The Interaction Site for Tamoxifen Aziridine with the Bovine Estrogen Receptor*

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Calf uterine estrogen receptor was covalently labeled with [3H]tamoxifen aziridine during affinity chromatography purification. After carboxymethylation, affinity labeled receptor was digested with trypsin under limit conditions and the labeled peptides were fractionated by reversed-phase high performance liquid chromatography into one major and two minor components. Sequence analysis of the dominant labeled fragment indicated the facile cleavage of label during Edman degradation but identified two peptides, both derived from the extreme carboxyl terminus of the steroid-binding domain. The 17 residues of one peptide were fully conserved in all estrogen receptors. This fragment contained five nucleophilic amino acids and was considered as the more favored interaction site for tamoxifen aziridine. A corresponding region of the glucocorticoid receptor has recently been identified as one of three major contact sites for glucocorticoids (Carlstedt-Duke, J., Strömstedt, P.-E., Persson, B., Cederlund, E., Gustafsson, J.-Å., and Jörnvall, H. (1988) J. Biol. Chem. 263, 6842-6845). A comparison of amino acid physical characteristics in the hormone-binding domains of human estrogen and glucocorticoid receptors demonstrated an excellent structural correlation between the two regions and delineated elements in the estrogen receptor which may be directly involved in estradiol binding.

The amino acid sequences for estrogen receptors from a number of species have been derived from their corresponding receptor cDNAs (1-6). All estrogen receptors (M, 66,000) have a structural organization similar to that initially described for human and chicken estrogen receptors by Krust et al. (3). The two functional regions, the DNA-binding domain and the steroid-binding domain, are highly conserved in all of these proteins (1-6). Deletion studies have defined a stretch of approximately 250 amino acids, toward the COOH terminus, as essential for the formation of the hydrophobic cavity involved in hormone binding (7). The size of this domain suggests the requirement of a correct tertiary structure for estrogen binding activity.

For the human estrogen and glucocorticoid receptors, considerable amino acid sequence similarity (30% identity) exists between the hydrophobic regions thought most likely to contain the hormone-binding domain (3, 8). Within the steroid-binding domain of the glucocorticoid receptor three amino acids, which interact with the steroid, have recently been identified by radiosequence analysis of photoaffinity labeled and affinity labeled receptor-hormone complexes (8-10). Photoinduced coupling located the interaction site for the A-ring of the steroid to 2 residues (methionine and cysteine) which, although proximal in the steroid-binding mode, are widely spaced (134 amino acids) along the polypeptide chain (8). The results suggest that the proline folds to accommodate this favored binding conformation (8). A cysteine residue, positioned between these photoaffinity labeled amino acids, was identified by alkylation with dexamethasone 21-mesylate (11) as the interaction site for the D-ring of the steroid (8-10).

The non-steroidal antiestrogen tamoxifen aziridine1 is an excellent labeling reagent for estrogen receptors (12). Evidence suggests that antiestrogens may compete allosterically rather than directly with estradiol for the hormone-binding site (13). An alternative mode of action may result in antiestrogen occupying the binding cavity but interacting with additional features in the hormone-binding domain to give a receptor with different conformational properties to that induced by estradiol (14). Despite the uncertainties in the mechanism of action of antiestrogens the highly selective, covalent interaction between tamoxifen aziridine and estrogen receptors has been of considerable utility in studies of receptor structure and function (15-19).

Two recent studies on the structural analysis of the hormone-binding region of the estrogen receptor have used controlled proteolysis of [3H]tamoxifen aziridine-labeled receptors (17, 18). The attachment site for the reagent was shown to be restricted to a small region of the protein near the carboxyl terminus (17). In this paper we describe an extension of these studies and report on the sequence analysis of [3H]

1The abbreviations and trivial names used are: tamoxifen aziridine, (Z)-1-[4-[2,N-aziridinyl]ethoxy]phenyl]-1,2-diphenyl-1-butene; dexamethasone 21-mesylate, 1,4-pregnadien-9α-fluoro-l6α-methyl-11β,17α,21-trihol-5,20-dione 21-methylbutanate; triamcinolone acetonide, 1,4-pregnadien-9α-fluoro-11β,16α,17α,21-tetrol-3,20-dione-16, 17-acetonide; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; hER, human estrogen receptor; hER, bovine estrogen receptor; PTH, phenylthiobutylacetate; hsp 90, heat shock protein of M, 90,000; TRTFPKC, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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tamoxifen aziridine-labeled peptides derived by limit trypsin digestion from pure affinity labeled receptors. Our results indicate that the major interaction site for \([3H]\)tamoxifen aziridine with the bovine estrogen receptor lies close to the COOH terminus of the hormone-binding domain.

**MATERIALS AND METHODS**

**RESULTS**

Purification of \([3H]\)Tamoxifen Aziridine-labeled Estrogen Receptors—Two different approaches were used for the preparation of highly purified \([3H]\)tamoxifen aziridine-labeled estrogen receptors prior to enzyme digestion and sequence analysis. Bovine estrogen receptor was purified initially by sequential chromatography on cellulose phosphate and heparin-Sepharose followed by affinity chromatography using \([3H]\)tamoxifen aziridine for receptor recovery (19). This method, which includes sodium molybdate in purification buffers, isolates the \(M_r \approx 65,000\) estrogen receptor associated with bovine heat shock protein hsp 90 (19–21). Small amounts of proteolyzed receptor, ranging in size from 50,000 to 60,000, are also present in the purified extracts, with the \(M_r \approx 50,000\) component usually being most abundant (19). After electrophoretic separation by preparative SDS-PAGE, bands corresponding to the \(M_r \approx 65,000\) and 50,000 receptors were excised and the proteins were recovered by electroelution (20). Assuming a single binding site/receptor monomer, tritium estimation revealed the recovered fractions to contain 388 and 470 pmol of the labeled \(65,000\) receptor and partially purified receptor, respectively. Much lower quantities of the Peak 2 labeled component were recovered, 17 pmol were derived from the \(65,000\) receptor fragment, respectively. Silver staining of analytical SDS-PAGE gels showed single bands for both receptor forms which coincided with the migration of protein-bound \([3H]\)tamoxifen aziridine (Fig. 1, A and B).

The affinity gel used in the above isolation experiments has recently been incorporated in a rapid, single-step procedure which, in the absence of molybdate buffers, provides untransformed, hsp 90-associated estrogen receptor of 5–15% purity.\(^3\)

In batchwise experiments, using \([3H]\)tamoxifen aziridine as eluting ligand, this alternative purification method gave 1092 pmol of covalently labeled estrogen receptor as the major labeled component usually being most abundant (19). After electroeloretic separation by preparative SDS-PAGE, bands corresponding to the \(M_r \approx 65,000\) and 50,000 receptors were excised and the proteins were recovered by electroelution (20). Assuming a single binding site/receptor monomer, tritium estimation revealed the recovered fractions to contain 388 and 470 pmol of the labeled \(65,000\) receptor and partially purified receptor, respectively. Much lower quantities of the Peak 2 labeled component were recovered, 17 pmol were derived from the \(65,000\) receptor fragment, respectively. Silver staining of analytical SDS-PAGE gels showed single bands for both receptor forms which coincided with the migration of protein-bound \([3H]\)tamoxifen aziridine (Fig. 1, A and B).

**Reversed-phase HPLC Purification of \([3H]\)Tamoxifen Aziridine-labeled Tryptic Peptides**—Tryptic peptides were separated initially by reversed-phase HPLC using a short microbore column (2.1-mm internal diameter) and a linear 0–60% (v/v) acetonitrile/water gradient delivered over 40 min at a flow rate of 1 ml/min. Trifluoroacetic acid (0.1% v/v) was included in the mobile phase. The radioactivity profiles obtained from the three digestion products were all closely comparable. Fig. 3A shows the profiles generated from the pure \(M_r \approx 65,000\) and 50,000 receptor digests. These profiles were characterized by a major peak of radioactivity (Peak 1) which was eluted with ~42% acetonitrile (Fig. 3A). A second, much smaller peak (Peak 2) was observed at slightly higher acetonitrile concentrations (Fig. 3A). Extended column elution with 60% acetonitrile, during fractionation of the \(M_r \approx 50,000\) protein digest, led to the isolation of a third radiolabeled component (Peak 3) (Fig. 3A). A similar profile pattern was produced during analytical reversed-phase HPLC of the trypsin digest derived from partially pure \(6H\)-labeled receptor (Fig. 3B). Column elution conditions were altered to a linear 0–100% (v/v) acetonitrile/water gradient applied over 60 min at 1 ml/min. The steeper gradient caused decreased resolution between the labeled peptides of Peaks 1 and 2. A broad peak of radioactivity (Peak 3) was attributed to the third labeled component which was recovered with ~70% acetonitrile (Fig. 3B). These elution conditions for the Peak 3 component suggested the presence of a \(6H\)-labeled peptide with strong hydrophobic interaction properties.

For the most abundant peptide fraction (Peak 1) tritium estimation indicated yields of 70, 80, and 120 pmol isolated from the pure \(M_r \approx 65,000\) and 50,000 receptor preparations and the partially purified receptor extract, respectively. Much lower quantities of the Peak 2 labeled component were recovered, 17 pmol were derived from the \(M_r \approx 65,000\) receptor monomer and 20 pmol from the \(M_r \approx 50,000\) receptor fragment. The Peak 3-labeled peptide of \(M_r \approx 50,000\) receptor origin amounted to 35 pmol.

Further purification of the isolated \(6H\)-labeled peptides was carried out using microbore reversed-phase HPLC with decreased flow rates and alternative mobile phase conditions. The peptides of Peak 1 (\(M_r \approx 65,000\) receptor) were fractionated using a low pH mobile phase (0.1% v/v aqueous trifluoroacetic acid) and then purified further by reversed-phase HPLC using a linear 0–20% acetonitrile/water gradient at a flow rate of 0.5 ml/min. The eluted fractions were concentrated and subjected to amino acid analysis. The radioactivity profiles obtained from the two purified peptides are shown in Fig. 3. The two labeled peptides were separated by reversed-phase HPLC, with the major peak eluting at 50% acetonitrile (Peak 1) and the minor peak eluting at 60% acetonitrile (Peak 2). The peptides were identified by amino acid analysis and sequence determination of trypsin-digested fragments. The results are summarized in Table I.
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Fig. 3. Reversed-phase HPLC of [3H]tamoxifen aziridine-labeled peptides. A, separation of radiolabeled peptides obtained from limit trypsin digestion of the pure M, ~ 65,000 (-----) and M, ~ 50,000 (· · · ·) affinity labeled receptors. Chromatographic conditions: column, Brownlee RP-300 (30 X 2.1-mm inner diameter), linear 40-min gradient from 0 to 100% B, where solvent A was 0.1% (v/v) trifluoroacetic acid and solvent B was 60% (v/v) acetonitrile, 40% water containing 0.1% trifluoroacetic acid, flow rate, 1 ml/min. Fractions were collected every 30 s, and 5-μl aliquots were withdrawn from each for tritium estimation. B, analytical separation of 3H-labeled peptides derived by limit trypsin digestion of affinity chromatography purified estrogen receptors covalently labeled with [3H]tamoxifen aziridine. Chromatographic conditions: column, Brownlee RP-300 (30 X 2.1-mm inner diameter), linear 60-min gradient from 0 to 100% B, where solvent A was 0.1% (v/v) trifluoroacetic acid and solvent B was acetonitrile containing 0.1% (v/v) trifluoroacetic acid, flow rate, 1 ml/min. Fractions were collected manually into counting vials every 30 s for tritium estimation.

Ninety two % of the applied radioactivity was recovered and eluted in two consecutive fractions (retention time 44.59 min), synchronous with a major peak of peptide absorbance (Fig. 4A). Under the same chromatographic conditions, fractionation of Peak 1 peptides, derived from partially pure estrogen receptor, gave a radioactivity profile which was superimposable on that obtained for the corresponding M, ~ 65,000 receptor fraction (Fig. 4B). The peptides of Peak 1 (M, ~ 50,000 receptor) were selected for further purification using a linear water-acetonitrile gradient with unbuffered sodium chloride (1%) as the mobile phase. Two major peptide peaks, eluting at 37.59 and 38.19 min, were resolved with this chromatographic dimension and the recovered radioactivity (61% of that applied to the column) was found to be evenly distributed between them (Fig. 5).

The above separation systems were also employed for the microbore HPLC fractionation of Peak 2 peptides combined from both radiolabeled M, ~ 65,000 and 50,000 estrogen receptors. The small, observed peak of radioactivity corresponded to only a minor peptide in the 215 nm absorbance profile (not shown) and the low levels of material precluded further analysis of the labeled fragment by Edman degradation. Similar results were obtained with Peak 3 peptides isolated from the M, ~ 50,000 proteolyzed receptor form.

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tides—HPLC-purified, radiolabeled peptides were loaded onto a polybrene-treated sequenator sample disc. Prior to Edman degradation, the sample was treated with trifluoroacetic acid, extracted with butyl chloride, precoupled with phenylisothiocyanate and trimethylamine and extracted with heptane and ethylacetate. Analysis of the major ^2H-labeled component isolated from the Peak 1 (M_r ~ 65,000 receptor) fraction (see Fig. 4A) identified a mixture of two peptides (T_1, T_2) (Table I and Fig. 6). The amino acid sequences of the two fragments showed close correspondence with two tryptic peptides predicted from the cDNA-derived amino acid sequence of the human estrogen receptor (1, 2) (Fig. 6). The residues of peptide T_2 are fully conserved in all estrogen receptors (1-6) (Fig. 6). Alignment of the T_1 and T_2 sequences with the cDNA-deduced amino acid sequence of the human estrogen receptor indicated that both peptides reside close to the COOH terminus of the hormone binding region of the protein (7).

The radioactivity monitored in PTH amino acid extracts, after each degradation cycle, was represented only a small fraction of the peptide-associated radioactivity applied to the sequencer. The bulk of the tritium label was located in the waste solvent container of the sequenator. These results suggested that the covalent link between the peptide and labeled tamoxifen aziridine was labile to the strongly acidic and/or basic conditions encountered during the Edman degradation. A small quantity of the equivalent, rechromatographed Peak 1 (partially purified receptor) fraction (see Fig. 4B) was loaded onto the sequencer and radioactivity was sampled from washes recovered after each predegradation treatment. Little radioactivity was collected after Edman degradation the sample was exposed to trifluoroacetic acid, which have no apparent sequence homology. A comparison of the primary structures of the human estrogen and glucocorticoid receptors showed only a 30% positional identity over the 250-residue long steroid-binding domain (5). Application of the physical parametric approach (33) to the sequence comparison of the two proteins in this region indicated an excellent predicted structural similarity between both receptors (Fig. 7).

In the rat glucocorticoid receptor, residues 622, 656, and 754 were found to be alkylated either by photoinduced reaction with the A-ring of triamcinolone acetonide (Met-622 and Cys-754) (8) or by electrophilic affinity labeling via the D-ring mesylate functionality of dexamethasone 21-mesylate (Cys-656) (8, 9). The labeled residues correspond to Met-604, Cys-638, and Cys-736 in the human glucocorticoid receptor (8, 34). As seen in Fig. 7, segments of the glucocorticoid

![Fig. 7. The summary of the smoothed amino acid physical characteristics plots for the human estrogen receptor (solid curve) and glucocorticoid receptor (dashed curve) versus the amino acid sequence number of steroid-binding domain residues.](image-url)
receptor which included these 3 residues gave parametric curves which were closely correlated with those of the estrogen receptor. The result suggests that these corresponding regions in the estrogen-binding protein may represent the equivalent contact domains for estradiol. Consistent with this proposal, human estrogen receptor peptides, which correspond to the sequenced fragments T₁ (equivalent to hER residues 504–515) and T₂ (hER residues 532–548), are shown in Fig. 7 to be located proximal to Cys-736 of human glucocorticoid receptor, the expected site for A-ring steroid interaction (8). The predicted secondary structure (35) in this region for both receptors is strikingly similar. Cys-736 in the glucocorticoid receptor marks the start of a β-sheet. For the estrogen receptor a predicted β-sheet structure begins at position 532 which corresponds to the first amino acid residue (asparagine) of peptide T₂.

**DISCUSSION**

In the present study we have shown that limit trypsin digestion of homogeneous and partially purified estrogen receptors, labeled covalently with [3H]tamoxifen aziridine, generates a consistent pattern of one major labeled fragment and two additional radiolabeled peptides present in lower concentrations. With the more abundant component, our strategy of using multidimensional microbore HPLC to isolate labeled peptides prior to radiosequence and amino acid sequence analysis was partially successful and provided sequence information on two tryptic fragments (T₁ and T₂) derived from the steroid-binding domain. While it is possible that both peptides play a role in the interaction of tamoxifen aziridine with the receptor only one (peptide T₂) is favored to contain the affinity labeled residue. Based on homologous sequences in steroidogenic enzymes, steroid receptors, and a steroid-binding protein a consensus sequence has recently been proposed which identifies the steroid binding region in these proteins (36). It is of note that the linear sequence of peptide T₂ overlaps this consensus site (36). Our evidence suggests that the interaction site for tamoxifen aziridine with the estrogen receptor is positioned close to the carboxyl terminus of the protein. This result is compatible with the photoinduced, covalent interaction of the corresponding region in the glucocorticoid receptor with the A-ring of the glucocorticoid derivative triamcinolone acetonide (8).

The covalent link between tamoxifen aziridine and estrogen receptors is stable to a wide range of denaturing conditions including hot solvent extraction, boiling in the presence of mercaptoethanol/sodium dodecyl sulfate, and acid fixation after polyacrylamide gel electrophoresis (12, 19). Our present results indicate the facile cleavage of the affinity label-peptide coupling by trimethylamine during routine precycling treatments carried out prior to Edman sequencing. It appears that the cleavage reaction generates an unaltered amino acid, and the previously labeled residue can then be sequenced in the normal mode. Several nucleophiles including aspartic acid, cysteine, histidine, lysine, methionine, and tyrosine have been implicated in the interaction of steroids with enzymes and steroid hormone-binding proteins (8, 37–44). In the estrogen receptor an early study by Ikeda (37) indicated that a sulfhydryl group proximates the D-ring of the steroid, and recent evidence suggests that a histidine residue or an unusually reactive tyrosine may also be important for steroid binding (45). Five nucleophilic residues, aspartic acid, glutamic acid, histidine, methionine, and tyrosine (all with a potential for affinity alkylation by tamoxifen aziridine) are present in peptide T₂, the fragment thought most likely to contain the labeled amino acid. Our initial hydrolytic experiments dis-
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Receptor assays: Receptor isolated from affinity chromatography with radiolabeled estradiol was estimated by an exchange assay [31] after adsorption of the steroid-receptor complex to hydroxyapatite.

Radioactivity measurements: Radioactivity was measured in a 14000 Becquerel liquid scintillation counter. Samples were counted in toluene-based three solution scintillation fluid (I:3:2). Quench correction was automatic after external standardization using the "B" quench method.

**Fig. 1** Analytical SDS-PAGE of [3H]-estradiol-labeled estrogen receptors prepared from preparation 1 by electrophoresis. Affinity labeled receptor, and non-affinity labeled receptor were prepared as described under Materials and Methods. After preparative SDS-PAGE the electrophoretic aliquots of the purified estradiol receptor (1 and 2) were electrophoresed through a polyacrylamide gel. Proteins were fixed and visualized by silver staining. Tritium content profiles were determined for both proteins as described under Materials and Methods. Standard proteins used as molecular weight markers were phosphorylase a, 97 kDa, bovine serum albumin, 67 kDa, ovalbumin, 43 kDa, carbonic anhydrase, 30 kDa, BSA - bromophenol blue.

**Fig. 2** Cation chromatography of estradiol-labeled estrogen receptors isolated from preparation 1. Chromatographic conditions were as described in Fig. 1 except that eluent A was 0.1 M sodium chloride and solvent B was 6 M urea in sodium chloride. The fraction indicated by the horizontal bar was taken for sequence analysis.

**Table 1**

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