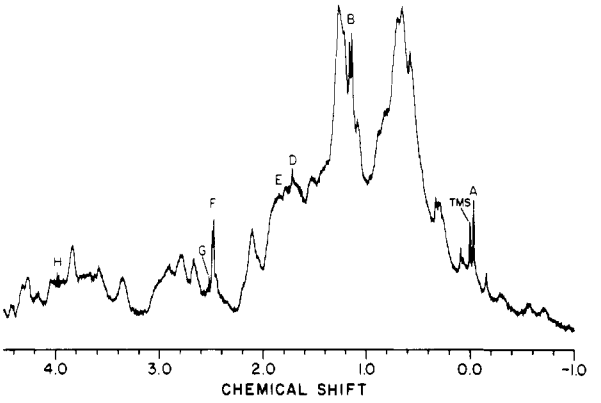


FIGURE 4: ^{1}H NMR spectrum of periodate-treated isomerase. The conditions of periodate treatment are described under Experimental Procedures. The enzyme concentration was 0.15 mM. 400 transients were collected for the spectrum.

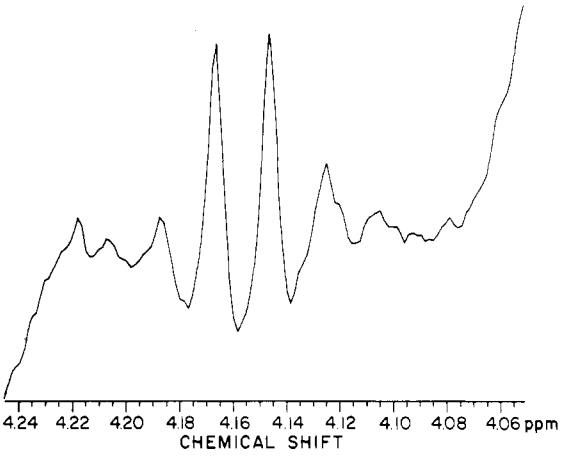


FIGURE 5: Resonance H of steroid isomerase. The isomerase concentration was 0.37 mM. 1760 free induction decays were used to obtain the spectrum.

Spin–Spin Coupling between Resonances B and H and the Multiplicity of Resonance H. The possibility existed that the multiple peaks of resonances B and H were the result of mutual spin–spin coupling since the separation of peaks B and peaks H were equal at 7.4 ± 0.2 Hz. This possibility was tested by decoupling experiments in which a saturating radio-frequency field was applied to resonances B and H. When the decoupler was placed at the frequency of resonance B, the multiplet H collapsed to a singlet. When the decoupler was adjusted to the frequency of H, the doublet B collapsed to a singlet. These results demonstrate that resonances B and H are, indeed, spin coupled.

The multiplicity of resonance H was determined by long-term spectral accumulation and by a decoupling experiment. A 0.37 mM solution of isomerase was subjected to 1760 pulse repetitions in order to improve the signal to noise ratio of the spectrum above that of the standard 400-pulse spectrum. Figure 5 shows the portion of the isomerase spectrum obtained between 4.06 and 4.24 ppm. The H resonance multiplet is seen to resemble a 1:3:3:1 quartet. Confirmation that resonance H was a quartet was furnished by a decoupling experiment in which the appearances of the H resonance in the presence and absence of a decoupling field applied at the frequency of the B resonance were compared. This comparison is displayed in Figure 6 in which the direct spectrum, A, the decoupled spectrum, B, and their difference, A − B, are presented. The

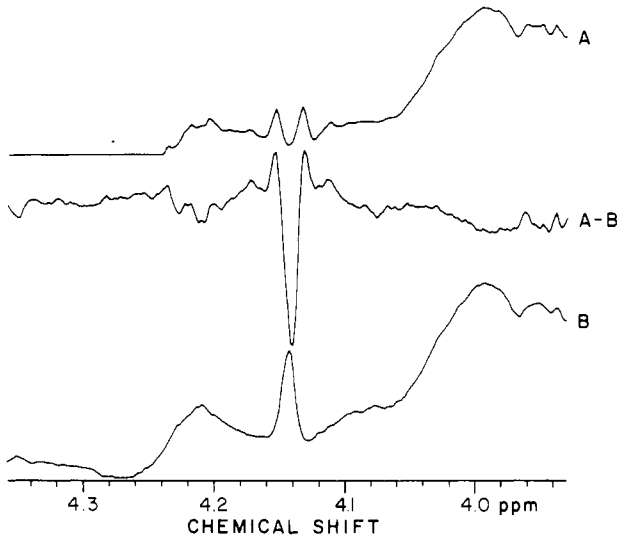


FIGURE 6: Decoupling of resonance H by a saturating radio-frequency field centered on resonance B. (A) Undecoupled spectrum. As a control, the decoupler was set 4000.0 Hz upfield from HDO. (B) Decoupled spectrum. The decoupler was positioned on the B-doublet, 1247.1 Hz upfield from HDO. (A − B) Difference spectrum. Spectrum A *minus* spectrum B.

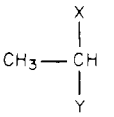
quartet of the direct spectrum is collapsed to a single resonance in the decoupled spectrum, confirming the assignment of all four peaks of the quartet to a single spin system coupled to resonance B. The difference spectrum, C, in which the decoupled spectrum is subtracted from the direct spectrum is fully consistent with the quartet to singlet collapse and is not consistent with the collapse of a doublet since the outer members of the H quartet are not present in the decoupled spectrum and are represented as positive peaks in the difference spectrum.

Discussion

Assignment of Resonances. Resonances 1, 2, and 3 having chemical shifts at pH 7.00 of 7.35, 7.76, and 8.38, respectively, can be assigned with confidence to the C-2 protons of the three histidines of the enzyme which are located at positions 6, 100, and 122 in the polypeptide chain. However, the assignment of the specific histidines to these resonances awaits further experimentation. The experimental justification for the assignment of these three resonances to histidine C-2 protons is provided by their chemical shifts, which are in the appropriate range for this class of protons, by the difference in limiting chemical shifts between at high pH and low pH of about 1 ppm, typical for histidine C-2 protons (Markley, 1975), and by the slow exchangeability of the protons of resonances 2 and 3, at least, for solvent deuterium (Markley & Kato, 1975).

As established by decoupling experiments, resonances B and H, a doublet and a quartet, are a spin-coupled system (J_{BH} = 7.4 ± 0.2 Hz), and therefore, they must be part of the same molecule. Several pieces of evidence point to the conclusion that resonances B and H arise from the isomerase itself. Both resonances are present at a constant abundance in all samples of enzyme examined by ^{1}H NMR by us. They are not diminished by extensive dialysis or by ultracentrifugal pelleting of the enzyme. They exhibit longitudinal relaxation times of 0.55 and 1.5 s, respectively. These T_1 values are within the range observed for protein resonances (Table II) and are significantly shorter than the T_1 values for free amino acids (Table II). Most significantly, the line widths of resonances B and H are sensitive to the degree of aggregation of the enzyme. At concentrations at which the enzyme is a dimer, these resonances are significantly narrower than they are at enzyme concentrations at which the enzyme has a higher molecular weight due to dimer aggregation (Benson et al., 1975; Tivol et al., 1975). Thus, all pieces of evidence suggest that resonances B and H arise from protons of the isomerase molecule and not from a low molecular weight contaminating species.

The appearance of resonances B and H in Figures 1 and 5 and the behavior of resonance H when decoupled from B show that resonance B is a doublet and resonance H is a quartet. Consequently they must arise from a structure of the type



where X and Y are neither hydrogen nor hydrogen-bearing groups in D_2O solution. Only one amino acid residue, alanine, satisfies this requirement. The chemical shift relative to DSS at neutral pH of resonance B is 1.34 ppm and that of resonance H is 4.17 ppm. These chemical shifts may be compared to those of the internal alanine residue and C-terminal alanine residue in tetrapeptides of the general structure Gly-Gly-X-Ala (in which X is any one of 20 different amino acid residues) which were studied by ^{1}H NMR by Bundi & Wuthrich (1979). When X = Ala, the α hydrogen of X had a chemical shift of 4.349 ppm while the methyl hydrogens had a chemical shift of 1.395 ppm. The C-terminal alanine of most of the tetrapeptides had an α-hydrogen chemical shift of 4.15 ppm and a methyl hydrogen chemical shift of 1.35 ppm, values which are in excellent agreement with the chemical shifts of resonances B and H. The C-terminal residue of the isomerase is alanine (Benson et al., 1971). Consequently the residue which best accounts for the chemical shift and multiplet structure of resonances B and H is the C-terminal alanine, residue 125. An alternative candidate responsible for resonances B and H is threonine. The methyl resonance of threonine is a doublet with a "random-coil" chemical shift of 1.23 (Bundi & Wuthrich, 1979). In this case, resonance H would be due to the threonine β proton, which absorbs at 4.22 ppm. Although the numerical agreement with the observed chemical shifts is not as good as is that of a C-terminal alanine, it is comparable to that of an internal alanine. However, the β proton of threonine in small peptides is split into a complex of eight peaks due to its spin couplings to the γ-methyl protons and the single α hydrogen. Decoupling of the β proton from the methyl protons by irradiation of the latter results in a doublet for β-proton resonance due to the remaining coupling to the α hydrogen. The decoupling experiment of Figure 6 results in a singlet for resonance H when resonance B was saturated. This is the result expected if resonance H were due to an alanine α hydrogen and contrary to the expected results if it were due to a threonine β hydrogen. The validity of this line of reasoning depends upon the precise conformation and conformational mobility of the residue responsible for resonances B and H. In a protein it is possible that the dihedral angle between the β hydrogen and α hydrogen of a threonine residue could be fixed at an angle between 60° and 120° resulting in a $J_{\alpha\beta}$ coupling constant close to 0 Hz. In such a case decoupling of the threonine methyl will result in the collapse of a β-hydrogen quartet to a singlet. However, in view of the narrow line width for resonances B and H, we must conclude that the residue from which they arise is highly mobile and thus should have coupling constants and chemical shifts for its protons which are typical of those for the same

Table IV: Titration Parameters of C-Terminal Alanine Peptides and Isomerase Resonances B and H

(poly)peptide	$\Delta\delta(\alpha\text{-H})$	$\Delta\delta(CH_3)$	pK_a'	reference
isomerase	0.156	0.063	3.7	this work
Gly-Gly-Glu-Ala		0.099	3.6	Bundi & Wuthrich (1979)
Gly-Ala	0.233	0.05		Sheinblatt (1966)
Ser-Ala	0.200	0.05		Sheinblatt (1966)
Phe-Ala	0.25	0.067		Sheinblatt (1966)
Pro-Pro-Ala	0.167	0.033		Sheinblatt (1966)
Gly-Ala-Ala	0.200	0.067		Sheinblatt (1966)
Pro-Gly-Ala		0.083		Sheinblatt (1966)

residue in small peptides. Bundi & Wuthrich (1979) reported that $J_{\alpha\beta}$ for the threonine residue in Gly-Gly-Thr-Ala is 5.0 Hz. Consequently, if a threonine residue were the source of resonances B and H, decoupling of the β protons should have produced a doublet at H separated by about 5.0 Hz. Since a singlet was obtained upon decoupling, we conclude that resonances B and H arise from an alanine residue. Moreover, the observed value of J_{BH} is about 7.4 Hz, a value which is typical for mobile alanine residues and substantially larger than that observed for mobile threonine (Campbell & Dobson, 1979).

Isomerase subunits contain 21 alanine residues, one of which is the C-terminal residue (Benzon et al., 1971). The chemical shifts of the α hydrogen and methyl protons of C-terminal alanine residues in peptides are pH dependent because of the titratable carboxyl group. Sheinblatt (1966) and Bundi & Wuthrich (1979) have reported the pK_a' and limiting chemical shifts of the α-hydrogen and methyl resonances of several C-terminal alanine peptides. Their data are compared to those we have obtained from the titration curves whose titration parameters are presented in Table I. For comparative purposes these data are collected together in Table IV. The total change in chemical shift of resonances B and H between the low and high pH limits is quantitatively similar to the total chemical shift changes for the methyl and α hydrogen of C-terminal alanine residues of the peptides examined by Bundi and Wuthrich and by Sheinblatt. Further, the pK_a' of the C-terminal carboxyl of the tetrapeptide Gly-Gly-Glu-Ala studied by Bundi and Wuthrich is very close to the pK_a' of the group influencing the chemical shifts of resonances B and H. The good agreement between the titration parameters of resonances B and H and those of the C-terminal alanine peptides strongly suggests the assignment of resonances B and H to the methyl and α hydrogen of the isomerase C-terminal alanine.

Resonances E, F_1, and F_2 are present in all enzyme samples at a constant level. The resonance line width of E is about 2 Hz, and those of the F resonances are close to 1 Hz, suggesting that the groups responsible for these resonances are more mobile than the bulk of the protein. Consequently, one expects that the chemical shifts of these resonances are close to those typical of the responsible amino acid residues in small, flexible peptides. With this assumption in mind, we have assigned resonance E to the protons of a methionine *S*-methyl group. Bundi & Wuthrich (1979) observed the *S*-methyl protons of Gly-Gly-Met-Ala at a chemical shift of 2.128 ppm which is close to the chemical shift of resonance E at pH 7.00 of 2.03 ppm. This assignment is confirmed by the effect of periodate treatment on the NMR spectrum and on the amino acid composition of the enzyme. The elimination of resonance E by periodate, an oxidizing agent which converts methionine to its sulfoxides (Yamasaki et al., 1982) under mild conditions, and the concomitant augmentation of resonances F_1 and F_2, which have chemical shifts close to that of methionine sulfoxide, support this assignment.

The *S*-methyl protons of the free amino acid L-methionine *d,l*-sulfoxide in D_2O solution appear in the ^1H NMR spectrum as a single peak, at 2.533 ppm, which was somewhat broader than the peak of the methyl protons of methionine. We presume that the single peak observed for the methyl protons of the sulfoxide is, in fact, two very close singlets arising from the two sulfoxide diastereoisomers. Resonances F_1 and F_2, thus, have chemical shifts close to that expected for the methyl protons of methionine sulfoxide and can be tentatively assigned to this modified amino acid. These assignments of resonances E, F_1, and F_2 were supported by amino acid analyses of alkaline hydrolysates of periodate-treated isomerase and an untreated control which showed that periodate treatment results in a decrease of methionine by 0.83 residue and an increase in methionine sulfoxides by 0.85 residue.

Pseudomonas testosteroni isomerase contains three methionines per subunit, one of which is the N-terminal residue. The chemical shift of the methyl resonance of the N-terminal methionine should be dependent upon pH since pH will affect the extent of protonation of the α-amino group of that residue. The pK_a' of the amino group of L-methionyl-L-methionine is reported to be 7.53 (Greenstein & Winitz, 1961). Consequently, if resonance E arose from the N-terminal methionine of isomerase, its chemical shift might have a pH dependence characterized by a similar pK_a'. This was, in fact, the case, the pK_a' for resonance E being 7.45 in the absence of 17β-estradiol. As expected from the periodate modifications, the chemical shifts of resonances F_1 and F_2 were also pH dependent and were characterized by pK_a' values of 7.00 and 6.90, respectively. The lower pK_a' for the sulfoxides is expected as a result of the more strongly electron-withdrawing power of the sulfoxide group compared to that of the thioether sulfur of methionine. Consequently, we assign resonance E to the methyl protons of the N-terminal methionine and resonances F_1 and F_2 to N-terminal methionine sulfoxides in a small fraction of the isomerase molecules. The sulfoxides might be due to air oxidation suffered by the enzyme during purification and storage.

Methionine sulfoxide has not been frequently reported as a component of proteins, probably because of the reduction of the sulfoxide derivative to methionine during the standard hydrochloric acid hydrolysis (Ray & Koshland, 1962). Nevertheless, several examples of methionine sulfoxide in proteins have been encountered. Bovine glomerular basement membrane protein has 30% of its total methionine as the sulfoxide (Hudson & Spiro, 1972). The hinge ligament protein of three species of surf clams has been examined for its methionine sulfoxide content by Kikuchi & Tamiya (1981), who found that nearly all of the total methionine of this methionine-rich protein is actually in the sulfoxide form. Even such well-studied proteins as bovine pancreatic ribonuclease and hen egg white lysozyme are found to contain 2–3% of their methionine as the sulfoxide (Hudson & Spiro, 1972). In these latter two cases, oxidants such as molecular oxygen are probably responsible for these small amounts of methionine sulfoxide, perhaps generated during purification procedures. The occurrence of protein-bound methionine sulfoxide in vivo may be phylogenetically widespread since Brot et al. (1981) have described an enzyme from *Escherichia coli* which catalyzes the reduction of methionine sulfoxide residues in proteins. This enzymatic activity was found also in extracts of rat tissue, two protozoans, HeLa cells, and spinach. The topic of methionine sulfoxide in proteins and its involvement in certain

diseases of humans has recently been reviewed by Brot & Weissbach (1982).

Functional Role of Histidine Residues. The pH dependence of the isomerase-catalyzed conversion of 5-androstene-3,17-dione to 4-androstene-3,17-dione has been investigated by Weintraub et al. (1970), who found that the reaction depended upon a group on the enzyme having a pK_a' of 4.7 which was increased to 5.6 upon formation of the enzyme–substrate complex. The chemical modification of the enzyme's histidine residues by dye-sensitized photooxidation (Colvin & Talalay, 1968) and by ethoxyformylation (Batzold et al., 1976), reactions which inactivate the enzyme, has raised the possibility that a histidine may be the base which abstracts the 4β proton of the substrate and transfers it to the 6β position of the product. The possibility has existed that the group whose pK_a' is increased form 4.7 to 5.6 when steroid binds is one of the three histidines of the enzyme subunit. However, the present ^{1}H NMR casts doubt on this view of the role of histidine in the catalytic mechanism. In the free enzyme the three histidine imidazole groups had pK_a' values of 4.68, 5.94, and 7.71 in 0.1 M potassium phosphate in D_2O. The most acidic of these imidazole groups has a pK_a' very near that of the catalytically relevant group of pK_a = 4.7 in the free enzyme. In the presence of the competitive inhibitor, 17β-estradiol, the pK_a' of this imidazole is raised only to 4.90. The other imidazole groups of the enzyme have pK_a' values which are far removed from the relevant pK_a' range, and these are virtually unaffected by 17β-estradiol binding. Also, it is noteworthy that the binding of 17β-estradiol has no significant effect on δ_{H^+} and δ_{H^0}, the low and high pH limiting chemical shifts, of any of the histidine C-2 protons. If 17β-estradiol binds to isomerase in the same orientation as does the substrate 5-androstane-3,17-dione, then the ring-current effect of the estradiol A ring should perturb these chemical shifts, and yet no significant effect on the limiting chemical shifts is evident, in the present study. Additional NMR studies of the effects of nonaromatic and ring A and B aromatic steroids on the titration parameters will be conducted in order to probe further the effects of steroid binding on the histidine C-2 proton resonances. But the present results on the effects of 17β-estradiol do not provide evidence for the involvement of a histidine imidazole as a catalytic group in the active site.

Perturbation of Aromatic Resonances by 17β-Estradiol. The most apparent effect of 17β-estradiol on the isomerase spectrum occurs in the aromatic region between 6.7 and 7.3 ppm. There is a diminution of the total area of the aromatic resonances between 6.9 and 7.3 ppm and an augmentation of the area between 6.7 and 6.9 ppm. A rough calculation (see Results) shows that the changes correspond to a loss of 4.8 protons from the region between 6.9 and 7.3 ppm and an increase of 7.7 protons in the region from 6.7 to 6.9 ppm. In order to interpret this perturbation of the spectrum, it would be necessary to know the chemical shifts of the three aromatic protons of the bound steroid; however, these data are not presently available. Nevertheless, it is possible to estimate the number of protein aromatic protons whose resonances are shifted upfield when 17β-estradiol binds. For this estimate we shall assume that the region between 6.7 and 7.3 ppm contains all of the carbon-bound aromatic proton resonances of isomerase and 17β-estradiol. There are 52 aromatic protons in the free enzyme subunit and 55 aromatic protons in the enzyme–17β-estradiol complex. If n estradiol aromatic protons absorb downfield of the isosbestic point at 6.9 ppm then 3 – n of these will absorb upfield of 6.9 ppm. Therefore, the loss of *protein* protons from the aromatic region downfield of 6.9 ppm is 4.8 + n, while the gain of *protein* protons to the aromatic resonances upfield of 6.9 ppm is 7.7 – (3 – n) = 4.7 + n. Thus, the number of protein protons lost from the low field aromatic region is nearly equal to the number gained by the high field aromatic region. Since n = 0–3, this number can be anywhere from 4.7 to 7.7 protons, equivalent to one to two aromatic rings. Of course these perturbed protons could be distributed among several aromatic systems. The results of this analysis are consistent with the important observation by Wang et al. (1963) of isomerase tyrosine fluorescence quenching by the competitive inhibitor 19-nortestosterone.

At the present time, the physical basis for the effect of 17β-estradiol on the aromatic resonances is uncertain. One possibility is that the aromatic ring current of the bound steroid produces an upfield shift of nearby protein aromatic protons. Another possibility is that the binding of the steroid results in a protein conformational change which is reflected by an upfield shift of aromatic protons which may or may not be near the bound steroid. Further studies involving both aromatic and nonaromatic steroids will be needed to distinguish between these alternatives.

Acknowledgments

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Phosphorus-31 Nuclear Magnetic Resonance Studies of the Two Phosphoserine Residues of Hen Egg White Ovalbumin†

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ABSTRACT: Ovalbumin contains two phosphoserine residues that give rise to two well-resolved resonances in a ^{31}P NMR spectrum. Ovalbumin samples that have been digested with a variety of phosphatases may give rise to only one phosphoserine resonance, indicating that one of the two phosphorylated sites is relatively inaccessible for phosphatase action. By comparison of the amino acid sequence of the peptide containing the nonsusceptible phosphate to the overall primary structure, we have assigned the resonances observed (pH 8.3) at 5.0 and 4.75 ppm to phosphoserines-68 and -344, respectively. pH titration behavior and susceptibility of the phosphoserine residues to phosphatases indicate that both are located on the surface of the protein. Both residues have a pK_a = 6.00–6.04. Analysis of the Hill coefficients measured for the pH titrations and the J_{PH} coupling constants indicate that neither residue interacts with other charged groups on the surface of the protein. Frequency dependence of ^{31}P NMR parameters shows that at higher magnetic field strengths the contribution of chemical shift anisotropy to the line width becomes very significant. We have calculated from the field-dependent terms that phosphoserine-344 is mobile with respect to the protein surface but that phosphoserine-68 is more restricted in its motion. The latter is also involved in a pH-dependent conformational change, since it is shielded from hydrolysis by phosphatases at higher pH. A comparison of the amino acid sequence of the phosphoserine-68 site shows that it has a striking homology to the active-site peptides of a wide variety of hydrolytic enzymes. Moreover, a comparison with the primary sequences of casein suggests that both proteins are phosphorylated by a protein kinase that specifically recognizes a Ser-X-Glu peptide.

Ovalbumin is the most abundant protein of egg white, from which it can be purified and crystallized in large quantities (Hofmeister, 1889). Although its properties have been frequently studied as those of a "model protein", very little is known about its biological function (Taborsky, 1974). Ovalbumin synthesis in the chick oviduct is under hormonal control and requires the presence of steroid sex hormones; this control has been extensively studied at the level of transcription as well as translation (Palmiter, 1975). Moreover, this protein has also proved to be of interest to students of eukaryotic gene organization (Breathnach et al., 1977).

The complete amino acid sequence of ovalbumin has been deduced both from the mRNA sequence (McReynolds et al., 1978) and by conventional amino acid sequencing techniques (Nisbet et al., 1981). The protein is a monomer comprised of 385 residues with a molecular weight for the polypeptide chain of 43 000. Unlike most other proteins that are translocated across a membrane, ovalbumin does not possess a hydrophobic N-terminal signal sequence but is believed to contain an internal signal sequence to facilitate its secretion (Lingappa et al., 1979). Most preparations of the protein have detectable heterogeneity, some of which arise from the genetic variants of the protein. The two variants that have been characterized have undergone only one base change, resulting in a replacement between an acid and its amide (Nisbet et al.,

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