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Isolation and Amino Acid Sequence Analysis of Bovine Adrenal 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase†

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ABSTRACT: 3β-Hydroxysteroid dehydrogenase/steroid isomerase has been purified to homogeneity from bovine adrenal glands. A single protein of molecular weight 42 090 ± 40 containing both enzyme activities has been isolated. Approximately 86% of the amino acid sequence of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase has been obtained by sequencing peptides isolated from digests with trypsin and lysyl endopeptidase and by chemical cleavage with CNBr. The sequence obtained is identical with that of the deduced amino acid sequence of the bovine ovarian 3β-hydroxysteroid dehydrogenase/steroid isomerase [Zhao et al. (1989) *FEBS Lett. 259*, 153-157], with the exception that the N-terminal methionine residue found in the bovine ovarian sequence is not present in the mature bovine adrenal enzyme. On the basis of the primary structure and comparisons with other NAD^+ binding proteins, we propose a structural model of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase localizing the NAD^+ binding site as well as the membrane-anchoring segment.

3β-Hydroxysteroid dehydrogenase and steroid isomerase catalyze consecutive steps in the steroid hormone biosynthesis pathway, namely, the conversion of pregnenolone to progesterone. The enzyme activities of 3β-hydroxy-5-ene steroid dehydrogenase and 5-ene-4-ene steroid isomerase are thought to be catalyzed by a single protein in mammalian tissues (Ford & Engel, 1974; Ishii-Ohba et al., 1986a,b, 1987; Lorence et al., 1990). In contrast, two distinct proteins catalyzing these reactions can be isolated from bacterial sources (Talalay & Wang, 1955; Batzold et al., 1976). The bifunctionality of the mammalian protein has led to interest in the nature of the active site, whether the same steroid binding site may be utilized for both enzyme activities or whether two distinct steroid binding sites exist. Recent reports tend to favor there being two distinct steroid binding sites (Blomquist et al., 1982; Thomas et al., 1990). The critical placement of this enzyme complex at the branch point of the pathway means that 3β-hydroxysteroid dehydrogenase/steroid isomerase plays a crucial role in the biosynthesis of all classes of steroid hormones. Congenital deficiencies or defects at this point in the pathway affect the biosynthesis of estrogens, androgens, and corticosteroids and consequently have far-reaching implications in disorders of steroid metabolism.

Because of the central importance of this enzyme in steroidogenesis, we undertook the complete purification and sequence analysis of the bovine adrenal enzyme. While this work was in progress the complementary DNAs (cDNAs) coding for the human placental and bovine ovarian 3β-hydroxysteroid dehydrogenase/steroid isomerase were cloned, and the amino

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acid sequences were deduced (Luu-The et al., 1989; Zhao et al., 1989).

This report describes the complete purification of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. Sequence analysis of the enzyme resulted in approximately 86% of the primary structure, and this is compared to the human placental and bovine ovarian 3β-hydroxysteroid dehydrogenase/steroid isomerase sequences, as well as to other NAD^+ binding proteins.

Experimental Procedures

Materials. Dehydroepiandrosterone (DHEA)[1] was obtained from Research Plus Laboratories, Inc., Denville, NJ. DEAE Toyopearl 650S was from Supelco Inc., Bellefonte, PA. Emulgen 913 was purchased from Kao Corp., Tokyo, Japan, and hydroxylapatite from Bio-Rad, Richmond, CA. Lysyl endopeptidase (from *Achromobacter lyticus*) was purchased from Wako Pure Chemical Industries Ltd., Dallas, TX.

Preparation of Bovine Adrenal Microsomes. Frozen bovine adrenal glands obtained from Pel-Freez Biologicals (Rogers, AR) were stored at −70 °C before use. Immediately prior to use the adrenal glands were trimmed of fat, the cortex was dissected from the medulla, and the latter was discarded. The tissue was cut into small pieces and homogenized in a Waring blender with 2.5 volumes of homogenization buffer (10 mM potassium phosphate buffer, pH 7.5 containing 0.25 M sucrose, 0.1 mM EDTA, and 0.1 mM DTT), for 20 s at low speed followed by 10 s at high speed. All the media used for the preparation of microsomes and the enzyme purification contained 2 μg/ml of both leupeptin and pepstatin A to minimize proteolysis of the enzyme.

The homogenate was centrifuged at 9950*g* for 15 min, and the resulting supernatant was centrifuged at 105000*g* for 70 min. The microsomal pellet was washed with homogenization buffer and then resuspended in 100 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT, diluted to a final protein concentration of 10 mg/mL, and stored at −70 °C.

Preparation of 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase. The purification method used is a modification of that described for rat adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase by Ishii-Ohba et al. (1986a). All steps in the purification of 3β-hydroxysteroid dehydrogenase/steroid isomerase were carried out at 0–4 °C, unless otherwise stated. 3β-Hydroxysteroid dehydrogenase/steroid isomerase was solubilized from the microsomes by the addition of 10% sodium cholate to a final concentration of 0.6%. Following a 30-min equilibration period, the solubilized proteins were obtained following centrifugation at 105000*g* for 70 min. The solubilized fraction was applied to a column (2.7 × 14 cm) of DEAE Toyopearl 650S equilibrated with 20 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.01 mM NAD^+, and 0.2% sodium cholate (buffer A). The column was washed with 100 mL of buffer A and then eluted with 20 mM potassium phosphate, pH 7.5, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.01 mM NAD^+, and 0.4% Emulgen 913. Fractions containing 3β-hydroxysteroid dehydrogenase/steroid isomerase activity were pooled and diluted with an equal volume of 20% glycerol. The diluted fraction was then applied to a column (1.8 × 28 cm) of hydroxylapatite, equilibrated with 10 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.01 mM NAD^+, and 0.2% Emulgen 913 (buffer B). The column was washed with 100 mL of buffer B before being eluted with a linear gradient from 10 to 70 mM potassium phosphate in buffer B. Fractions containing 3β-hydroxysteroid dehydrogenase/steroid isomerase activity were pooled and concentrated to a final volume of 10 mL in an Amicon ultrafiltration apparatus with a PM10 membrane filter (Amicon, Beverly, MA).

Enzyme Assays. 3β-Hydroxysteroid dehydrogenase activity was determined spectrophotometrically by measuring the increase in absorbance at 340 nm at 25 °C, due to the conversion of NAD^+ to NADH. The assay mixture (1 mL) contained 100 mM potassium phosphate buffer (pH 7.5), 20% glycerol, 0.1 mM EDTA, 1 mM NAD^+, and 0.1 mM DHEA. The DHEA was added as 10 μL of a 10 mM solution made up in ethanol. The reaction was initiated by the addition of the substrate, DHEA. A molar extinction coefficient for NADH of 6200 was used, and the enzyme activity was expressed as nanomoles of NADH formed per minute.

The steroid isomerase activity was assayed at 25 °C by measuring the time-dependent increase in absorbance at 248 nm, due to the formation of 4-ene-3-oxosteroid (progesterone). The assay mixture (1 mL) contained 100 mM potassium phosphate (pH 7.5), 20% glycerol, 0.1 mM EDTA, 0.1 mM NAD^+, and 20 μM 5-progesterone. The reaction was started by the addition of 10 μL of 2 mM 5-progesterone in ethanol. The enzyme activity was expressed as nanomoles of progesterone formed per minute, with a molar extinction coefficient of 16 300 used for progesterone.

The protein concentrations were measured by the method of Bradford (1976), with bovine serum albumin as a standard.

HPLC Purification of 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase. Enzyme that was required for protein sequencing work was further purified by reverse-phase HPLC to remove the glycerol and detergents that were present during the purification procedure. The protein was loaded onto a Brownlee C-4 column (4.6 × 30 mm), and eluted by using a linear gradient from 100% solvent I (0.1% TFA) to 100% solvent II (0.1% TFA and 90% acetonitrile) over a period of 60 min. The protein, detected at 214 nm, was manually collected and lyophilized prior to reduction and alkylation.

Reduction and Alkylation of 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase with 4-Vinylpyridine. The protein was dissolved in 0.25 M Tris-HCl buffer (pH 8.5) containing 1 mM EDTA and 6 M guanidine hydrochloride, prior to the addition of 70 mM β-mercaptoethanol. Reduction was allowed to occur in the dark under argon for 2 h, prior to the addition of a 3-fold molar excess over the total thiol groups of 4-vinylpyridine. Alkylation was allowed to proceed in the dark for 2 h under argon. The alkylated protein was then desalted by reverse-phase HPLC on a Brownlee C-4 column (4.6 × 30 mm), as previously described.

Reduction and Alkylation of 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase with Iodoacetic Acid. The protein was dissolved in 0.25 M Tris-HCl buffer (pH 8.5) containing 1 mM EDTA and 6 M guanidine hydrochloride, prior to the addition of DTT (0.05 μg/mg of protein). Reduction was allowed to occur in the dark under argon for 2 h, prior to the addition of a 3-fold molar excess over the total

[1] Abbreviations: DHEA, dehydroepiandrosterone; HPLC, high-performance liquid chromatography; ODS, octadecylsilane; TPCK, L-1-tosylamino-2-phenylethyl chloromethyl ketone; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; HFA, hexafluoroacetone trihydrate; TFA, trifluoroacetic acid; CNBr, cyanogen bromide; FAB, fast atom bombardment; FABMS, fast atom bombardment mass spectrometry; DMSO, dimethyl sulfoxide.

Table I: Purification of 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase from Bovine Adrenal Microsomes

		dehydrogenase activity		isomerase activity	
purification step	tot. protein (mg)	tot. act. (nmol/min)	sp act. (nmol min^{-1} mg^{-1})	tot. act. (nmol/min)	sp. act. (nmol min^{-1} mg^{-1})
microsomes	714	2767	3.9	17309	24.2
cholate extract	420	1170	2.8	15016	35.8
DEAE Toyopearl	25.2	1146	45.5	9954	395.0
hydroxylapatite	13.8	1032	74.8	7430	538.4

thiol groups of iodoacetic acid. Alkylation was allowed to proceed in the dark for 45 min under argon. The reaction was then terminated by the addition of an excess of β-mercaptoethanol. The alkylated protein was then desalted by reverse-phase HPLC on a Brownlee C-4 column (4.6 × 30 mm), as previously described.

Tryptic Digestion. Tryptic digestion was carried out on 3β-hydroxysteroid dehydrogenase/steroid isomerase following either alkylation with 4-vinylpyridine or with iodoacetic acid. The lyophilized protein was dissolved in 20 μL of hexafluoroacetone trihydrate (HFA), followed by 1 mL of 100 mM ammonium bicarbonate. The pH was adjusted to 8.5 prior to the addition of TPCK-treated trypsin in an enzyme:substrate ratio of 1:100 (w/w). Digestion was allowed to proceed for 18 h at 37 °C.

Lysyl Endopeptidase Digestion. The lyophilized protein was dissolved in 20 μL of HFA, followed by 500 μL of 100mM Tris HCl, pH 9.0. If necessary the pH was adjusted to 9.0 prior to the addition of the lysyl endopeptidase, in an enzyme to substrate ratio of 1:200 (mol/mol). Digestion was carried out for 6 h at 30 °C.

CNBr Cleavage. The lyophilized alkylated protein was dissolved in 20 μL of HFA, followed by 500 μL of 70% TFA. CNBr (100-fold molar excess over the total methionine content) was then added, and the reaction was allowed to occur overnight under argon at room temperature.

Analysis and Separation of Fragments by HPLC. Peptides were separated by reverse-phase HPLC performed on a Vydac C-18 column (4.6 × 250 mm), using a linear 2-h gradient from 100% solvent I to 100% solvent II. Peptides were detected by absorbance at 214 nm and were manually collected.

Microsequence Analysis. Approximately 50-200 pmol of peptide was subjected to automated Edman degradation, performed on a gas-phase peptide/protein microsequencer (Hawke et al., 1985). The phenylthiohydantoin amino acid derivatives were identified by reverse-phase HPLC on a Beckman Ultrasphere ODS column (2.0 × 250 mm, San Ramon, CA) and were quantitated by integration with Perkin-Elmer LIMS software.

Mass Spectral Analysis. Positive-ion fast-atom bombardment (FAB) mass spectra were obtained with a JEOL HX-100HF high resolution, double-focusing, magnetic-sector mass spectrometer operating at 5 kV accelerating potential and a nominal resolution of 3000. Sample ionization was accomplished by using a 6-keV Xe atom beam. A JEOL DA5000 data system was used to control instrument parameters and collect the spectral data. Peptides were dissolved in 2 μL of DMSO and added to 1 μL of sample matrix [dithiothreitol–dithioerythritol (5:1) (Witten et al., 1984) and camphor sulfonic acid (6mM) (DePauw et al., 1984)] on a 1.5 × 6 mm stainless steel sample stage.

Time of flight positive-ion mass spectra were obtained by using a Shimadzu LAMS-50KS laser desorption mass spectrometer, with a Q-switched Quanta-Ray DCR-11 neodymium/yttrium aluminium garnet laser (355 nm, 5–6-ns output pulse). Protein samples were prepared for laser desorption analysis by the method of Beavis and Chait (1990).

Circular Dichroism Studies. 3β-Hydroxysteroid dehydrogenase/steroid isomerase was purified as described above, except that for the hydroxylapatite column 0.2% Lubrol PX was substituted for the Emulgen 913, in order to reduce the absorbance contribution of the detergent. This did not result in any loss of enzyme activity or change in yield. Prior to CD analysis, DTT was added to the preparation to achieve a 1 mM concentration. DTT was included in the preparation since it was found that in the absence of DTT the enzyme underwent rapid inactivation during the CD scanning procedure, which could be fully reversed by the addition of DTT.

Circular dichroism spectra were recorded at 0.2-nm intervals over the wavelength range 190–250 nm by using a Jasco J-600 spectropolarimeter. Samples (1 mg/mL) were placed in a 0.1-mm cell and scanned at 25 °C at a rate of 20 nm/min. All spectra are the average of eight scans, with the appropriate buffer baseline subtracted.

Results

Purification of 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase. 3β-Hydroxysteroid dehydrogenase/steroid isomerase was purified from bovine adrenal microsomes by first solubilizing the enzyme with cholate, followed by a combination of ion-exchange chromatography on DEAE Toyopearl 650S and adsorption chromatography on hydroxylapatite. Table I shows a typical purification scheme of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. The overall yield was about 37%, and the specific activities of the 3β-hydroxysteroid dehydrogenase and the steroid isomerase were 74.8 and 538.4 nmol min^{-1} mg^{-1}, respectively. During the purification procedure, 3β-hydroxysteroid dehydrogenase activity and that of steroid isomerase were copurified. The purified 3β-hydroxysteroid dehydrogenase/steroid isomerase gave a single protein band on SDS–polyacrylamide gel electrophoresis and was confirmed as being a homogeneous protein by N-terminal sequence analysis. The molecular weight of the protein was estimated to be 43 300 ± 500 by SDS–polyacrylamide gel electrophoresis and 42 090 ± 40 by laser desorption mass spectrometry (Figure 1).

Sequencing. N-terminal sequence analysis and FABMS were carried out on peptides isolated from tryptic digests of 3β-hydroxysteroid dehydrogenase/steroid isomerase (alkylated either with 4-vinylpyridine or with iodoacetic acid), peptides from a lysyl endopeptidase digest, and peptides resulting from cleavage of the enzyme with CNBr. Figure 2 shows the amino acid sequences, and the monoisotopic masses (MH^+) of the analyzed peptides, aligned by using the sequence overlaps wherever possible and by sequence homology with the human placental and bovine ovarian sequences (Luu-The et al., 1989; Zhao et al., 1989) where overlaps did not occur. Sequencing of the isolated tryptic peptides resulted in the attainment of a major portion of the primary structure of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. A number of peptides isolated were the result of incomplete cleavage by trypsin (T-82, T-84, T-107, T-109, T-116B, T-123B, T-126, T-131). The failure of trypsin to cleave after the lysine at position 244 in the peptide T-123B was probably due to the

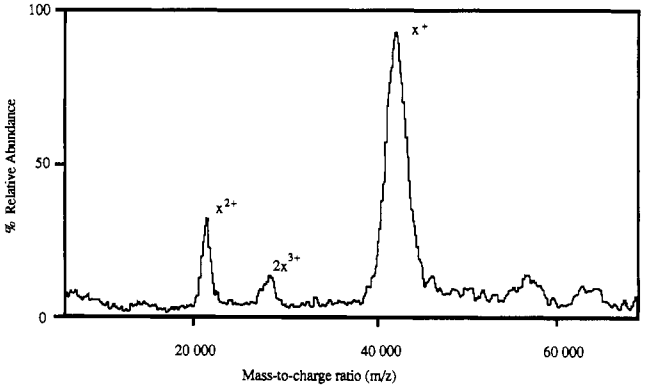

FIGURE 1: Mass spectrum of purified bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. The peaks labeled as x^+ and x^{2+} refer to the singly and doubly protonated ion species respectively, while $2x^{3+}$ refers to the triply charged dimer species.

presence of the double basic sequence ^{243}KK^{244}, since it is well known that the presence of a double basic sequence such as this often results in incomplete cleavage by trypsin. Of the other peptides generated by incomplete cleavage by trypsin, all but one (T-131) have at least one acidic residue adjacent to the arginine or lysine; incomplete cleavage by trypsin for these sequences is not uncommon. Of the tryptic peptides isolated from the enzyme alkylated with iodoacetic acid, several were the result of chymotryptic cleavage (F-74, F-79, F-85), despite the trypsin being TPCK-treated and repurified prior to use. However, the limited chymotryptic cleavages were of some benefit since sequence was obtained for peptides that otherwise would have been too long for complete sequence analysis. Digestion with lysyl endopeptidase, which cleaves specifically on the carboxy-terminal side of lysine residues, resulted in several overlaps being obtained. Several CNBr peptides were isolated, but most were only partially sequenced due to their excessive length (1–97, 98–187, 201–283, 284–372). The peptide composed of residues 284–372 was not isolated, perhaps due to its hydrophobic nature.

Mass spectral analysis was carried out on most of the isolated peptides, resulting in the monoisotopic masses shown in Figure 2. In a few cases, however, no molecular ions could be distinguished; this was usually the result of the chemical nature of the peptide or due to the high mass of the peptide (in general peptides > 3000).

It is clear from Figure 2 that several portions of the sequence were not sequenced. A number of these were single amino acid residues or dipeptides, which were unlikely to be isolated under the HPLC conditions used. The two most notable sections that were not sequenced are residue numbers 121–134 and 283–309. Residues 121–134 correspond to the C-terminal portion of the tryptic peptide that begins at residue 103. It is most likely that this portion of the sequence was isolated as part of T-137A but that, due to the length of the peptide (32 residues) as well as its amino acid composition, the peptide could not be completely sequenced. Determination of a monoisotopic mass by FABMS was also unsuccessful, probably due also to the peptide length. Residues 283–309 represents a 27-residue tryptic peptide that, upon consideration of the sequence in this region from the bovine ovarian and human placental sequences, is clearly very hydrophobic. It is therefore possible that due to the hydrophobic nature of this peptide region it was not eluted from the reverse-phase column under the HPLC conditions used.

Approximately 86% of the amino acid sequence of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase was obtained by combining the results of N-terminal sequence

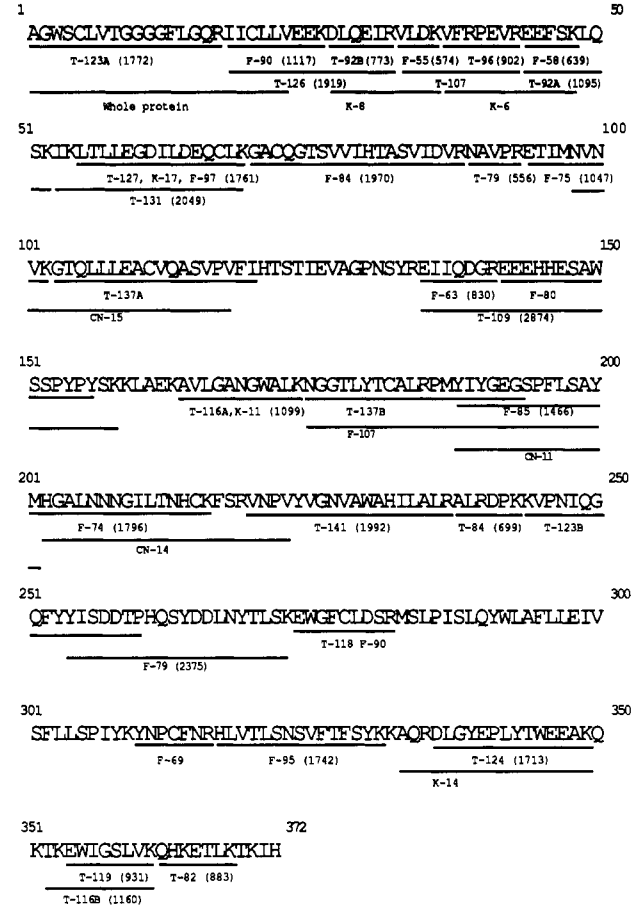

FIGURE 2: Amino acid sequence of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. All the peptides analyzed are shown with continuous lines and accompanying monoisotopic molecular weights (MH^+). T- and F- indicate peptides generated by tryptic digestion of the enzyme following alkylation with 4-vinylpyridine and iodoacetic acid, respectively. K- indicates peptides generated by lysine-specific cleavage, and CN- indicates CNBr peptides. Approximately 100–200 pmol of sample was used for sequencing, while 20–40 pmol was used for FABMS analysis.

analysis of the native protein and peptides generated by proteolytic cleavage of the alkylated protein with trypsin and lysyl endopeptidase and by chemical cleavage with CNBr. On comparison to the bovine ovarian sequence deduced from the cDNA, no differences were observed.

Circular Dichroism Studies. A typical CD spectrum for 3β-hydroxysteroid dehydrogenase/steroid isomerase is shown in Figure 3. The spectrum displays a high positive value at 198 nm, a crossover at 201 nm, and two minima at approximately 209 and 218 nm, with a plateau in between.

The fractional compositions of secondary structure present in 3β-hydroxysteroid dehydrogenase/steroid isomerase were estimated by using an algorithm based on Greenfield and Fasman (1969) reference data of poly-(L-lysine). This method calculates a nonrestrained least-squares fit for α-helix, β-sheet, and random coil, optimizing the curve shape over the CD intensity. For three sets of spectra, each an average of eight scans, the estimated percentage of α-helix is 80%, β-sheet is 5%, and random conformation is 15%, with an average correlation coefficient of 0.986.

Discussion

A single protein bearing both 3β-hydroxysteroid dehydrogenase and steroid isomerase activities has been purified from bovine adrenal microsomes. This is in contrast to a report by Hiwatashi et al. (1985), who used a combination of poly-

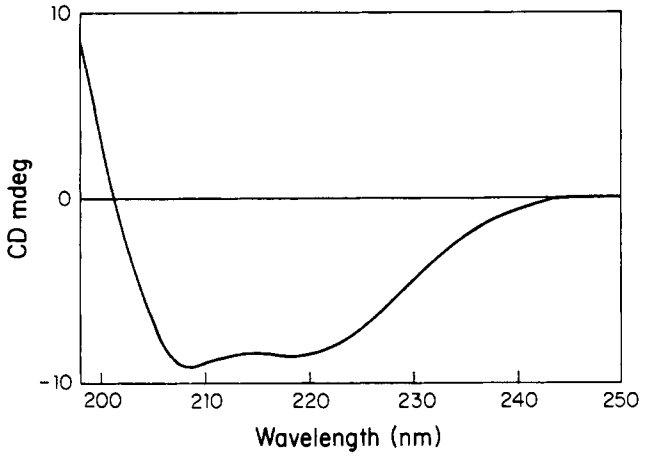

FIGURE 3: Circular dichroism spectrum of 3β-hydroxysteroid dehydrogenase/steroid isomerase. The spectrum is a signal average of eight scans and is baseline corrected. The protein (1 mg/mL) was prepared in 10 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.01 mM NAD^+, and 0.2% Lubrol PX.

(ethylene glycol) precipitation, ion-exchange chromatography, and affinity chromatography (Matrix red and blue) and reported the purification of a protein from bovine adrenal microsomes bearing only 3β-hydroxysteroid dehydrogenase activity. It is most likely that their failure to detect steroid isomerase activity was due to the absence of NAD^+ during purification; NAD^+ has since been found to be a potent allosteric activator essential for steroid isomerase activity (Ishii-Ohba et al., 1986a,b; Brandt & Levy, 1989; Thomas et al., 1988). Our result is in agreement with a number of reports from different species claiming a single protein contains both enzyme activities. The yield from our preparation at 37% is somewhat higher than from a number of reports in the literature, where yields range from 4 to 17% (Ishii-Ohba et al., 1986a; Thomas et al., 1988; Hiwatashi et al., 1985). The specific activity of our enzyme preparation, at 74.8 and 538 nmol min^{-1} mg^{-1} for the 3β-hydroxysteroid dehydrogenase and steroid isomerase activities, respectively, is slightly higher than that reported for the rat adrenal enzyme, 37.5 and 464 nmol min^{-1} mg^{-1} (Ishii-Ohba et al., 1986a), but significantly lower than the 677 nmol min^{-1} mg^{-1} reported for the 3β-hydroxysteroid dehydrogenase activity of the bovine adrenal enzyme (Hiwatashi et al., 1985). The estimated molecular weight of 42090 ± 40 determined by laser desorption mass spectrometry is similar to that of 42126 and 42093 calculated from the deduced amino acid sequences of the human placental (Luu-The et al., 1989) and bovine ovarian (Zhao et al., 1989) enzymes, respectively, and helps in confirming the primary structure of the protein.

The amino acid sequence of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase determined in this study aligned with the cDNA deduced amino acid sequences of the human placental and bovine ovarian enzymes is shown in Figure 4. The N-terminal sequence of the mature protein lacks the N-terminal methionine residue; otherwise the amino acid sequence obtained for the bovine adrenal enzyme is in complete agreement with the deduced sequence of the bovine ovarian enzyme (Zhao et al., 1989). This is not surprising since there is evidence that the same structural gene encode both testicular and adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase in mice (Stalvey et al., 1987).

A search for sequence homology in the protein data base of known protein primary structures against the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase sequence did not result in any significant homology with other proteins. Baker et al. (1990) have described an alignment of bovine ovarian 3β-hydroxysteroid dehydrogenase/steroid isomerase with plant dihydroflavonol reductase, while Thomas et al. (1989) compared the human placental 3β-hydroxysteroid dehydrogenase/steroid isomerase and estradiol 17β-dehydrogenase sequences. Since *Pseudomonas putida* (Linden & Benisek, 1986) and *Pseudomonas testosteroni* steroid isomerase (Benson et al., 1971) catalyze the same isomerization of Δ^5-androstene-3,17-dione to Δ^4-androstene-3,17-dione as the mammalian 3β-hydroxysteroid dehydrogenase/steroid isomerase, it is of interest to see if there is any sequence homology between the bacterial isomerase steroid binding site and the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase sequence. This is especially true since it appears likely that the mammalian 3β-hydroxysteroid dehydrogenase/steroid isomerase contains two distinct steroid binding sites, one associated with dehydrogenase activity and the other with isomerase activity (Blomquist et al., 1982; Thomas et al., 1990). It is interesting to note that the sequence identity between the two bacterial isomerases is relatively low at 35%. However, residues Asp-38, Tyr-14, and Tyr-55, which have been implicated in the catalytic mechanism by site-directed mutagenesis in the *P. testosteroni* enzyme, are conserved (Kuliopulos et al., 1989). Comparison of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase sequence with the steroid binding region of the *P. testosteroni* and *P. putida* isomerase shows approximately 46% sequence homology (Figure 5), with 10 residues being identical and seven being conservative amino acid replacements within the 37 residues compared. Among the conserved residues are the three residues implicated in the bacterial steroid isomerase binding site, with D-38 in the bacterial sequence corresponding to D-241 in the bovine sequence, Y-55 to Y-253, and Y-14 to F-217, with allowances made for insertions and deletions. The sequence homology between the two bacterial isomerases is slightly higher around these three essential residues than in other portions of the molecule. This is also apparent in the bovine enzyme with P-39 corresponding to P-242, F-54 to F-252, and R-13 to K-216 in the bovine enzyme. These residues are also conserved in the human placental sequence (Luu-The et al., 1989). Therefore, it is possible that this region of the bovine adrenal enzyme may represent a portion of the isomerase steroid binding site of the enzyme.

A direct comparison of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase sequence with those of other dehydrogenases reveals that it is distantly but clearly related to a family of short-chain nonmetalloenzyme dehydrogenases, which are characterized by the presence of the sequence YXXXK occuring around position 150 of the enzyme (Krook et al., 1990). This family includes insect alcohol dehydrogenase (Jörnvall et al., 1981), bacterial ribitol dehydrogenase (Jörnvall et al., 1984), two bacterial steroid dehydrogenases (Krook et al., 1990), 17β-hydroxysteroid dehydrogenase, and 15-hydroxyprostaglandin dehydrogenase amongst others. The fact that the tyrosine and lysine residues are conserved in the majority of these enzymes suggests that they may be of some functional importance, and investigation into the role of these residues in one member of this enzyme family may provide insight into its role in the other family members.

It has been shown in many cases that the nucleotide binding site of kinases and dehydrogenases contains a so-called "glycine-rich" sequence, GXGXXG, which maintains the appropriate secondary or tertiary structure for nucleotide

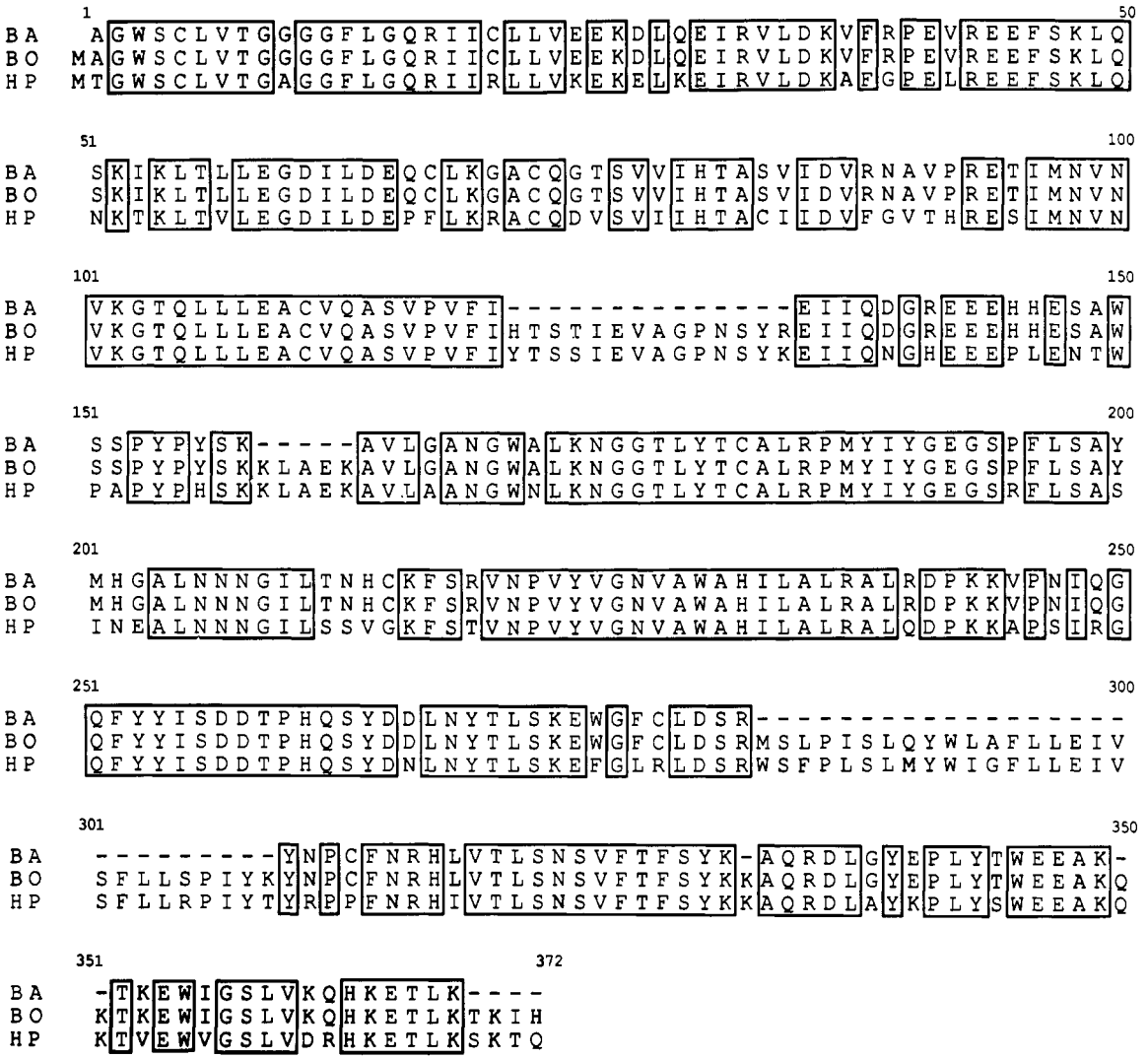

FIGURE 4: Sequence comparison among 3β-hydroxysteroid dehydrogenase/steroid isomerase from bovine adrenal gland (BA), bovine ovary (BO) (Zhao et al., 1989), and human placenta (HP) (Luu-The et al., 1989). Dashes (–) represent residues that were not determined in the sequence.

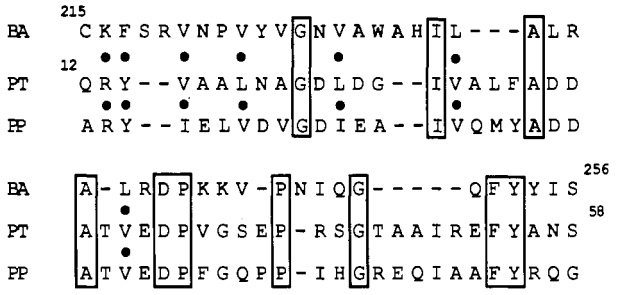

FIGURE 5: Alignment of the steroid binding site of *P. testosteroni* (PT) (Kuliopulos et al., 1989) and *P. putida* steroid isomerase (PP) (Benson et al., 1971) with bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. Identical residues are shown in boxes, while conservative amino acid replacements are indicated with dots (●).

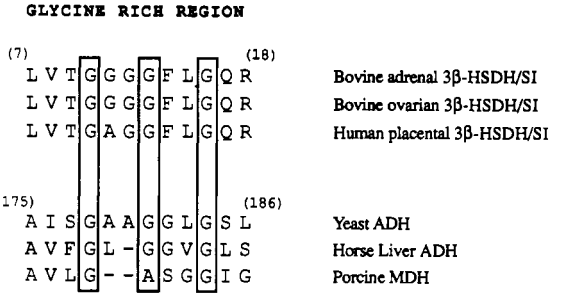

FIGURE 6: Sequence homology of the N-terminal glycine-rich region of bovine adrenal, bovine ovarian, and human placental 3β-hydroxysteroid dehydrogenase/steroid isomerase. The amino acid sequences of the N-terminal regions of human placental (Luu-The et al., 1989) and bovine ovarian (Zhao et al., 1989) 3β-hydroxysteroid dehydrogenase/steroid isomerase (3β-HSDH/SI) are compared to the bovine adrenal sequence, and the glycine rich regions of yeast alcohol dehydrogenase (ADH) (Jörnvall, 1977b), horse liver alcohol dehydrogenase (ADH) (Borrás et al., 1989), and porcine malate dehydrogenase (MDH) (Birktoft et al., 1982).

binding. This region is usually located adjacent to the binding region for the nicotinamide ribose diphosphate moiety. Analysis of the amino acid sequence of bovine ovarian 3β-hydroxysteroid dehydrogenase/steroid isomerase deduced from the cDNA sequence (Zhao et al., 1989) and of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase obtained by direct protein sequencing shows a peptide segment (residues 10–16) that exhibits the classic glycine-rich sequence (Figure 6). In contrast, the amino acid sequence of human placental 3β-hydroxysteroid dehydrogenase/steroid isomerase deduced from the cDNA sequence (Luu-The et al., 1989) does not display the same glycine-rich sequence, with the Gly being substituted for an Ala at position 11 in the human placental enzyme. One possibility is that residue 11 be considered an insertion, as has been described for yeast alcohol de-

hydrogenase (Jörnvall, 1977b), where Ala-179 is considered to be an insertion, with the Gly occuring at position 178 believed to be the first in the glycine-rich sequence (Figure 6). Thus Ala-11 of the human placental 3β-hydroxysteroid dehydrogenase/steroid isomerase may also be considered an insertion and the Gly at position 10 would then be the first essential glycine residue, thus satisfying the glycine-rich sequence pattern.

A common feature in the family of FAD-binding and NAD^+-binding domains is the βαβ unit, which in all the known complexes has been found to bind the ADP moiety of the dinucleotide in the same manner (Wierenga et al., 1985). A "fingerprint" describing the essential amino acid types that consistently occur at specific positions within the ADP binding βαβ-fold has been proposed (Wierenga et al., 1986). This fingerprint has provided a set of rules by which a particular sequence can be tested to see if it may possibly fold into a βαβ unit with ADP-binding properties. When this set of rules is used to test residues 5–36 of the bovine ovarian and bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase sequence, the maximum possible score of 11 is obtained by the insertion of one residue at the end of the first β-strand and having five residues in the variable length loop between the α-helix and the second β-strand. Therefore, it is possible that this region (residues 5–36) represents the NAD^+ binding site of bovine ovarian and adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. When the same alignment is used for the human placental 3β-hydroxysteroid dehydrogenase/steroid isomerase, a score of 10 is obtained, since the first essential Gly has been substituted for an Ala in this case. However, if residue 11 is considered to be an insertion as described above, then a perfect score of 11 is obtained for the human placental enzyme. However, even with a score of 10 and the absence of the first essential glycine residue, it is still likely that this region will fold into the βαβ fold representing the NAD^+-binding site of the protein: first, because a score of 10 is sufficient to predict with reasonable confidence the βαβ fold, and second, because in both the cases of yeast alcohol dehydrogenase (Jörnvall, 1977a) and cytoplasmic porcine malate dehydrogenase (Birktoft et al., 1982) one of the essential glycines in the fingerprint has been substituted for an alanine residue, yet both are still thought to fold into a βαβ unit.

Calculation of the secondary structure of the region proposed to be the NAD^+ binding site, according to the method of Chou and Fasman (1978), reveals that residues 4–10 are most likely to adopt a β-sheet conformation, thus forming the first β-sheet in the βαβ unit. For residues 13–25 the secondary structure prediction is not as conclusive, since the first part of this region is more likely to fold into β-sheet, while the second part is more likely to adopt an α-helical conformation. Likewise, for residues 30–35 the results are not as conclusive, with a similar probability of this region folding into either α-helix or β-sheet. Such secondary structure predictions, although not conclusive, are consistent with the presence of a βαβ unit.

Since the segment consisting of residues 5–36 contains a glycine-rich sequence, has a high score in the βαβ fingerprint test, and from secondary structure predictions may well fold into such a unit, we propose that this segment comprises at least a part of the NAD^+ binding site of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. It is also interesting to note that, of the known structures with βαβ structural units, a number of these occur near the N-terminus of the nucleotide binding domain (Wierenga et al., 1986), as does the proposed NAD^+ binding site of the mammalian 3β-hydroxysteroid dehydrogenase/steroid isomerase. It has been proposed (Schulz & Schumer, 1979) that the βαβ fold may function as a nucleation center for the complete folding of the domain, with the βαβ fold forming first and the remaining polypeptide folding around this core to complete the dinucleotide binding domain.

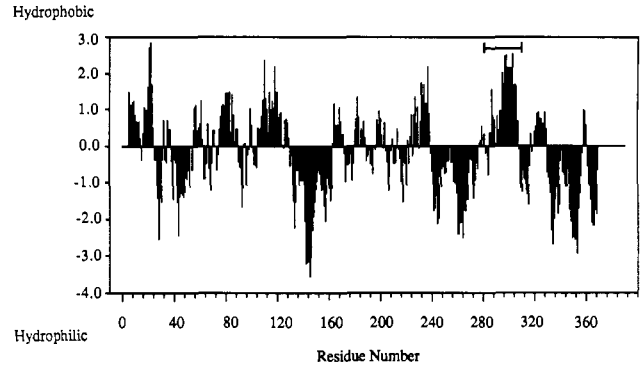

FIGURE 7: Hydrophobicity profile of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. The plot is according to Kyte and Doolittle (1982) with a seven-residue window size.

From the primary structure of 3β-hydroxysteroid dehydrogenase/steroid isomerase it is possible to postulate the location of the membrane binding site by using the hydrophobicity plot of Kyte and Doolittle (1982). From Figure 7 it can be seen that the 26-residue segment from Met 283 to Tyr 308 is extremely hydrophobic and was calculated to be 65% and 69% hydrophobic (Val, Met, Ile, Leu, Tyr, Phe, and Trp assessed as hydrophobic) for the bovine and human sequences, respectively. With the exception of this segment, the length and degree of hydrophobicity of the other hydrophobic regions are insufficient to penetrate a membrane. Membrane-spanning α-helices are usually 18–20 residues in length (Engelman et al., 1980), connected by a varying number of intervening residues. The 26-residue hydrophobic segment in the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase is clearly not long enough to span the membrane twice in an α-helical conformation. Therefore two possibilities arise: either this segment spans the membrane in a 3_{10} helix formation, which with a rise of 2 Å/amino acid residue would require 15 residues to span a membrane layer (Engelman & Steitz, 1981), or this segment acts as a membrane anchor by passing only partially into the membrane layer before looping back to return the C-terminal segment to the same side of the membrane as the N-terminus.

Both the bovine and human putative membrane-anchoring segments have a glutamic acid residue at position 298, and the human enzyme has an arginine at position 305. The occurrence of charged residues in membrane spanning segments is not unusual; for example, both bacteriorhodpsin and porin transmembrane segments (Engelman et al., 1980; Paul & Rosenbusch, 1985) have a number of charged residues within the membrane, particularly located in the first 5–6 residues within the membrane, where interaction with the polar head group may be expected to occur. On either side of the hydrophobic domain of the putative membrane anchoring segment of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase lie several charged residues, which may reside at the endoplasmic reticulum–cytosol interface, perhaps interacting with the polar head groups.

The CD spectrum of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase indicates the presence of approximately 80% α-helix and 5% β-sheet. However, the secondary structure prediction methods indicate 35% α-helix and 28% β-sheet. Therefore, it would appear that the sec-

ondary structure prediction methods are somewhat unreliable for this protein. The high α-helical content of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase is similar to other microsomal proteins such as cytochrome P-450c and P-450d, at 50 and 60%, respectively (Haniu et al., 1986).

From the primary structure of bovine 3β-hydroxysteroid dehydrogenase/steroid isomerase we have proposed a possible location for the NAD^+ binding site and the membrane-spanning region of the protein, and by homology with the bacterial isomerase enzyme we have suggested a possible location for the isomerase steroid binding site. On the basis of these proposed sites, we propose a model for the structure of bovine 3β-hydroxysteroid dehydrogenase/steroid isomerase. Our model predicts two globular domains on the cytoplasmic side of the membrane, a large one (residues 1–282) and a small one (residues 309–372), separated by a membrane-anchoring segment (residues 283–308). The first large domain contains the so-called glycine-rich sequence (residues 10–16), which binds to the nicotinamide ribose diphosphate moiety, while residues 5–36 form the βαβ fold characteristic of many ADP and NAD^+ binding proteins. Residues Asp-241, Tyr-253, and Phe-217 may lie at the isomerase steroid binding site and be responsible for the catalysis of isomerase activity.

Since in bacteria there are two distinct enzymes catalyzing the 3β-hydroxysteroid dehydrogenase and steroid isomerase reactions, it is tempting to suggest that a distant ancestor of the mammalian 3β–hydroxysteroid dehydrogenase/steroid isomerase arose from the fusion of the two bacterial enzymes, yielding a single enzyme exhibiting dual activity and having two steroid binding sites. This and the individual bacterial enzymes then underwent considerable mutation such that the only remaining resemblances occur at the steroid binding sites. Under such a scheme half of the mammalian 3β-hydroxysteroid dehydrogenase/steroid isomerase would represent the dehydrogenase activity and the other half the isomerase activity; in the case of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase, it would appear that the N-terminal half has the dehydrogenase activity while the C-terminal half contains the proposed isomerase steroid binding site. Since the mammalian 3β-hydroxysteroid dehydrogenase/steroid isomerase appears to be active as a dimer (Ishii-Ohba et al., 1986a), the conversion of pregnenolone to progesterone may involve both subunits such that the dehydrogenation occurs on one subunit before the intermediate passes to the adjacent isomerase steroid binding site on the other subunit.

In conclusion, we have purified and determined the amino acid sequence of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. Based on the available information and comparison of its sequence to those dehydrogenases with known three-dimensional structures, a model of this enzyme is presented. This will be the basis for our future structure–function studies of this enzyme.

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Affinity Labeling of Bovine Adrenal 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase by 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine†

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ABSTRACT: Incubation of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA) results in the inactivation of the 3β-hydroxysteroid dehydrogenase enzyme activity following pseudo-first-order kinetics. A double-reciprocal plot of $1/k_{obs}$ versus 1/[5′-FSBA] yields a straight line with a positive *y* intercept, indicative of reversible binding of the inhibitor prior to an irreversible inactivation reaction. The dissociation constant (K_d) for the initial reversible enzyme–inhibitor complex is estimated at 0.533 mM, with $k_2 = 0.22$ min^{-1}. The irreversible inactivation could be prevented by the presence of NAD$^+$ during the incubation, indicating that 5′-FSBA inactivates the 3β-hydroxysteroid dehydrogenase activity by reacting at the NAD$^+$ binding site. Although the enzyme was inactivated by incubation with 5′-FSBA, no incorporation of the inhibitor was found in labeling studies using 5′-[*p*-(fluorosulfonyl)benzoyl][^{14}C]adenosine. However, the inactivation of 3β-hydroxysteroid dehydrogenase activity caused by incubation with 5′-FSBA could be completely reversed by the addition of dithiothreitol. This indicates the presence of at least two cysteine residues at or in the vicinity of the NAD$^+$ binding site, which may form a disulfide bond catalyzed by the presence of 5′-FSBA. The intramolecular cysteine disulfide bridge was found between the cysteine residues in the peptides ^{274}EWGFCLDSR^{282} and ^{18}IICLLVEEK^{26}, by comparing the [^{14}C]iodoacetic acid labeling before and after recovering the enzyme activity upon the addition of dithiothreitol.

The 3β-hydroxysteroid dehydrogenase/steroid isomerase enzyme complex (EC 1.1.1.51, EC 5.3.3.1) catalyzes consecutive steps in the conversion of pregnenolone to progesterone. NAD$^+$ is the cofactor for the dehydrogenase activity, and this nucleotide has also been shown to be a potent allosteric activator of the steroid isomerase activity (Ishii-Ohba et al., 1986a,b, 1987; Brandt & Levy, 1989; Thomas et al., 1988). Therefore, NAD$^+$ plays an important role in both the dehydrogenation and isomerization reactions catalyzed by this enzyme complex.

To date, the nucleotide binding site of 3β-hydroxysteroid dehydrogenase/steroid isomerase has not been identified, although the primary structure of the enzyme has been determined by cloning (Luu-The et al., 1989; Zhao et al., 1989) and by protein sequencing (Rutherfurd et al., 1991). Affinity labeling studies can provide information as to the importance and role of regions in the active site of the enzyme. Previous studies have shown that 5′-[(fluorosulfonyl)benzoyl]adenosine (5′-FSBA), an adenine analogue that has proven to be a useful affinity label for studies of many adenine nucleotide and nicotinamide-dependent enzymes (Colman, 1983; Chen et al., 1986; Liu et al., 1989), inactivates rat testicular and adrenal 3β-hydroxysteroid dehydrogenase activities (Ishii-Ohba et al., 1986a,b). The modified residue(s) in these enzymes were not determined (Ishii-Ohba et al., 1986a,b). As a first step in determining the structure of the NAD$^+$ binding site of 3β-hydroxysteroid dehydrogenase/steroid isomerase, an affinity labeling study using 5′-FSBA has been carried out in this laboratory. Results presented here show that 5′-FSBA can be used to modify the NAD$^+$ binding site of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase, resulting in the inactivation of the 3β-hydroxysteroid dehydrogenase activity. Kinetically, 5′-FSBA fulfills all of the criteria of an affinity label for 3β-hydroxysteroid dehydrogenase/steroid isomerase, but our data suggest that the inactivation caused by 5′-FSBA is due to the formation of a disulfide bond that is catalyzed by 5′-FSBA.

Experimental Procedures

Materials. 5′-FSBA, NAD$^+$, and trypsin (TPCK-treated) were obtained from Sigma Chemical Co. (St. Louis, MO). 5′-[(Fluorosulfonyl)benzoyl][*adenine*-8-^{14}C]adenosine (53.6 mCi/mmol) was obtained from New England Nuclear Corp. (Boston, MA). [1-^{14}C]Iodoacetic acid (6.25 mCi/mmol) was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA).

Enzyme Preparation. 3β-Hydroxysteroid dehydrogenase/steroid isomerase was purified to homogeneity from bovine adrenal glands by a combination of ion-exchange chromatography with DEAE Toyopearl 650S and adsorption

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