Specific Photoaffinity Labeling of Tyr-49 on the Light Chain in the Steroid-Combining Site of a Mouse Monoclonal Anti-Estradiol Antibody Using Two Epimeric 6α- and 6β-(5-Azido-2-nitrobenzoyl)amidoestradiol Photoreagents[†,||]

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ABSTRACT: A mouse monoclonal anti-7-(*O*-carboxymethyl)oximinoestradiol antibody was photoaffinity labeled with two cross-reactive 6α- and 6β-(5-azido-2-nitrobenzoyl)amido[17α-^{3}H]estradiol photoreagents (6α- and 6β-ANBA-[17α-^{3}H]estradiol). Covalently bound radioactivity was found exclusively on the light chain. The maximal level of specific incorporation was 0.18 mol of label per mole of antibody for both photoreagents. In both cases, tryptic digestion of the photolabeled light chain, immunopurification with the immobilized antibody, reverse-phase liquid chromatography, and Edman degradation showed the presence of radioactive peptide GLM-([^{3}H]X)-HGNTLEDGIPSR derived from peptide 46−61 of the light chain sequence (determined from cDNA) in which the unidentified amino acid corresponding to X is a Tyr residue. Two other radioactive peptides were also isolated, one corresponding probably to the methionine sulfoxide derivative of the peptide 46−61 photolabeled with the 6β-reagent and the other to the N-terminal tetrapeptide 46−49 of the peptide 46−61 photolabeled with the 6α-reagent. In all cases, the main peak of radioactivity was released at the fourth Edman cycle, thus suggesting that the same Tyr-49 residue on the light chain was photolabeled. This residue is contiguous to the N-terminal amino acid of the second hypervariable complementary determining region 50−56 of light chain. Covalent labeling was confirmed by mass spectrometry of photolabeled peptides which showed molecular ion values corresponding to the addition of the photoactive 6α- or 6β-ANBA-estradiol nitrene derivatives to the peptide.

Anti-estradiol antibodies are widely employed for immunoassays of estradiol in clinical biology. Much effort has been devoted to developing monoclonal anti-steroid antibody reagents, but the access to binding specificities and affinities suitable for direct and sensitive immunoassays, such as that of estradiol, proved to be difficult. In the course of investigations currently made in our laboratory on the antibody response to structural modifications of the steroid hapten structure, monoclonal antibodies have been obtained against a relatively rigid 7-CMO-estradiol[1] hapten, among which the antibody 15H11 showed a high specificity for the phenolic A ring of estradiol and a much lower specificity for modifications at the 6 and 16 positions.

The present study was undertaken with the goal of localizing the part of the anti-estradiol antibody-combining site in the vicinity of the hapten link which may also cause the low specificity for modifications at the 6 position. The identification of amino residues in this region by affinity labeling [cf. Givol (1977), Lindeman et al. (1975), and Richards (1980)] could provide useful information for site-directed mutagenesis experiments (Schildbach et al., 1993) aimed at modifying the specificity for the steroid B ring and the covalent links inserted between the steroid and labeled molecules in steroid tracers. In this work, the identification of interacting amino acid residues was undertaken by photoaffinity labeling with a 5-azido-2-nitrobenzoyl chromophore coupled through an amide bond to radioactive 6α- or 6β-aminoestradiol.

EXPERIMENTAL PROCEDURES

Chemicals and Buffers. [2,4,6,7,16,17-$^3H_6$]Estradiol (159 Ci/mmol) and [^{3}H]$NaBH_4$ (5.8 Ci/mmol) were purchased

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[||] The sequences have been deposited with GenBank (L chain, BankIt 111346 AF 001866; H chain, BankIt 111352 AF 001867).

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[1] Abbreviations: 6-ANBA-estradiol, 6-(5-azido-2-nitrobenzoyl)-amidoestradiol; ANB-NOS, *N*-[(5-azido-2-nitrobenzoyl)oxy]succinimide; CDR, complementary determining region; 6- or 7-CMO-estradiol, 6- or 7-*O*-(carboxymethyl)oximinoestra-1,3,5(10)-triene-3,-17β-diol; CNBr, cyanogen bromide; DCC, dextran-coated charcoal; DHAS, dehydroepiandrosterone sulfate; DHT, 5α-dihydrotestosterone; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; LSIMS, liquid secondary ion mass spectrometry; FR, framework region; H chain, heavy chain; HPLC, high-performance liquid chromatography; L chain, light chain; LMP-agarose, low-melting point agarose; mAb, monoclonal antibody; OD, optical density; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PTH, phenylthiohydantoin; PVDF, polyvinylidene difluoride; RIA, radioimmunoassay; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TLC, thin-layer chromatography.

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from Amersham. L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone−trypsin (TPCK−trypsin) was from Sigma. The following were also used: Tris-HCl at pH 9.0 [0.05 M tris(hydroxymethyl)aminomethane hydrochloride at pH 9.0], Tris-HCl at pH 8.0 (0.1 M Tris-HCl at pH 8.0), Tris-gelatin (0.05 M Tris-HCl and 0.1% gelatin at pH 9.0), Tris-urea (0.1 M Tris-HCl and 6 M urea at pH 8.0), PBS (0.01 M sodium phosphate buffer and 0.15 M NaCl at pH 7.4), and DCC (5 g of Norit A and 0.5 g of dextran T-70 in 1 L of PBS).

Synthesis of Radioactive Photoaffinity Labeling Reagents. A mixture of 6α- and 6β-aminoestrone epimers (1.5 mg or 5.3 μmol in 100 μL of ethanol containing 10 μL of pyridine), prepared by chromic oxidation (Collins et al., 1968) of the 17-hydroxy group of unseparated 6-aminoestradiol epimers (Luppa et al., 1994), was reduced with a solution of [^{3}H]-$NaBH_4$ in ethanol (30 μL, 15 mCi) for 48 h at 4 °C. The reaction mixture was purified by TLC on silica gel (Macherey-Nagel, Alugram SIL G/UV$_{254}$, 100:20:5 chloroform−CH_3OH−NH_4OH) to give an unseparated mixture of 6α- and 6β-amino[17α-^{3}H]estradiol epimers (specific activity of ∼1.45 Ci/mmol, estimated as $^1/_4$ of that of the commercial [^{3}H]$NaBH_4$). N-Acylation of this mixture of amino precursors (250×10^6 cpm in 150 μL of pyridine) with *N*-[(5-azido-2-nitrobenzoyl)oxy]succinimide (ANB-NOS reagent purchased from Sigma) overnight, in the dark, led to the 6α- and 6β-ANBA-[17α-^{3}H]estradiol photoreagents (∼1.45 Ci/mmol, radiochemical purity of >95%) which were separated by reverse-phase HPLC on a C_{18} column (Shandon Kromasil 5 μm, 250 × 4.6 mm, 70:30, methanol−water, retention times of 6 and 7 min, respectively) using a radiochromatography detector (Flo-one, Packard Instruments). The radioinert 6α- and 6β-ANBA-estradiol analogs were characterized by UV absorbance [λ_{max}(EtOH) at 291 nm ($\epsilon = 11\,100$ M^{-1} cm^{-1}) and 305 nm ($\epsilon = 11\,000$ M^{-1} cm^{-1}) for both epimers], LSIMS ([M + H]$^+$ at *m/z* 478.4), and nuclear magnetic resonance [^{1}H-NMR (CD_3OD) δ (6α-isomer): 0.81 (3H, s, CH_3-18), 3.67 (1H, t, J = 8 Hz, H-17), 5.28 (1H, dd, J = 11 Hz and J = 6 Hz, H-6β), 6.65 (1H, dd, J = 8.4 Hz and J = 2.5 Hz, H-2), 6.89 (1H, d, J = 2.5 Hz, H-4), 7.15 (1H, d, J = 8.4 Hz, H-1), 7.22 (1H, d, J = 2.5 Hz, H-6′), 7.35 (1H, dd, J = 8.9 Hz and J = 2.5 Hz, H-4′), 8.20 (1H, d, J = 8.9 Hz, H-3′). ^{1}H-NMR (CD_3OD) δ (6β-isomer): 0.78 (3H, s, CH_3-18), 3.67 (1H, t, J = 8 Hz, H-17), 5.13 (1H, pseudo-d, J = 3.3 Hz, H-6α), 6.68 (1H, dd, J = 8.5 Hz and J = 2.6 Hz, H-2), 6.78 (1H, d, J = 2.6 Hz, H-4), 7.09 (1H, d, J = 2.5 Hz, H-6′), 7.18 (1H, d, J = 8.5 Hz, H-1), 7.30 (1H, dd, J = 8.9 Hz and J = 2.5 Hz, H-4′), 8.16 (1H, d, J = 8.9 Hz, H-3′).

Production and Purification of the Monoclonal Anti-Estradiol Antibody 15H11. Ten male, 8-week-old, BALB/c mice (IFFA-CREDO, L'Arbresle, France) were immunized with a 7-CMO-estradiol−BSA immunogen. The spleen cells of the selected donor mouse were then fused with P3/X 63Ag8.653 nonsecreting myeloma cells (Radbruch et al., 1980), following essentially a previously described methodology (Parvaz et al., 1989). The cell culture supernatants were screened by RIA for their binding activity for a tritiated estradiol tracer. One hybridoma 15H11, showing the highest binding activity, was selected and expanded in ascitic fluid obtained from nude mice. The immunoglobulin class was determined from culture supernatants by an ELISA method using a commercial anti-mouse immunoglobulin kit (Pierce 37501).

The ascitic fluid was purified on Protein G−agarose (Pierce kit 44441), diafiltered with a 50 kDa centrifugal concentrator (Macrosep, Pall-Filtron Technology Corp., Northborough, MA) against a 0.001 M phosphate buffer to reach a final antibody concentration of 10 mg/mL, as measured by UV absorption at 280 nm using the reported OD of 1.35 for 1 mg of IgG/mL (Harlow & Lane, 1988), and frozen.

cDNA Sequence Determination. Total cellular RNA was extracted from hybridoma 15H11 cells. cDNA was synthesized by reverse transcriptase (CM-MLV at 200 units/μL, GIBCO-BRL-Life Technologies) from 10 μg of RNA complexed with an oligo(dT) 12−18 (USB 71540, United States Biochemicals) and was purified by electrophoresis on a LMP-agarose gel. Extracted cDNA (100 μg) was tailed with a poly(dG) anchor (Frohman et al., 1988; Plaza et al., 1991; Marche et al., 1992; Churchill et al., 1994) using terminal deoxynucleotidyl transferase and dGTP. cDNA−poly(dG) (0.5 μg) was amplified by PCR (DNA thermal cycler from Perkin-Elmer Cetus) in the presence of the universal poly(dC) primer (5′ATGCATGCCGCGGCCGC-CCCCCCCCCCC3′) and one of two antisense primers specific either for κ L chains (5′TAGCGGCCGCTCACTG-GATGGTGGGAAGATGG3′) or for γ_1 H chains (5′AT-GCGGCCGCTCCAGGGGCCAGTGGATAGACA3′), both containing a *Not*I site (underlined). The DNA of the appropriate size was isolated on a LMP-agarose gel, digested with the *Not*I enzyme (Boehringer), inserted in the plasmid pBlueScript SK$^+$ (Stratagene), and cloned into *Escherichia coli* JM 101 cells (Stratagene). Amplified DNA was sequenced by standard methods (Sambrook et al., 1989) with T7 polymerase (Amersham kit) and T3 and T7 as primers, either directly on extracted double-stranded plasmids or on single-stranded DNA induced with phage helper M13K07.

Antibody Binding Characteristics. Antibody solutions in Tris-gelatin buffer (200 μL) were incubated for 15−16 h at 4 °C with [^{3}H]estradiol (20 000 dpm, 57×10^{-3} pmol in 100 μL of the same buffer), in the absence (for titration curves) or in the presence (for displacement curves) of increasing amounts of radioinert steroids (seven concentrations ranging from a 0.5- to 25-fold molar excess per steroid binding site), after adjustment of the total incubation volume at 800 μL with Tris-gelatin buffer. The unbound steroid fraction was separated by incubation with 1 mL of DCC suspension for 10 min at 4 °C and centrifugation (3000 rpm for 15 min at 4 °C). Radioactivity of supernatants was measured in 4 mL of scintillation fluid (Scintillator Plus, Packard).

Association constants were estimated by equilibrium dialysis using Scatchard plots (Scatchard, 1949).

Size-Exclusion HPLC, Chromatofocusing, and Gel Electrophoresis. Size-exclusion HPLC was performed on a Superdex 200 HR 10/30 column (Pharmacia) equilibrated and run with either 0.05 M sodium phosphate buffer and 0.15 M NaCl at pH 7.0 (for antibody purification) or Tris-urea and 0.1 M β-mercaptoethanol buffer (for H and L chain separation), at a flow rate of 0.5 mL/min.

Chromatofocusing was performed on a Mono P HR 5/20 column (Pharmacia) using a 0.025 M bis-Tris-HCl start buffer at pH 7.1 and a 1:9 Polybuffer 74 (Pharmacia)−water

elution mixture adjusted to pH 5 with HCl, at a flow rate of 1 mL/min.

Separations by SDS−PAGE and detection of radioactive bands by fluorography (autoradiography) were performed as described previously (Grenot et al., 1994). Radioactive protein bands were quantified with an image analysis system (SAMBA 2005, Alcatel TITN, Meylan, France). Electroblotting of the peptides separated by gel electrophoresis was performed on Immobilon-P transfer membranes (PVDF filters of 0.45 mm, Millipore), according to a standard procedure (Matsudaira, 1987).

Photoaffinity Labeling. The purified antibody (5 mg, 33.3 nmol, i.e. 66.6 nmol of steroid binding sites) was incubated with 6α- and/or 6β-ANBA-estradiol (66.6 nmol), containing the corresponding 6α- and/or 6β-ANBA-[17α-^{3}H]estradiol derivative (20×10^6 dpm, 6.5 nmol), in 3.33 mL of Tris-HCl buffer at pH 9.0 in the absence or in the presence of estradiol (266 nmol) overnight at 4 °C in the dark under an argon atmosphere. The unbound steroid was separated by treatment of the incubation mixture for 10 min at 4 °C in the dark under an argon atmosphere with dry DCC (prepared by centrifugation of 2 mL of the DCC suspension), followed by centrifugation (4000 rpm for 10 min at 4 °C). The supernatant was degassed with argon and transferred into 0.4 × 12 cm glass tubes (0.8 mL per tube) disposed at a distance of 3 cm from the external wall of a refrigerated high-pressure mercury lamp (Hanovia, 450 W). The solutions were maintained at 4 °C under an argon atmosphere and irradiated at a λ of >300 nm using a 2 mm thick Pyrex filter.

Separation of H and L Chains from Photoaffinity-Labeled Antibody. The irradiated mixture was dried using a Speed-Vac concentrator, and the antibodies (5 mg, 33.3 nmol) were completely reduced and alkylated after treatment with DTT (5 mg, 32.4 μmol) in 500 μL of Tris-urea, for 1 h at 60 °C, and iodoacetamide (10 mg, 54 μmol), for 20 min at room temperature, in the dark under an argon atmosphere. Reduced and alkylated H and L chains were separated by size-exclusion chromatography (Superdex 200 HR 10/30 column with Tris-urea and 0.1 M β-mercaptoethanol buffer at 0.5 mL/min). The isolated chains were diafiltered with a 10 kDa centrifugal concentrator against 3 × 2.5 mL of Tris-urea to reach a final volume of 0.4 mL and precipitated with a 4-fold volume of cold acetone for 15 min at 4 °C. After centrifugation (3000 rpm for 15 min at 4 °C), the residue was dissolved in 0.4 mL of Tris-urea, precipitated as before, and dried under a nitrogen stream.

CNBr Cleavage of the Photoaffinity-Labeled Antibody L Chain. The isolated photoaffinity-labeled antibody L chain sample recovered after the diafiltration step was dialyzed against 0.2 M NH_4HCO_3 at pH 8.2 and lyophilized. The dry residue (200 μg) was dissolved in 70% formic acid (200 μL) and treated with an excess of CNBr (4 mg) for 16 h at room temperature in the dark under a nitrogen atmosphere (Lifter et al., 1974). The reaction mixture was diluted 10 times with water and lyophilized.

Tryptic Cleavage of the Photoaffinity-Labeled Antibody L Chain. The isolated photoaffinity-labeled antibody L chain was suspended in 200 μL of Tris-HCl buffer at pH 8.0. Enzymatic cleavage was performed by two successive incubations with TPCK−trypsin (10% enzyme:protein ratio w:w) for 5 h at 37 °C, which progressively solubilized the precipitate until complete hydrolysis could be achieved (controlled by SDS−PAGE).

Immunopurification and HPLC Purification of Photoaffinity-Labeled Tryptic Peptides. The crude trypsin hydrolysate was centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was diluted to a volume of 5 mL with Tris-HCl buffer and incubated overnight at 4 °C, with gentle stirring in the presence of an immunoaffinity gel (1 mL) prepared by covalent coupling of the purified anti-estradiol antibody 15H11 (7 mg) to CNBr-activated Sepharose 4B (1 mL). The gel was rinsed with 1 M NaCl (4 × 2 mL) and distilled water (2 × 2 mL), and the retained peptides were eluted with a 7:3 water−dioxane (v:v) mixture (10 × 2 mL).

The mixture of peptides recovered from the immunoaffinity column was separated by reverse-phase HPLC on a C_{18} column (Macherey-Nagel Nucleosil-100 Å, 5 μm, 150 × 4.6 mm) using an aqueous acetonitrile gradient (0−4 min with 0% acetonitrile, 4−9 min with 0 to 25% acetonitrile, and 10−50 min with 40 to 100% acetonitrile) in the presence of 0.1% TFA. Peptide profiles were monitored by UV absorption at 220 nm, and radioactivity profiles were determined on-line with a Flo-one Packard radiodetector. The fractions containing radioactive peptides were evaporated under a stream of nitrogen and stored at 4 °C in 200 μL of water.

Edman Sequence Determinations. Automated Edman degradation of the N-terminal part of antibody L chain and of photolabeled peptides was performed in a gas-phase sequencer equipped with an on-line phenylthiohydantoin (PTH) analyzer (Applied Biosystems, model 470 sequencer). Peptides electroblotted on Immobilon-P membranes were sequenced directly from the membrane.

Mass Spectrometry. Electrospray and liquid secondary ion mass spectra of the steroid−peptide conjugates photolabeled with 6α- and 6β-ANBA-[17α-^{3}H]estradiol (∼200 pmol) were recorded with Hewlett-Packard 5989 and VG ZAB 2SEQ mass spectrometers, respectively, as reported (Grenot et al., 1994), using a capillary exit voltage of 300 V and a quadrupole scanning from *m/z* 250 to 1500 for the electrospray technique and a glycerol−1% TFA matrix for liquid secondary ion mass spectrometry.

RESULTS

Production, Purification, and Characterization of the Monoclonal Anti-Estradiol Antibody 15H11. The mouse monoclonal anti-7-CMO-estradiol antibody 15H11 was characterized as an IgG_1 of the κ class and was therefore purified from mouse ascites fluid by chromatography on immobilized Protein G. The purified immunoglobulin was controlled by size-exclusion HPLC, which showed essentially a single peak representing more than 97% of the total absorbance at 280 nm by SDS−PAGE. Chromatofocusing revealed a single peak corresponding to an isoelectric point of 5.15. Completely reduced and alkylated H and L chains were separated by size-exclusion HPLC as two distinct peaks (UV detection at 280 nm) with retention times of 19 and 24 min, respectively. The L chain was characterized by Edman sequencing of 15 amino acid residues (DIVMTQSPASIS-VSL) of the N-terminal part. The H chain could not be sequenced, owing to the presence of an N-terminal Gln residue (Doolittle et al., 1972).

cDNA Sequence Determination. The nucleotide sequences for H and L chain variable regions (Figure 1) were

15H11 Light chain sequence:

1 FR1																		
gac	att	gtg	atg	acc	caa	tct	ccg	gcc	tcc	ata	tct	gtg	tct	ctg	gga	gac	aca	gtc	agc
D	I	V	M	T	Q	S	P	A	S	I	S	V	S	L	G	D	T	V	S
21			CDR1											FR2					
atc	act	tgc	cat	gca	agt	cag	gga	att	aac	agt	aat	ata	ggg	tgg	ttg	cag	ttg	aag	cca
I	T	C	**H**	**A**	**S**	**Q**	**G**	**I**	**N**	**S**	**N**	**I**	**G**	W	L	Q	L	K	P
41									CDR2							FR3			
ggg	aag	tca	ttt	aag	ggc	cta	atg	tat	cat	gga	aat	acc	ttg	gag	gat	gga	att	cca	tca
G	K	S	F	K	G	L	M	Y	**H**	**G**	**N**	**T**	**L**	**E**	**D**	G	I	P	S
61																			
agg	ttc	agt	ggc	agt	gga	tct	gga	gga	att	tat	tct	ctc	acc	ata	aat	agc	ctg	gaa	ttt
R	F	S	G	S	G	S	G	G	I	Y	S	L	T	I	N	S	L	E	F
81								CDR3									FR4		
gaa	gat	ttt	gcg	gtc	tat	tat	tgt	gtt	cag	tat	gct	cag	ttt	cct	cgg	acg	ttc	ggt	gga
E	D	F	A	V	Y	Y	C	**V**	**Q**	**Y**	**A**	**Q**	**F**	**P**	**R**	**T**	F	G	G
101						107													
ggc	acc	agg	ctg	gag	atc	aaa													
G	T	R	L	E	I	K													

15H11 Heavy chain sequence:

1 FR1																		
cag	gtt	ctc	cta	caa	cag	cct	ata	act	gag	ctg	gcg	agg	cct	ggg	act	tca	gtg	aaa	ctg
Q	V	L	L	Q	Q	P	I	T	E	L	A	R	P	G	T	S	V	K	L
21										CDR1					FR2				
tcc	tgc	aag	gct	tct	ggg	tac	gcc	ttt	cca	agc	cat	gtt	atg	agc	tgg	gtg	aaa	cag	ata
S	C	K	A	S	G	Y	A	F	P	**S**	**H**	**V**	**M**	**S**	W	V	K	Q	I
41									CDR2										
agt	gga	cag	ggc	ctt	gag	tgg	att	gga	gaa	atc	tat	ccg	aga	agt	gga	aat	tct	tat	tac
S	G	Q	G	L	E	W	I	G	**E**	**I**	**Y**	**P**	**R**	**S**	**G**	**N**	**S**	**Y**	**Y**
61						FR3													
aat	gag	aac	ttc	aag	gac	agg	gcc	aca	ctg	act	gca	gac	aga	tcc	tcc	aat	aca	gtc	tac
N	**E**	**N**	**F**	**K**	**D**	R	A	T	L	T	A	D	R	S	S	N	T	V	Y
81														CDR3					
atg	gag	gtc	agc	agc	ctg	acg	act	gaa	gac	tca	gca	gtc	tat	ttc	tgt	ggg	ttc	gga	aag
M	E	V	S	S	L	T	T	E	D	S	A	V	Y	**F**	**C**	**G**	**F**	**G**	**K**
101		FR4									112								
gag	tgg	ggc	cag	ggc	acc	act	ctc	aca	gtc	tcc	tca								
E	**W**	G	Q	G	T	T	L	T	V	S	S								

FIGURE 1: Nucleotide sequences of L and H chain variable regions (see Experimental Procedures). The CDR regions of each chain are underlined (nucleotides) or in bold characters (amino acids).

Table 1: Binding Specificity of Purified Monoclonal Anti-Estradiol Antibody 15H11 for Steroid Analogs

estradiol derivatives	cross-reaction[a] (%)	estradiol derivatives	cross-reaction (%)	other steroids	cross-reaction (%)
estradiol	100	7-oxo-	24	estrone	3
3-sulfate	0.2	7-CMO-	45	estrone 3-sulfate	<0.001
3-glucuronide	0.2	11α-OH-	<0.001	cortisol	<0.001
6-oxo-	71	16α-OH-(estriol)	38	testosterone	0.02
6-CMO-	19	17-hemisuccinate	4	progesterone	<0.001
6α/β-amino-	24	17-glucuronide	<0.001	DHT	0.02
6α-ANBA-	43			DHAS	0.45
6β-ANBA-	60			21-deoxycortisol	<0.001

[a] The percent cross-reactions were determined as the ratio of the moles of radioinert estradiol (×100) to the moles of competing steroids required to displace 50% of the [^{3}H]estradiol tracer.

determined using anchored PCR (Frohman et al., 1988; Plaza et al., 1991; Marche et al., 1992; Churchill et al., 1994). The $V_H/D/J_H$ region of the γ_1 H chain used the J558 family (Brodeur & Ribbet, 1984) or Kabat's group V_H-IIA (Kabat et al., 1987) gene of the murine V_H region, associated with a D segment showing a slight homology with the DHFL16 1 family, and a J_H-2 segment. The variable region of the H chain was found homologous (88%) with that of a Mus musculus anti-DNA murine antibody mRNA, clone BWBs3-Vkappa (Ohnishi et al., 1994). The V_K/J_K region of the L chain was found to correspond to the subgroup V of the murine V_K region, associated with a J_K-1 segment (Kabat et al., 1987). The variable region of the L chain was found to have a very high homology (91.2%) with that of a murine monoclonal antibody directed against the Tac protein of the human interleukin-2 receptor (Kaluza et al., 1991).

Antibody Binding Characteristics. The cross-reactions of estrogenic competitors (Table 1) revealed a relatively high specificity for the 3-hydroxy group toward 3-sulfate and 3-glucuronide conjugates, a partial lack of specificity for the 17β-hydroxy group toward 17-oxo and 17-hemisuccinate analogs, but a much lower specificity for the 16 position and particularly for the B ring through which the 7-CMO-estradiol hapten was linked to the immunogenic protein. The two 6α- and 6β-ANBA-estradiol photoreagents displayed significant cross-reactions (43 and 60%, respectively) and were displaced (up to 90−95%) in the presence of a 200-fold molar excess of estradiol.

The association constants for 6α- and 6β-ANBA-[17α-^{3}H]estradiol photoreagents (5 and 6×10^9 M^{-1}, respectively), estimated from Scatchard plots established by equilibrium dialysis experiments at 4 °C with purified antibody (Figure 2), were significantly lower than that measured similarly for estradiol ($\sim 2.4 \times 10^{10}$ M^{-1}). The number of binding sites per molecule of antibody was 1.5 and 1.6 for 6α- and 6β-ANBA-[17α-^{3}H]estradiol photoreagents, respectively, and 1.9 for estradiol.

Time Course of Photolysis of the Photoaffinity Labeling Reagents and of Unbound Antibody 15H11. Photolysis of 6α- and 6β-ANBA-estradiol photoreagents in a 9:1 Tris-

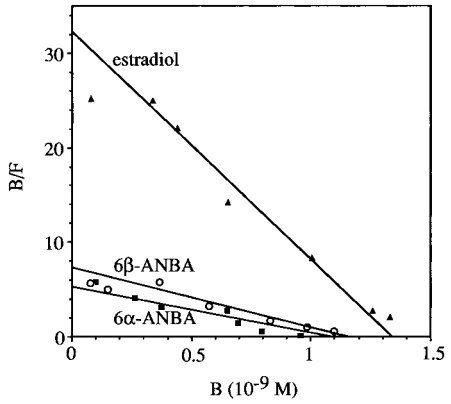

FIGURE 2: Scatchard plots for the binding of estradiol (▲), and 6α- (■) and 6β-ANBA-estradiol (○) photoreagents with purified monoclonal anti-estradiol antibody 15H11. The antibody solution at the dilution corresponding to the titer (0.7 pmol in 1 mL of Tris-HCl buffer at pH 9.0) was dialyzed for 48 h at 4 °C against increasing amounts of tritiated estradiol or photoreagents (0.2−10-fold molar excess per steroid binding site) placed outside the dialysis bag, in 10 mL of the same buffer.

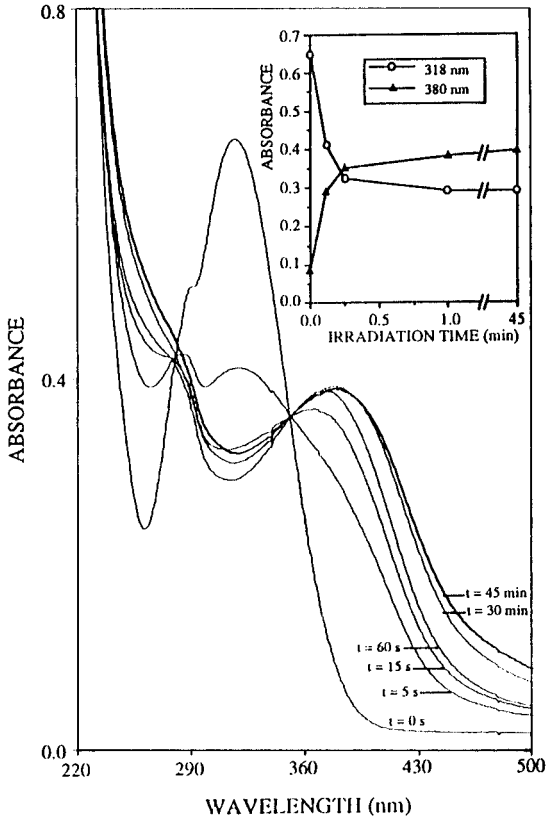

FIGURE 3: Time course of photolysis of 6β-ANBA-estradiol. Aliquots (1 mL) of a solution of 6β-ANBA-estradiol (30 μg/mL) in 0.05 M Tris buffer (pH 9.0) containing 10% ethanol were irradiated at 4 °C, under an argon atmosphere, as described in Experimental Procedures. Absorbance spectra were recorded between 220 and 500 nm immediately after irradiation, following exposure at a λ of >300 nm for 0 s, 5 s, 15 s, 1 min, 30 min, and 45 min.

HCl buffer (pH 9.0)−ethanol mixture, at a λ of >300 nm, using a 2 mm thick Pyrex filter (Taylor et al., 1980) was complete by about 10−15 s. Only the UV absorption spectra recorded for the more soluble 6β-epimer are shown (Figure 3) since no significant differences with respect to those of the 6α-epimer were observed.

Irradiation of unbound antibody 15H11 at a λ of >300 nm, for up to 30 min at 4 °C, did not alter the binding capacity for estradiol, while no significant photolytic cleavage of H and L chains was observed after SDS−PAGE separation.

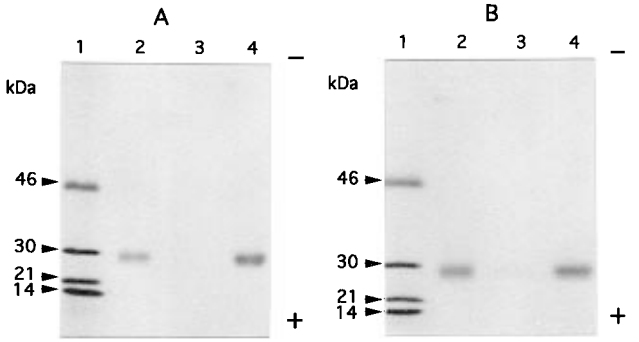

FIGURE 4: SDS−PAGE (10% acrylamide) analysis of H and L chains of completely reduced and alkylated anti-estradiol antibody 15H11 photolabeled with 6α- and 6β-ANBA-[17α-^{3}H]estradiol photoreagents. (A) Antibody 15H11 photolabeled with 6α-ANBA-[17α-^{3}H]estradiol, revealed by fluorography: ^{14}C molecular mass markers (Amersham) lysozyme, trypsin inhibitor, carbonic anhydrase, and ovalbumin (lane 1), an unseparated mixture of H and L chains (116 pM) (lane 2), separated H chain (232 pM) (lane 3), and separated L chain (232 pM) (lane 4). (B) Antibody 15H11 photolabeled with 6β-ANBA-[17α-^{3}H]estradiol, revealed by fluorography: ^{14}C molecular mass markers (lane 1), an unseparated mixture of H and L chains (116 pM) (lane 2), separated H chain (232 pM) (lane 3), and separated L chain (232 pM) (lane 4).

Photolabeling of Antibody 15H11 with 6α- and 6β-ANBA-[17α-^{3}H]Estradiol Photoreagents. Purified antibody 15H11 incubated with photoreagent (2 mol per mole of antibody) was irradiated at a λ of >300 nm, for 30 min at 4 °C, as reported elsewhere (Grenot et al., 1992, 1994), and completely reduced and alkylated. With both 6α- and 6β-ANBA-[17α-^{3}H]estradiol photoreagents, the covalently bound radioactivity was found exclusively on the L chain as shown by SDS−PAGE autoradiograms (Figure 4).

The time course of photoaffinity labeling was studied by measuring the radioactivity recovered from the tryptic fragments of the isolated L chains photolabeled with each of the 6α- and 6β-ANBA-[17α-^{3}H]estradiol epimers (*vide infra*). The maximal levels of covalently attached radioactivity (0.18 mol of label per mole of antibody in both cases) were reached after irradiation times ranging from 5 to 15 min, well beyond the time required for complete photolysis of photoreagents in the absence of antibody (<15 s). No further changes were observed after irradiation for up to 30 min. Control experiments without irradiation showed the absence of irreversibly bound radioactivity, thus indicating that photoactivation is necessary for covalent labeling. No residual nonspecific covalent labeling was detected by irradiation of the antibody incubated with either photoreagent (2 mol per mole of antibody) in the presence of an excess of estradiol (8 mol per mole of antibody), without prior charcoal treatment. Moreover, no photolabeling was detected in similar conditions after irradiation of the antibody incubated with tritiated estradiol (data not shown), thus ruling out a mechanism of covalent photoattachment of estradiol through a phenoxyl radical [cf. Sawada et al. (1993)].

CNBr Cleavage of the Photoaffinity-Labeled Antibody L Chain. The isolated antibody L chain photoaffinity-labeled with a 45:55 mixture of 6α- and 6β-ANBA-[17α-^{3}H]estradiol was cleaved by CNBr. After separation by SDS−PAGE (Figure 5), the bulk of the radioactivity was found to be concentrated at the level of the intermediate band at 14 kDa

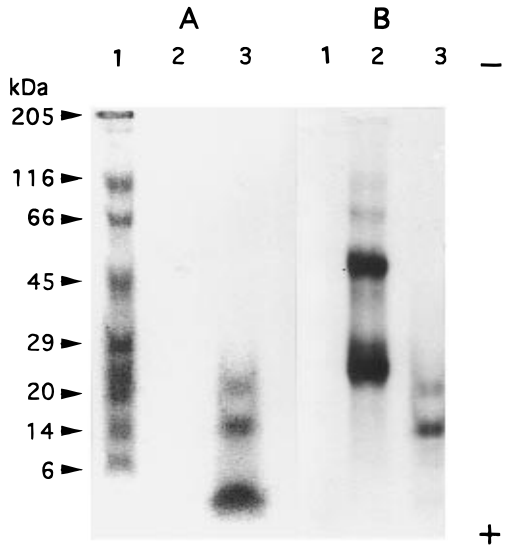

FIGURE 5: SDS−PAGE analysis of CNBr peptide fragments of the L chain of completely reduced and alkylated antibody 15H11 photolabeled with a 45:55 mixture of 6α- and 6β-ANBA-[17α-^{3}H]estradiol photoreagents. (A) CNBr peptide fragments of the L chain of antibody 15H11 photolabeled with a mixture of 6α- and 6β-ANBA-[17α-^{3}H]estradiol, revealed by staining with Coomassie blue: molecular mass markers (Sigma) aprotinin, α-lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, β-galactosidase, and myosin (lane 1), [^{14}C]IgG marker (lane 2), and CNBr peptide fragments of the L chain (lane 3). (B) Same gel revealed by fluorography.

assigned to the largest CNBr fragment 49−175 while a trace of radioactivity was observed at the level of the minor band at 25 kDa corresponding to the residual untransformed L chain.

Tryptic Cleavage of the Photoaffinity-Labeled Antibody L Chain. The tryptic cleavage was performed on completely reduced and alkylated L chains photoaffinity-labeled with 6α- or 6β-ANBA-[17α-^{3}H]estradiol. In all cases, the HPLC radioactivity profiles of the tryptic digests showed only two main radioactive peaks which could not be purified in acceptable yields by HPLC in the absence of a prior immunopurification step (*vide infra*).

Immunopurification and HPLC Purification of Photoaffinity-Labeled Tryptic Peptides. The radioactive tryptic peptides photolabeled with 6α- and 6β-ANBA-[17α-^{3}H]-estradiol were immunopurified (Wilchek et al., 1971) with the anti-estradiol antibody 15H11 immobilized on Sepharose 4B. Elution of the retained radioactive peptide fraction was performed with a 7:3 water−dioxane (v:v) mixture. The percentage of the recovery of the radioactivity for the immunopurified photolabeled peptides ranged from 75 to 92%.

The HPLC profile of the immunopurified tryptic peptides photolabeled with 6α-ANBA-[17α-^{3}H]estradiol showed the presence of two prominent radioactive tryptic peptide peaks, a minor one (retention time of 28 min) and a major one (retention time of 34 min) which was found to be either strongly diminished or absent when shorter incubation times were employed for enzymatic cleavage. The HPLC profile of the immunopurified tryptic peptides photolabeled with 6β-ANBA-[17α-^{3}H]estradiol showed a major radioactive tryptic peptide peak (retention time of 27.5 min) and a less-retained radioactive peptide (retention time of 25 min) which was found to increase with the time of storage in the presence of air, at the expense of the major peak. A third small radioactive peak was also found to be present between the two latter peaks, but the amount of radioactivity was too small for further characterization and could not be augmented by increasing the incubation time with trypsin. All photolabeled peptides showed a strong UV absorption at 405 nm, characteristic of the covalently attached 6α- or 6β-label which, in this work, could replace radioactivity for monitoring HPLC purifications.

Edman Sequencing of Photoaffinity-Labeled Peptides. Edman degradation of the N-terminal part of the major radioactive CNBr−peptide obtained from the photolabeled L chain revealed the sequence Tyr-His-Gly-Asn-Thr-Leu-Glu-Asp-Gly-Ileu-Pro-Ser-Arg, corresponding to the largest CNBr−peptide 49−175, while a major peak of radioactivity was eluted at the first cycle, thus suggesting the photolabeling of the Tyr-49-L residue.

The sequence determination of the less-retained tryptic peptide photolabeled with 6α-ANBA-[17α-^{3}H]estradiol and of the more-retained tryptic peptide photolabeled with 6β-ANBA-[17α-^{3}H]estradiol (Table 2) revealed in both cases the same single sequence Gly-Leu-Met-([^{3}H]X)-His-Gly-Asn-Thr-Leu-Glu-Asp-Gly-Ileu-Pro-Ser-Arg identified as the photolabeled peptide 46−61 of the L chain in which X corresponds to a Tyr residue. The more-retained radioactive tryptic peptide recovered after photolabeling with the 6α-reagent was found to correspond to the N-terminal tetrapeptide 46−49 resulting from subcleavage of peptide 46−61. On the other hand, the minor radioactive tryptic peptide isolated after photolabeling with the 6β-reagent showed the same sequence and radioactivity profile as peptide 46−61. For these four photolabeled peptides, no known amino acid residue could be identified at the fourth Edman cycle while the major peak of radioactivity (Table 2) was also eluted at this cycle, thus confirming a specific labeling of the same Tyr-49-L amino acid residue by the two 6α- and 6β-photoreagents.

Mass Spectrometry. To confirm the identity of the covalently attached steroid photolabels, the molecular masses of the steroid−peptide conjugates were determined by electrospray ionization mass spectrometry. The characteristic protonated $[M + 3H]^{3+}$ and $[M + 2H]^{2+}$ ions of the peptide 46−61 photolabeled with either 6α- or 6β-ANBA[17α-^{3}H]-estradiol appeared at *m/z* 737 and 1105, respectively, corresponding to a $[M + H]^+$ molecular ion value of 2209 identical to the calculated $[M + H]^+$ molecular ion value for the peptide Gly-Leu-Met-(*Tyr)-His-Gly-Asn-Thr-Leu-Glu-Asp-Gly-Ileu-Pro-Ser-Arg covalently photolabeled with the nitrene generated from the corresponding 6α- or 6β-photoreagents. The $[M + H]^+$ ion of the minor steroid−peptide conjugate photolabeled with 6β-ANBA-[17α-^{3}H]-estradiol was determined by electrospray ionization mass spectrometry at *m/z* 2225, showing an increment of 16 mass units, as compared with the corresponding major steroid−peptide conjugate, thus suggesting an oxidation of this latter peptide, presumably of methionine. The $[M + H]^+$ ion of the more-retained steroid−peptide conjugate with 6α-ANBA-[17α-^{3}H]estradiol at *m/z* 932 was in agreement with the calculated $[M + H]^+$ molecular ion values for the tetrapeptide Gly-Leu-Met-(*Tyr) photolabeled with the nitrene generated from the 6α-ANBA-[17α-^{3}H]estradiol.

Since recent reports have shown that electrospray ionization mass spectrometry could in some cases detect nonco-

Table 2: Amino Acid Sequence Analysis of Photolabeled Tryptic Peptides

		6α-ANBA-estradiol−peptides				6β-ANBA-estradiol−peptides			
		peptide 46−61		peptide 46−49		peptide 46−61		oxidized peptide 46−61	
		(60 394 dpm) 190 pmol[a]		(30 000 dpm) 95 pmol[a]		(21 105 dpm) 200 pmol[a]		(20 500 dpm) 200 pmol[a]	
cycle no.	amino acid	PTH derivative (pmol)	radioactivity (dpm)	PTH derivative (pmol)	radioactivity (dpm)	PTH derivative (pmol)	radioactivity (dpm)	PTH derivative (pmol)	radioactivity (dpm)
1	G	39	420	46	141	49	243	107	411
2	L	26	375	56	216	49	300	124	396
3	M	24	342	47	201	66	519	122	282[b]
4	X	(15)[c]	4701	(4)[c]	1092	(47)[c]	4962	(16)[c]	1638
5	H	3	2841		816	31	2091	15	867
6	G	7	1842		753	44	1155	59	609
7	N	8	1197			51	831	56	510
8	T	2	963			14	468	17	435
9	L	7	846			38	423	65	396
10	E	4	861			27	447	29	435
11	D	3	483			26	315	12	357
12	G	3	663			15	306	30	285
13	I	5	537			19	198	35	315
14	P	4	468			21	189	31	219
15	S	1	336			nd[d]	171	4	192
16	R	0.5	387			nd	168	2	222
17			300				111		798
18			264				135		243
filter			4147		nd		1272		2056

[a] Picomoles of labeled peptide applied to the sequencer calculated from the specific activity of the corresponding radioactive photoreagent. [b] Probably methionine sulfoxide residue, as suggested by mass spectrometry (see Results). [c] Picomoles of amino acid estimated from the radioactivity eluted at the fourth Edman cycle. [d] Not determined.

valent conjugates [cf. Siudzak (1994)], a control was also performed on the major peptide conjugate photolabeled with the 6β-photoreagent and on its minor oxidized contaminant, using liquid secondary ion mass spectrometry, which led to the same molecular ion values as those determined by electrospray ionization.

DISCUSSION

The monoclonal anti-estradiol antibody 15H11 was produced from a mouse immunized with a BSA−7-CMO-estradiol conjugate. The choice of a 7-CMO-estradiol hapten rather than of the more usual 6-CMO-hapten for undertaking these structural studies was made in an attempt to minimize stereoelectronic interactions between the hapten link and the phenolic A ring of estradiol.

In this study, affinity labeling of the combining site of monoclonal anti-estradiol antibody 15H11 was undertaken with a photoreactive chromophore introduced at the end of an amino function at the C-6 position rather than at the extremity of the 7-CMO link which may orient the chromophore outside the combining site. The two epimeric 6α- and 6β-ANBA-[^{3}H]estradiol derivatives were selected as photoaffinity reagents owing to their high cross-reactivity with antibody 15H11 and to the stability of their amide bond. The differences in orientations and conformations between the 6α- or 6β-amido links of these two photoreagents, as well as the relatively short distance between the photoreactive chromophore and the steroid molecule, may also increase the probability of a contact between the reactive nitrene and an amino acid residue in the immediate vicinity of the combining site for the steroid part of the hapten.

The L chain photolabeled with either 6α- or 6β-ANBA-estradiol was cleaved with TPCK−trypsin which led to a mixture of two radioactive peptides identified by Edman sequencing as the photolabeled peptide 46−61 in both cases, and either a subcleaved tetrapeptide 46−49, for the 6α-epimer, or the oxidized peptide 46−61, for the 6β-epimer. However, no radioactive subcleaved peptide 50−61 complementary to tetrapeptide 46−49 could be detected. The presence of the tetrapeptide photolabeled with 6α-ANBA-estradiol was due probably to the combined effects of some residual chymotryptic activity of TPCK−trypsin (Kerwin & Yount, 1992) and of the unusually large amount of enzyme required to obtain a complete tryptic digestion of the L chain, although the absence of a significant similar subcleavage for the peptide 46−61 photolabeled with 6β-ANBA-estradiol suggests that the stereochemistry of the covalently bound photoaffinity reagent may also play a role. The minor peptide photolabeled with 6β-ANBA-estradiol was found to correspond to an oxidized form of the major peptide, as confirmed by mass spectrometry, resulting probably from conversion of the Met-48 residue to methionine sulfoxide, although a less probable oxidative transformation of the photolabel cannot be excluded. Similarly, the identification of the major radioactive CNBr−peptide 49−175 obtained from the L chain of antibody 15H11 photolabeled with a 45:55 mixture of 6α- and 6β-photoreagents was confirmed by partial Edman degradation which revealed the N-terminal sequence Tyr-His-Gly-Asn-Thr-Leu-Glu-Asp-Gly-Ileu-Pro-Ser-Arg.

The elution of the major radioactive peak at the first cycle of Edman degradation of the photolabeled CNBr−peptide 49−175 from the L chain suggested the covalent photolabeling of the corresponding first Tyr-49 residue, but this localization could not be confirmed since a PTH derivative of Tyr was still identified in the absence of a prior immunopurification step.

For the two tryptic peptides 46−61 photolabeled respectively with 6α- and 6β-ANBA-estradiol, the amounts of PTH derivative of the modified Tyr, as estimated from the

radioactivity eluted at this fourth cycle, were lower but correspond in magnitude with the amounts of PTH derivatives measured at the preceding cycle, whereas much lower amounts of radioactivity were found at the fourth cycle of Edman degradation of the tetrapeptide or of the oxidized peptide. The relatively low levels of radioactivity recovered at the first three Edman cycles for all photolabeled peptides confirm that the covalently bound radioactivity found at the fourth cycle and at the subsequent cycles is rather stable under the conditions of Edman degradation. Therefore, a large part of the decreasing amounts of radioactivity observed after the fourth cycle probably corresponds to "carryover" effects due to incomplete Edman cleavage of the labeled Tyr residue. A marked decrease in the yields of PTH amino acid derivatives recovered after the fourth cycle was observed for the peptide 46−61 photolabeled with 6α-ANBA-estradiol, thus suggesting that the covalently bound 6α-photoreagent may partially decrease the rate of Edman degradation. The presence of radioactivity after the fourth Edman degradation step of the tetrapeptide 46−49 photolabeled with 6α-ANBA-estradiol and the absence of a radioactive complementary peptide 50−61 both point to the conclusion that the radioactivity and the low yields of PTH derivatives recovered after the fourth Edman cycle for the uncleaved peptide 46−61 do not reflect the presence of partial labeling of the corresponding amino acids with 6α-ANBA-estradiol photoreagents. Moreover, mass spectrometry showed that only 1 mol of steroid per mole of peptide is added for all four photolabeled peptides. Therefore, all these observations support the hypothesis of the presence of the two 6α- and 6*β*-photolabels exclusively at the level of Tyr-49-L.

The specific photoaffinity labeling of the same Tyr-49-L by the two 6α- and 6*β*-photoreagents suggests that Tyr-49-L was in both cases within reach of the azide group of each epimer. Therefore, the binding of the same Tyr-49-L by the two epimeric photoreagents could be explained by the conformational mobility of the chromophore. Nevertheless, owing to the differences between the structures of the two 6α- and 6*β*-amido photoreagents and that of 7-CMO-estradiol−hapten, it cannot be ascertained whether the photolabeled Tyr-49-L can be considered as a part of the binding site for the hapten link or represents only a residue in the vicinity of the binding site. The syn orientation of the 7-CMO toward the C-6 position (Mappus et al., 1975) may however confer some spatial proximity between 6-amido reagents and the 7-CMO link.

The photolabeled Tyr-49-L corresponds to the C-terminal residue of FR_2-L contiguous to the highly variable first residue of CDR_2-L (Padlan et al., 1995). In several instances, framework residues flanking the CDRs were found to be directly involved as contact residues with the antigen, as reported previously for the same Tyr-49-L in the anti-lysozyme D1.3 antibody (Amit et al., 1986; Fischman et al., 1991; Padlan, 1994) and in the anti-neuraminidase NC 41 antibody (Tulip et al., 1992). The CDR_2-L is rarely used in binding to small antigens as illustrated in the three-dimensional structure of the 26-10 Fab−digoxin complex (Jeffrey et al., 1993) but was found to be important in interactions with larger antigens as reported in the case of the anti-neuraminidase NC 41 antibody [cf. Davies and Padlan (1990) and Wilson and Stanfield (1994)].

Direct evidence supporting the existence of a specific combining subsite for the hapten link was afforded by recent structural studies which established that the link could be partially buried in the combining site, as shown by X-ray crystallography of the Fab′ complex of the monoclonal anti-progesteron antibody DB3 with its corresponding highly cross-reactive 11α-hydroxyprogesterone hemisuccinate hapten (Arevalo et al., 1994). On the other hand, the three-dimensional structure of the 26-10 Fab−digoxin complex (Jeffrey et al., 1993) showed that the digoxose part of the hapten was not significantly buried in the combining site, thus explaining the lack of specificity of this antibody for this group through which the digoxin was coupled to the carrier protein in the immunogen.

In the antibody 15H11, the presence of two relatively short CDR_1-L and CDR_3-H containing respectively only 11 and 8 amino acid residues suggests a flat binding site (De la Paz et al., 1986; Jackson et al., 1992). This flat shape may explain the high cross-reactivity of structural congeners bearing large modifications at C-6 and C-7 positions. However, the limited cross-reactivity of the two 7-oxo- and 7-CMO-estradiol analogs indicates that some large subsite may well subsist around the C-7 position.

Molecular models have been reported for an anti-testosterone antibody (Jackson et al., 1992) and for two anti-cortisol antibodies (Le Calvez et al., 1995). Attempts to obtain a three-dimensional structure by molecular modeling of antibody 15H11 are in progress which may also facilitate the structural design of other affinity labeling reagents.

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