Affinity Radiolabeling Identifies Peptides Associated with the Isomerase Activity of Human Type I (Placental) 3β-Hydroxysteroid Dehydrogenase/Isomerase[†]

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ABSTRACT: 3β-Hydroxysteroid dehydrogenase and steroid $\Delta^{5\rightarrow4}$-isomerase (3β-HSD/isomerase) were purified as a single protein from human term placenta. The affinity alkylator, 5,10-secoestr-4-yne-3,-10,17-trione (secosteroid), was incubated with the purified enzyme (30/1 secosteroid/enzyme molar ratio) to produce an 80% loss of initial isomerase activity over 90 min in a time-dependent, irreversible manner. The secosteroid inactivated 3β-HSD by only 20% during the same 90 min. Incubations containing the isomerase substrate steroid, 5-androstene-3,17-dione, completely protected the isomerase activity from inactivation by the secosteroid and did not slow the inactivation of 3β-HSD. The enzyme containing covalently bound steroid was separated from unreacted secosteroid by reversed phase HPLC. Ketones on the protein-bound secosteroid were radiolabeled by reduction with sodium boro[^{3}H]hydride (specific radioactivity 50 μCi/μmol for the transferred tritium). After removal of the unreacted sodium boro[^{3}H]-hydride, the affinity-radiolabeled enzyme was digested with trypsin-TPCK, and the peptides were isolated by reversed phase HPLC. The radiolabeled peptide fractions were sequenced. The secosteroid alkylated three tryptic peptides: ^{251}GQFYYISDDTPHQSYDNLNYTLSK^{274}, tritiated His^{262}; ^{176}NGGTLYTCALR^{186}, tritiated Cys^{183}; and ^{353}TVEWVGSLVDR^{363}, tritiated Trp^{356}. Coincubation with the isomerase substrate blocked the labeling of these three peptides and shifted the alkylation by secosteroid to a single tryptic peptide (^{135}EIIQNGHEEEPLENTWPAPYPHSK^{159}, tritiated His^{142}). Using substrate protection to validate specificity, the affinity labeling secosteroid has identified peptides in the enzyme that are associated with isomerase activity.

Human placental 3β-hydroxysteroid dehydrogenase (3β-HSD,[1] EC 1.1.1.145) and steroid $\Delta^{5\rightarrow4}$-isomerase (EC 5.3.3.1) catalyze the sequential conversion of 3β-hydroxy-5-ene steroids (pregnenolone, dehydroepiandrosterone) to 3-keto-4-ene steroids (progesterone, androstenedione) on a single enzyme protein. The enzyme is the gatekeeper of steroid hormone biosynthesis in all tissues that produce steroid hormones. Two tissue-specific genes encode the type I enzyme (placenta, skin), and the type II enzyme (gonads, adrenals) in the human. 3β-HSD/isomerase has been copurified from human term placenta (Thomas et al., 1988, 1989; Luu-The et al., 1990), bovine adrenals (Rutherford et al., 1991), rat adrenals (Ishii-Ohba et al., 1986a), and rat testis (Ishii-Ohba et al., 1986b).

The secosteroid, 5,10-secoestr-4-yne-3,10,17-trione, is an active site-directed affinity alkylator of the isomerase activity of human placental 3β-HSD/isomerase (Thomas et al., 1992), bovine adrenal Δ^5-3-ketosteroid isomerase (Penning & Covey, 1982), and Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* (Penning et al., 1981a). Acetylenic secosteroids have been shown to covalently bind to nucleophilic amino acids in *Pseudomonas testosteroni* (Penning et al., 1981b; Penning & Talalay, 1981). Hence, 5,10-secoestr-4-yne-3,10,17-trione is used in the current study to localize the isomerase site in the known primary structure of 3β-HSD/isomerase (Luu-The et al., 1989).

EXPERIMENTAL PROCEDURES

Materials. Steroid hormones and pyridine nucleotide (NAD^+) were purchased from Sigma Chemical Co. (St. Louis, MO); trypsin-TPCK from Worthington Biochemical Co. (Freehold, NJ); HPLC-grade trifluoroacetic acid (TFA) from Pierce Chemical Co. (Rockford, IL); sodium boro[^{3}H]-hydride ([^{3}H]$NaBH_4$, 14.08 Ci/mmol, functional specific radioactivity 3.52 Ci/mmol for the transferred tritium) from Dupont New England Nuclear (Boston, MA); HPLC-grade water and acetonitrile, analytical grade solvents, and reagent grade salts from Fisher Scientific Co. (Pittsburg, PA); Brownlee and Vydac HPLC columns from P. J. Cobert Associates (St. Louis, MO).

Synthesis of the Secosteroid. 5,10-Secoestr-4-yne-3,10,-17-trione was prepared by the five-step synthesis previously described (Covey & Parikh, 1982). The physical properties of the pure product agreed with literature values. The secosteroid eluted as a single sharp peak (214 nm) during reversed-phase HPLC using a Brownlee BU-300 Aquapore Butyl cartridge (0.46 × 3.0 cm) with a linear gradient of 0.1% TFA in H_2O to 0.1% TFA in 90% acetonitrile over 90 min at 1.0 mL/min.

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[1] Abbreviations and trivial names: secosteroid, 5,10-secoestr-4-yne-3,10,17-trione; 2α-BAP (2α-bromoacetoxyprogesterone), 2α-bromoacetoxy-4-pregnene-3,20-dione; pregnenolone, 3β-hydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; androstenedione, 4-androstene-3,17-dione; PTH, phenylthiohydantoin; TPCK, L-(1-tosylamino)-2-phenylethyl chloromethyl ketone; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; DPM, disintegrations per minute.

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Enzyme Purification. 3β-HSD and steroid Δ-isomerase were copurified from human placental microsomes by our published method (Thomas et al., 1988, 1989). The purified enzyme is a homogeneous, dimeric protein (monomeric M_r = 42 000) according to NH_2-terminal sequence analysis, SDS−polyacrylamide gel electrophoresis, and fractionation by gel filtration chromatography (Thomas et al., 1989). Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Enzyme Inactivation and Assay. Purified enzyme (1.0 μM, 4.0 mg, 4.74 nmol of enzyme dimer) was incubated with the secosteroid, 5,10-secoestr-4-yne-3,10,17-trione (40.0 μM, 4% final methanol concentration), in 0.2 M potassium phosphate buffer, pH 7.0, 20% glycerol, and 0.1 mM EDTA (buffer A) at 22 °C. In the substrate-protection study, identical incubations contained 5-androstene-3,17-dione (0.15 mM). Smaller volume (1.0 mL) but otherwise identical control mixtures contained cortisol in place of the alkylating steroid and included 5-androstene-3,17-dione in the protection study. After incubation with the secosteroid (90 min), 2-mercaptoethanol (0.2 mM) was added as a scavenger nucleophile to prevent further alkylation of the enzyme.

Assays that monitored the loss of 3β-HSD or isomerase activity during enzyme inactivation were performed in duplicate according to our published conditions (Thomas et al., 1990). The slope of the initial linear increase in absorbance at 340 nm (due to NADH production) per unit time was used to determine 3β-HSD activity. Isomerase activity was measured by the initial absorbance increase at 241 nm (due to androstenedione formation from 5-androstene-3,17-dione) as a function of time. Blank assays (zero-enzyme, zero-substrate) assured that specific isomerase activity was measured as opposed to nonenzymic "spontaneous" isomerization (Thomas et al., 1990). Changes in absorbance were measured with a Varian (Palo Alto, CA) Cary 219 recording spectrophotometer.

Purification and Sequencing of Radiolabeled Tryptic Peptides. Nonionic detergent (Genapol C-100), glycerol, and unbound steroid were removed from the secosteroid-alkylated enzyme on a Beckman (Fullerton, CA) Model 338 gradient HPLC using the Brownlee BU-300 cartridge and gradient elution that was described above for the characterization of secosteroid purity. Fractions containing detergent-free, alkylated enzyme were pooled, concentrated under a stream of nitrogen gas to remove acetonitrile, and adjusted to pH 8.0 with NaOH. A 10-fold molar excess of iodoacetic acid was added, and the mixture was stirred under nitrogen for an additional 30 min. After adding 2-mercaptoethanol (5-fold excess relative to iodoacetic acid), the mixture (5.0 mL) was incubated for two 20 min intervals with [^{3}H]$NaBH_4$ (200 molar excess to enzyme, specific radioactivity of 50 μCi/μmol of transferred tritium). After the treatment, the pH was lowered to 5.0, and the degraded tritium gas was evaporated in a fumehood approved by the Division of Radiation Safety at this institution. Trace unbound radioactivity was removed by HPLC (Brownlee BU-300 cartridge). This procedure was based on the [^{3}H]$NaBH_4$ reduction of keto groups on enzyme-bound 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-triphosphate that identified a cysteinyl peptide in the active site of rabbit muscle pyruvate kinase (Vollmer & Colman, 1990). The secosteroid was not radiolabeled prior to enzyme alkylation because ^{14}C on the steroid nucleus is diluted during the five-step synthesis to an unusable specific radioactivity.

The stoichiometry for the binding of tritium to enzyme inactivated by the secosteroid in the presence or absence of isomerase substrate was then determined using quantitative amino analysis (Thomas et al., 1993). Control enzyme that had not been alkyated by the secosteroid was identically treated with [^{3}H]$NaBH_4$ to test for radiolabeling of the enzyme protein itself. Amino acid analysis cannot identify the radioalkylated residues because the entire steroid is covalently bound to the nucleophilic amino acid (Penning et al., 1981b).

Trypsin-TPCK (4% w/w) was incubated with the radiolabeled enzyme in 0.05 M ammonium bicarbonate, pH 8.1, for 1 h at 22 °C, followed by incubation at 37 °C overnight. The digested enzyme preparation was frozen, lyophilized, and washed 3 times with water to remove salt. The mixture of radiolabeled tryptic peptides was resuspended in 0.1% aqueous TFA (1.0 mL). The peptides were separated by reversed-phase HPLC using a Vydac C-18 column (0.46 × 25.0 cm) with a linear gradient of 0.1% TFA in water to 0.1% TFA in 90% acetonitrile over 120 min at 0.5 mL/min. Peptides detected by the UV absorbance at 214 nm were matched with fractions collected at 0.5 min intervals using a programmed interface system. Single fractions containing the maximal radioactivity (DPM) associated with each radiolabeled peptide were dried under nitrogen. The peptides were sequenced at the Protein and Nucleic Acid Chemistry Laboratories at Washington University on a Perkin Elmer Applied Biosystems (Foster City, CA) Model 477A automated protein microsequencer using pulsed liquid-phase chemistry. An on-line Perkin Elmer Applied Biosystems Model 120A analyzer with UV detection was used to identify phenylthiohydantoin (PTH) amino acid derivatives at each cycle. PTH analysis was performed on 40% of the available sequencer fraction, and the remaining 60% was used for the measurement of ^{3}H-radioactivity on a Packard Model 2500TR liquid scintillation analyzer.

RESULTS

Inactivation of 3β-HSD/Isomerase. The secosteroid (30/1 alkylator/enzyme molar ratio) inactivated the purified enzyme to 20% of control isomerase activity in a time-dependent, irreversible manner over 90 min (Figure 1A). When the isomerase substrate, 5-androstene-3,17-dione, was incubated with an identical mixture of secosteroid and enzyme, the isomerase activity was completely protected from inactivation (Figure 1A). In contrast, 3β-HSD was inactivated to only 80% of the initial activity during the same 90 min, and 5-androstene-3,17-dione did not slow the inactivation of 3β-HSD (Figure 1B). In our previous kinetic study, the 3β-HSD substrate, pregnenolone, also failed to diminish the rate of dehydrogenase inactivation by the secosteroid (Thomas et al., 1992).

Enzyme alkylated by secosteroid in the presence or absence of isomerase substrate was separated from unbound secosteroid, and the keto groups on the enzyme-bound steroid were then reduced and radiolabeled by [^{3}H]$NaBH_4$. After removal of the unbound tritium, the stoichiometry of binding was determined to be 1.1 mol of tritium/mol of enzyme dimer (based on a functional specific radioactivity of 50 μCi/μmol of [^{3}H]$NaBH_4$ for the transferred tritium). Enzyme alkylated

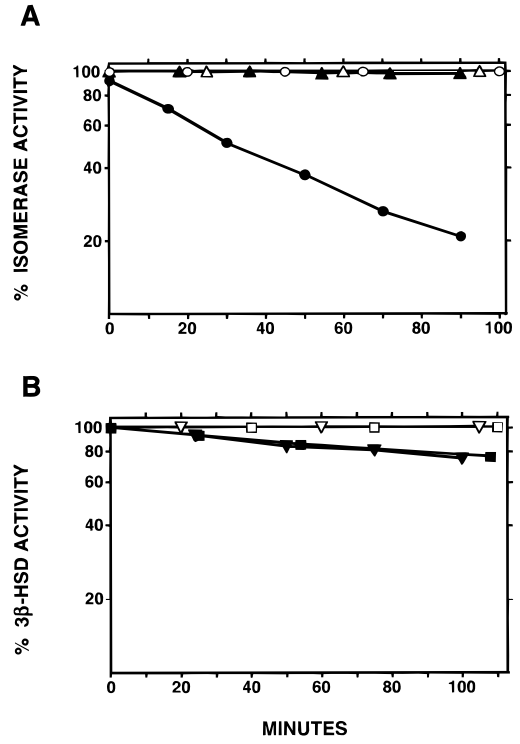

FIGURE 1: Inactivation of isomerase and 3β-HSD by 5,10-secoestr-4-yne-3,10,17-trione. The enzyme (1.0 μM, 4.0 mg) was incubated with the secosteroid (30.0 μM) in the presence or absence of the isomerase substrate, 5-androstene-3,17-dione (150.0 μM). Panel A: The loss of isomerase activity was measured in the absence (●) or presence (▲) of the protecting substrate steroid. Isomerase activity was also assayed in identical control mixtures that contained cortisol in place of the secosteroid (○) or cortisol plus 5-androstene-3,17-dione as the protection control (△). Panel B: Inactivation of 3β-HSD was measured in the absence (■) or presence (▼) of the isomerase substrate. 3β-HSD activity was also assayed in identical control mixtures containing cortisol (□) or cortisol plus isomerase substrate (▽). The percent of initial (zero-time) enzyme activity is plotted on a logarithmic scale along each ordinate, and time is represented by the linear scale on each abscissa.

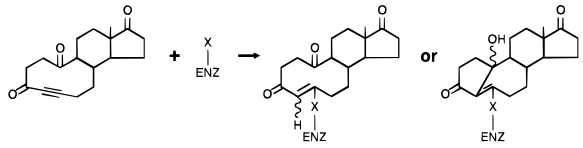

FIGURE 2: Reaction of 5,10-secoestr-4-yne-3,10,17-trione with a nucleophilic residue (X) on 3β-HSD/isomerase (ENZ). This reaction scheme was graciously provided by Dr. Douglas F. Covey, who also synthesized the secosteroid (Covey & Parikh, 1982).

by secosteroid in the presence of isomerase substrate steroid was decreased to 0.5 mol of tritium/mol of enzyme dimer. In the identically treated control that had not been inactivated by the secosteroid, the stoichiometry was 1.0×10^{-4} mol of tritium/mol of enzyme dimer. Because the protein-bound secosteroid exists in two forms, one with 3- and 10-keto groups and another with 3-, 10-, and 17-keto groups (Figure 2), it is impossible to determine a definitive secosteroid/enzyme stoichiometry. In addition, the possibility of a kinetic isotope effect (^3H vs ^1H) in the reduction of the ketones by [^3H]$NaBH_4$ dictates that the stoichiometric measurements are reported relative to each other. The stoichiometry of binding data shows that nonspecific radiolabeling of amino acids in the enzyme by tritium is insignificant during the reduction of the secosteroid-alkylated enzyme with [^3H]$NaBH_4$.

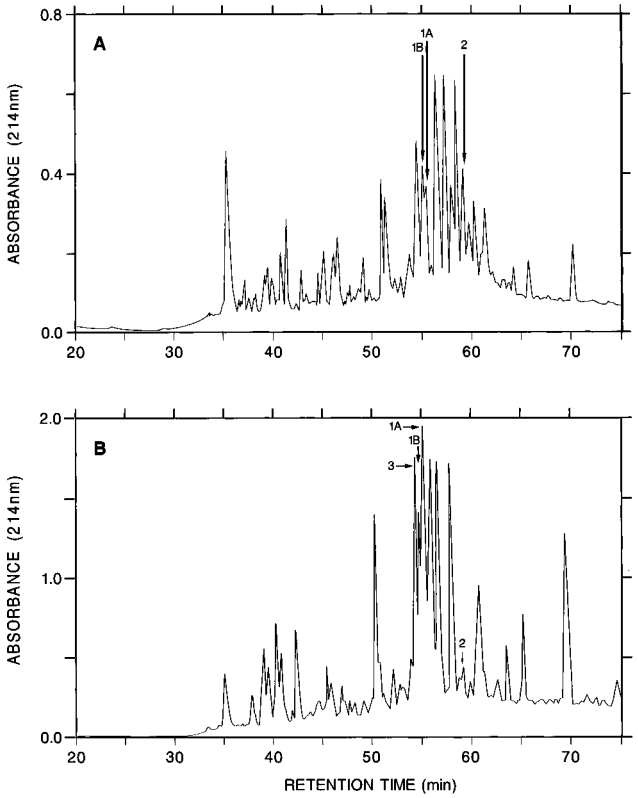

FIGURE 3: Tryptic peptide maps of 3β-HSD/isomerase that had been affinity radiolabeled by the secosteroid. Panel A: Enzyme alkylated in the absence of isomerase substrate was digested with trypsin-TPCK and separated by reversed phase HPLC (Vydac C-18 column). Panel B: Enzyme alkylated in the presence of the isomerase substrate was digested and separated by HPLC in an identical manner. The tryptic peptides were detected by the ultraviolet absorbance at 214 nm. The radioactive peptide peaks are labeled. The profiles of eluted tritium are shown in Figure 4 for clarity.

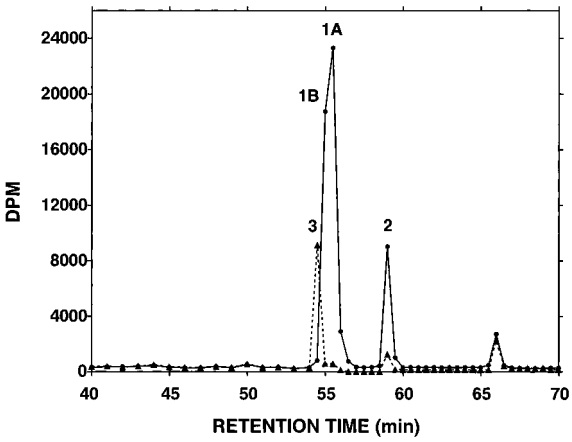

FIGURE 4: Profiles of radioactivity eluted during the HPLC separation of peptides from the unprotected and substrate-protected enzyme. The DPM of tritium was measured in each fraction (0.25 mL) collected during the HPLC runs shown in Figure 3. The radioactive profile obtained for enzyme alkylated by secosteroid in the absence of isomerase substrate (●, solid line) was plotted versus the retention time of the eluted peptides. The radioactive profile obtained for enzyme alkylated in the presence of isomerase substrate (▲, dashed line) was plotted in the same manner. The retention times of the identified peptides in the two HPLC runs were identical according to our Beckman System Gold software.

Tryptic Peptide Map. As shown in Figure 3A and Figure 4, separation of the tryptic peptides of the unprotected, secosteroid-alkylated enzyme revealed major peaks of ra-

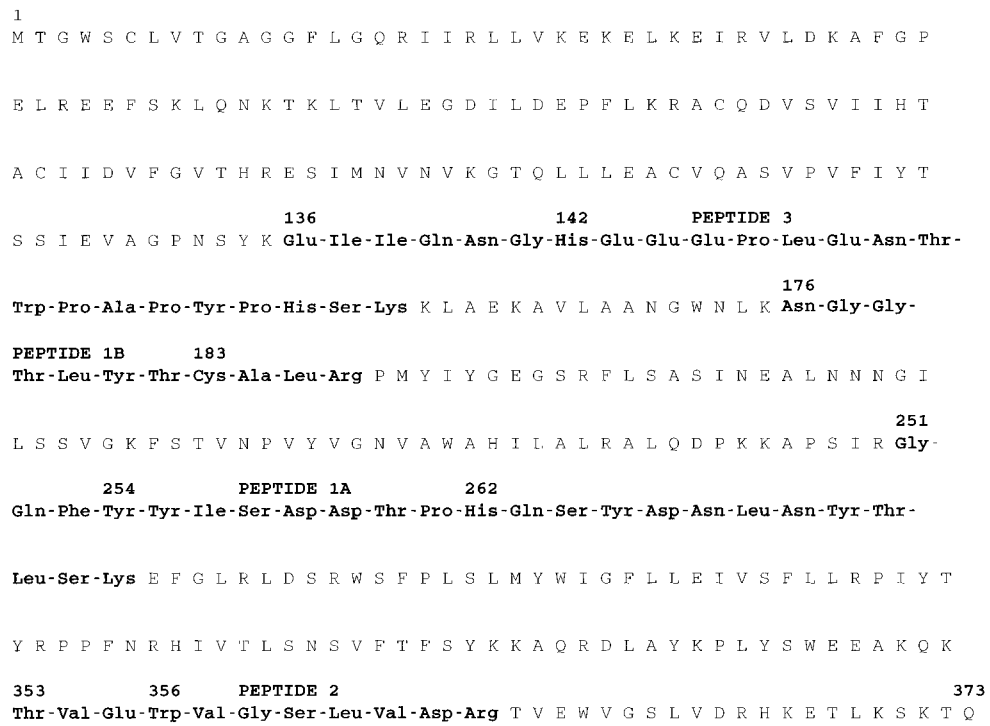

FIGURE 5: Tryptic peptides radiolabeled by the secosteroid are localized in the primary structure of human placental 3β-HSD/isomerase. The sequences of the radiolabeled peptides were determined by automated Edman degradation. Amino acids in the identified peptides are represented by three-letter abbreviations in boldface type. Position numbers are noted for the radiolabeled amino acids (His^{142}, Cys^{183}, His^{262}, Trp^{356}), a potentially critical residue (Tyr^{254}), the initial residue of each radiolabeled tryptic peptide, and the NH_2- or COOH-terminal amino acid. Enzyme sequences between the identified tryptic peptides are represented by single-letter amino acid abbreviations that are not in boldface type.

dioactivity at retention times 55.0 min (peak 1B), 55.5 min (peak 1A), and 59.0 min (peak 2). In the substrate-protected enzyme, a new major peak of radioactivity eluted at 54.5 min (peak 3), and the radioactivity associated with each of the peaks 1A, 1B, and 2 was greatly diminished (Figure 3B and Figure 4). The peak of radioactivity at 66.0 min was identified as tritiated, free secosteroid according to the elution profile of an authentic secosteroid/2-mercaptoethanol complex, which absorbs strongly at 214 nm (ϵ 9900). The radiolabeled secosteroid peak was the same in the unprotected and substrate-protected HPLC profiles (Figure 4).

Affinity Radiolabeled Peptides. As shown in Figures 4 and 5, radioactive peaks 1A and 1B contained the tryptic peptides ^{251}GQFYYISDDTPHQSYDNLNYTLSK^{274} (peptide 1A) and ^{176}NGGTLYTCALR^{186} (peptide 1B). These two peptides did not separate well in the unprotected HPLC run (Figure 3A) when each was covalently bound to the secosteroid. However, peptides 1A and 1B did separate during the HPLC elution of the substrate-protected enzyme (Figure 3B). The unprotected peptide 1A was identified by sequence analysis of cycles 1−16 (Table 1). Cycle 12 was radiolabeled and contained an unidentified residue because an amino acid bound to a [^{3}H]steroid cannot be identified by Edman degradation. In the substrate-protected peptide 1A (Table 2), cycle 12 could be identified as His^{262} because the residue was not bound to the secosteroid. The unprotected peptide 1B was identified by sequence analysis of cycles 1−11, and cycle 8 was radiolabeled (Table 1). The substrate-protected peptide 1B had no radiolabel at cycle 8 (Table 2). Cycle 8 contained Cys^{183} according to the known primary structure (Luu-The et al., 1989). Cys cannot be identified by Edman degradation in either the steroid-modified or the umodified form.

Table 1: Sequence Analysis of the Unprotected Affinity Radiolabeled Peptides[a]

	peptide 1A			peptide 1B			peptide 2		
cycle	residue	pmol	DPM	residue	pmol	DPM	residue	pmol	DPM
1	Gly	19	5	Asn	17	5	Thr	15	3
2	Gln	12	4	Gly	13	4	Val	15	4
3	Phe	10	4	Gly	12	4	Glu	14	3
4	Tyr	11	4	Thr	12	4	X	−	54
5	Tyr	10	4	Leu	11	4	Val	15	6
6	Ile	10	3	Tyr	11	3	Gly	15	2
7	Ser	11	3	Thr	11	3	Ser	15	2
8	Asp	11	*[b]	X	−	134	Leu	13	3
9	Asp	8	5	Ala	7	5	Val	13	4
10	Thr	6	2	Leu	5	2	Asp	12	4
11	Pro	5	1	Arg	4	1	Arg	4	4
12	X	−	280						
13	Gln	5	7						
14	Ser	4	0						
15	Tyr	4	0						
16	Asp	3	2						
	17−24	ND							

[a] Pure 3β-HSD/isomerase was inactivated by the secosteroid in the absence of isomerase substrate. [b] Cycle 8 had 134 DPM due to the mixture of peptides 1A and 1B. The asterisk (*) was used to avoid confusion in the table. The recovery of applied radioactivity from the automated protein microsequencer is low (<5%).

Peak 2 contained ^{353}TVEWVGSLVDR^{363} and is labeled peptide 2 in Figure 5. Peptide 2 was identified by Edman degradation of cycles 1−11 with an unidentified, radiolabeled residue in cycle 4 (Table 1). Substrate-protected peptide 2 (Table 2) had no radiolabel at cycle 4, which was identified as Trp^{356}.

The radiolabeled peptide 3 (^{135}EIIQNGHEEEPLENTW-PAPYPHSK^{159}) was alkylated by the secosteroid only in the presence of the isomerase substrate (Figure 5). Sequence analysis of cycles 1−10 (Table 2) revealed a radiolabel at

Table 2: Sequence Analysis of the Substrate-Protected Peptides[a]

	peptide 1A			peptide 1B			peptide 2			peptide 3		
cycle	residue	pmol	DPM	residue	pmol	DPM	residue	pmol	DPM	residue	pmol	DPM
1	Gly	15	3	Asn	13	5	Thr	13	6	Glu	14	3
2	Gln	10	4	Gly	10	4	Val	12	4	Ile	10	2
3	Phe	8	2	Gly	9	0	Glu	12	3	Ile	10	2
4	Tyr	8	1	Thr	8	2	Trp	11	7	Gln	9	1
5	Tyr	8	2	Leu	7	3	Val	10	2	Asn	7	2
6	Ile	7	4	Tyr	7	3	Gly	9	0	Gly	7	2
7	Ser	7	0	Thr	7	0	7−11	ND		X	−	139
8	Asp	8	3	X	−	4				Glu	7	9
9	Asp	7	0	Ala	7	2				Glu	6	1
10	Thr	7	0	Leu	6	2				Glu	6	0
11	Pro	7	4	11	ND					11−24	ND	
12	His	7	3									
13	Gln	6	4									
14	Ser	4	2									
	15−24	ND										

[a] Pure 3β-HSD/isomerase was inactivated by the secosteroid in the presence of the isomerase substrate, 5-androstene-3,17-dione. A similar amount of each corresponding peptide was sequenced for Tables 1 and 2 according to estimates based on the peak areas (214 nm) measured by Beckman System Gold during the HPLC separations.

cycle 7, which was determined to be His^{142} from the known primary structure.

DISCUSSION

The affinity-alkylating secosteroid, 5,10-secoestr-4-yne-3,10,17-trione, was designed to bind at the substrate site of Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni* (Penning et al., 1981a). In our extensive kinetic study with human placental 3β-HSD/isomerase (Thomas et al., 1992), the inactivation of isomerase by the secosteroid was slowed dramatically by the isomerase substrate, 5-androstene-3,17-dione, but 3β-HSD was not protected from inactivation by its substrate, pregnenolone. The current study uses the secosteroid to identify peptides at the isomerase site of the bifunctional placental enzyme. Three tryptic peptides (1A, 1B, and 2) were first alkylated by the secosteroid and then protected from alkylation by coincubation of the secosteroid with the isomerase substrate steroid.

Evidence from clinical studies suggests that peptide 1A is critical to enzyme activity. Tyr^{255} (numbering from the initial Met residue) was identified as a mutation (Tyr255Asp) in the type II 3β-HSD gene of a female patient with nonsalt-losing 3β-HSD/isomerase deficiency. Tyr^{254} (Figure 5) was mutated (Tyr254Asn) in the type II 3β-HSD gene in a family with the salt-losing form of 3β-HSD deficiency (Simard et al., 1995). Our affinity radiolabeled peptide data obtained with the isomerase-directed secosteroid indicate that peptide 1A, which contains Tyr^{254} and Tyr^{255}, is associated with the isomerase activity. A Tyr residue participates in the isomerase reaction mechanism of Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni* (Kuliopulos et al., 1990).

The same two tryptic peptides (peptide 1A and peptide 1B) were radiolabeled in this study by the isomerase-directed secosteroid and in our previous study using the 3β-HSD-directed alkylator, 2α-bromo[2′-^{14}C]acetoxyprogesterone (2α-BAP) (Thomas et al., 1993). Similar to the isomerase substrate protection of these peptides in the current study, pregnenolone protected 1A and 1B from alkylation by 2α-BAP. This raises the following question: Why does the secosteroid not inactivate isomerase and 3β-HSD at the same rates if both activities reside at the same site on the protein?

We answered that question in our previous kinetic and substrate-protection study of the inactivation of 3β-HSD/isomerase by the secosteroid (Thomas et al., 1992). As the concentration of secosteroid increases, progressively more molecules of enzyme are converted from the 3β-HSD conformation into the isomerase form. Enzyme alkylated in the isomerase form retains significant 3β-HSD activity when diluted 10-fold into the 3β-HSD assay mixture that favors the 3β-HSD conformation. In contrast, enzyme alkylated in the isomerase conformation has no activity in the isomerase assay because the enzyme conformation is not shifted to the 3β-HSD form under these incubation conditions. At the 30 μM concentration of secosteroid used in this affinity radiolabeled peptide study, most of the enzyme is in the isomerase conformation. Hence, the secosteroid inactivates the isomerase activity by 80% and the 3β-HSD activity by only 20% over the time period used to generate the affinity-alkylated enzyme. The phenomenon of a ligand-induced conformational change was validated by biophysical methods in a related study of 3β-HSD/isomerase (Thomas et al., 1995).

The radiolabeling of peptide 2 at Trp^{356} was not seen in our studies with 2α-BAP (Thomas et al., 1993, 1994). Michael addition to this weak nucleophilic residue by the secosteroid suggests that the Trp^{356} is in close proximity to the active site when the enzyme is in the isomerase conformation.

Coincubation of the enzyme with both the secosteroid and 5-androstene-3,17-dione shifted alkylation from peptides 1A, 1B, and 2 to His^{142} of tryptic peptide 3. Since 5-androstene-3,17-dione prevented the secosteroid from gaining access to the isomerase site, His^{142} apparently lies outside of the isomerase site. His^{142} of peptide 3 was also alkylated by 2α-BAP in the presence of NADH as a protecting ligand (Thomas et al., 1994). Peptide 3 was not alkylated by 2α-BAP either alone or in the presence of the 3β-HSD substrate, pregnenolone (Thomas et al., 1993). Since NADH and the secosteroid induce the enzyme to assume the isomerase conformation (Thomas et al., 1995), the cofactor may block access to the isomerase active site similarly to 5-androstene-3,17-dione when the enzyme is in that form. Pregnenolone does not induce the isomerase conformation, so peptide 3 is

not oriented favorably for alkylation by 2α-BAP when the enzyme is in the 3β-HSD form (Thomas et al., 1993).

These observations indicate that the 3β-HSD and isomerase activities are associated with peptides 1A and 1B of the bifunctional enzyme protein. The different catalytic mechanisms of the 3β-HSD and isomerase reactions require that different catalytic amino acids carry out the 3β-HSD hydride ion transfer and the 5→4-ene double bond shift within this region. Site-directed mutagenesis of possible catalytic amino acids identified by the secosteroid is underway to test our conclusions on the primary structure/function relationships of 3β-HSD/isomerase.

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