Redox-Dependent DNA Binding of the Purified Androgen Receptor: Evidence for Disulfide-Linked Androgen Receptor Dimers†

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ABSTRACT: Full-length histidine-tagged, dihydrotestosterone-bound human androgen receptor (AR) was purified to homogeneity by affinity and gel-filtration chromatography for antibody production and analysis of AR dimerization and DNA binding properties. A monoclonal antibody was raised that recognized human and rat AR epitope ^{360}ArgAspTyrTyrAsnPheProLeuAla^{368} in the NH_2-terminal domain and slowed migration of AR−DNA complexes in mobility shift assays. AR binding to androgen response element DNA had a K_d of 2.0 nM and a Hill coefficient of 2.1, indicating high-affinity, cooperative binding. AR solution dimerization was detected only at ≥0.2 μM AR, and DNA binding increased dimerization up to 30-fold. Slow- and fast-migrating AR−DNA complexes were detected under different reducing conditions that differed 5-fold in their dissociation rates from DNA. Treatment with the sulfhydryl oxidizing reagent diamide formed the faster migrating, slower dissociating complex, indicating it represents disulfide-linked AR dimers bound to DNA. The results indicate that high concentrations of purified AR are required for solution dimerization and that cooperative DNA binding stabilizes two dimer forms that differ in redox state.

Cloning of complementary DNA for the human androgen receptor (AR)[1] (*1*−*3*) has facilitated its structural and functional analysis. High-affinity androgen binding stabilizes the AR protein against degradation (*4*) and targets it for translocation to the nucleus (*5*). While stabilization is limited to high-affinity, slow-dissociating ligands such as testosterone, dihydrotestosterone, and several synthetic androgens (*6*), AR nuclear transport is effected by a variety of ligands that bind with moderate affinity (*4*). High-affinity androgen binding is required for AR binding as a homodimer to androgen response element DNA (*7*); however, AR dimerization in solution has not been demonstrated. Androgen-induced AR dimerization in association with DNA binding appears to involve an antiparallel orientation of monomers (*8, 9*), which is consistent with crystal structure predictions of a symmetrical, head-to-head orientation of DNA binding domain monomers, as shown for the glucocorticoid receptor (*10*). A role for an intermolecular AR NH_2/carboxyl-terminal interaction in AR dimerization and DNA binding (*8, 9, 11, 12*) was recently suggested to facilitate AR-mediated gene activation at low physiological androgen concentrations (*13*). A number of nuclear receptor coactivators have been identified that influence transcriptional regulation of steroid hormone receptors (*14*−*16*). Interactions between AR, coactivators, and the basal transcriptional machinery contribute to selective gene transcription.

Purification of the full-length AR is complicated by its low abundance, instability in the absence of androgen and difficulty in separation from other nuclear proteins. Recombinant AR has been overexpressed in its native and protein-tagged forms in *Escherichia coli* (*17*), insect (*11, 18, 19*), and mammalian cells (*20*). Approaches for nuclear receptor purification have included hormone, DNA, and antibody affinity chromatography. Denatured AR was purified after expression from baculovirus and subsequently renatured to bind DNA (*19*). Purification with histidine affinity-tagged proteins is advantageous because, unlike protein tags such as glutathione S-transferase, short histidine sequences have minimal effects on protein structure and function and efficiently bind metal chelating columns largely independent of protein conformation (*21, 22*).

Here we report the purification of histidine-tagged, full-length dihydrotestosterone-bound human AR. A mouse monoclonal antibody was raised that reacts with amino acid residues 360−368 in the human AR NH_2-terminal domain. Immunoblot analysis indicates AR solution dimerization occurs only at high AR concentrations in the absence of DNA and that dimerization is cooperative with DNA binding. Purified AR binds androgen response element DNA with high affinity in oxidized and reduced forms that differ in dissociation rate from DNA, raising the possibility that AR activity is influenced by cellular redox state.

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[1] Abbreviations: AR, androgen receptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; DHT, dihydrotestosterone.

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EXPERIMENTAL PROCEDURES

Baculovirus Expression. Full-length human AR cDNA was cloned in the polylinker region of baculovirus transfer vector $pAcC_4$ (Cetus) as previously described (*7*) except with an alanine−6-histidine NH_2-terminal tag. Recombinant plaques were purified and human AR was expressed in *Spodoptera frugiperda* (Sf9) insect ovary cells cultured in 5 L bioreactor preparations obtained from the University of Colorado Denver Cancer Center tissue culture core facility. Cells were infected at a multiplicity of infection of 1 and incubated for 48 h at 27 °C, with the last 24 h in the presence of 1 μM dihydrotestosterone. Cells were washed and stored as 10 frozen pellets for later extraction. Typically two pellets [$(1.6-2.8) \times 10^9$ infected Sf9 cells] were extracted by three cycles of freeze/thaw in 100 mL of 0.5 M NaCl in buffer A: 10% glycerol, 0.02% NP-40, 20 mM Tris-HCl, pH 7.5 (pH determined at room temperature), 5 mM β-mercaptoethanol [1 mM dithiothreitol (DTT) in steps after metal ion affinity chromatography], 1 μM dihydrotestosterone, 0.5 mM phenylmethanesulfonyl fluoride, 20 μM leupeptin, 10 μM pepstatin A, and 20 mM ϵ-amino-*n*-caproic acid. Lysates were incubated for 1 h on ice and centrifuged at 25000*g* for 1 h, and supernatants were stored in 25 mL aliquots at −80 °C.

AR Purification. Cell lysates (100 mL) were dialyzed against buffer A (4 L) containing 0.15 M NaCl and 5 mM imidazole and were clarified by centrifugation at 25000*g* for 40 min. Samples were subsequently incubated for 1 h at 4 °C with 4 mL of the cobalt affinity Talon resin (Clontech) in two 50 mL tubes with gentle agitation. The mixture was transferred to two 2 mL columns and washed with 10 column volumes of buffer A containing 0.15 M NaCl and 5 mM imidazole. AR was eluted with 40 mL of buffer A containing 0.5 M NaCl and 100 mM imidazole and stored frozen at −80 °C. Further chromatographic procedures were performed with Pharmacia FPLC systems.

Cobalt affinity column elution fractions were applied to 10 mL phenyl-Sepharose columns (Pharmacia) equilibrated in 0.5 M NaCl in buffer B (buffer A containing 0.5 mM EDTA but lacking NP-40). The column was washed with buffer B, buffer B containing 0.1 M NaCl, and buffer B containing 4 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). AR was eluted with buffer B containing 25 mM CHAPS. Elution fractions were applied to 5 mL HiTrap heparin−Sepharose columns (Pharmacia) equilibrated in 0.1 M NaCl in buffer B. After washing, AR was eluted with 0.3 M NaCl in buffer B and samples were stored at −80 °C. Heparin−Sepharose peak fractions of 15−20 mL were diluted to 0.1 M NaCl in buffer B and reapplied to a heparin−Sepharose column to concentrate AR by eluting in approximately 8 mL of 0.6 M NaCl in buffer B. Concentrated AR fractions were applied to 2.6 × 60 cm HiLoad 26/60 Superdex 200pg columns (Pharmacia) equilibrated in 0.4 M NaCl, 10% glycerol, 0.5 mM EDTA, 1 mM DTT, 1 μM dihydrotestosterone (DHT), 0.5 mM phenylmethanesulfonyl fluoride, and 20 mM Tris-HCl, pH 7.6. For DNA binding studies with concentrated AR, peak fractions eluting in 25 mL at a Stokes radius of 58 Å were rechromatographed on 1 mL HiTrap heparin−Sepharose columns and eluted in 2 mL of 0.5 M NaCl, 10% glycerol, 1 mM DTT, 1 μM DHT, and 20 mM Tris, pH 7.5.

Monoclonal Antibody Production and Epitope Mapping. Purified AR (10−100 μg) in phosphate-buffered saline was injected into a mouse every 2 weeks for 5 months in the hybridoma facility at North Carolina State University College of Veterinary Medicine. Cell fusions were performed following standard procedures with P3 myeloma cells, which do not secrete IgG. Initial screening by enzyme-linked immunosorbent assay (ELISA) was against purified AR, which was dialyzed against 0.5× phosphate-buffered saline containing 1 μM dihydrotestosterone for 2 h at 4 °C. AR (0.1−0.2 μg in 100 μL) was added per well of 96-well microtiter plates and air-dried at room temperature overnight. Antigen was fixed with 100 μL of methanol for 5 min and washed 3 times with washing buffer (phosphate-buffered saline containing 0.1% Tween 20). Plates were blocked for 2 h with 130 μL/well of blocking buffer [0.12 M NaCl, 0.05% Tween 20, 1 mM EDTA, 0.4% bovine serum albumin (BSA), and 0.17 M boric acid, pH 8.5] and washed 3 times with washing buffer. Hybridoma supernatants (100 μL/well; 768 wells/fusion) were added and incubated at room temperature for 2 h and the plates were washed 3 times. Horseradish peroxidase-conjugated goat anti-mouse γ-IgG (Calbiochem, 1 mg/mL) was diluted 2000-fold into blocking buffer; 100 μL/well was added and the plates were incubated at room temperature for 2 h and washed 3 times. *o*-Phenylenediamine substrate (100 μL; four 2 mg tablets and 30 μL of 30% H_2O_2 in 50 mL of 0.05 M sodium citrate, 0.15 M NaH_2PO_4, pH 6) was added and incubated for 15 min and the reaction was stopped with 50 μL/well 4 M H_2SO_4. Optical density was determined at 495 nm in a multiwell plate reader. Positive samples were expanded for clonal isolation. ELISA positive hybridoma supernatants were further tested against purified AR on 8% acrylamide gels containing SDS and transferred to Immobilon-P transfer membranes (Millipore). Strips were reacted with AR52 antipeptide antibody to locate the position of AR. Filters were placed in 24-well tissue culture plates, incubated with 50−100 μL of hybridoma supernatant, and developed for chemiluminescence by the ECL method (Amersham).

The AR epitope recognized by AR-M1 was determined by ELISA, immunoblot analysis, and peptide competition. AR deletion mutant expression vectors (*12, 23*) were expressed in COS-1 cells transfected with DEAE-dextran and 10 μg of expression vector DNA. Immunoblots were performed on 8% acrylamide gels containing SDS. Three 12 amino acid peptides corresponding to AR amino acid residues 357−368, 384−395, and 402−413 in a 63 amino acid NH_2-terminal region recognized by AR-M1 were synthesized on a Rainin Symphony multiple peptide synthesizer by Fmoc chemistry and purified by reverse-phase HPLC with 0.1% trifluoroacetic acid/acetonitrile gradients on semipreparative (10 × 250 mm) C18 columns. Purity was assessed by analytical HPLC and time-of-flight analysis on a Kratos MDI III mass spectrometer.

Oxidation/Reduction Studies. Purified AR (0.0125−1.5 μM) with or without unlabeled androgen response element DNA was incubated with 0−20 mM diamide (azodicarboxylic acid bis[dimethylamide]) (Sigma) as indicated for 5 min at 4 °C or with 10 mM glutaraldehyde (Sigma) for 30 min at room temperature. After glutaraldehyde treatment, reactions were stopped with 2% hydrazine (Sigma) as previously described (*24*). Samples were subsequently treated without

or with increasing concentrations of DTT. Denaturing buffer was added prior to electrophoresis to a final concentration of 2% SDS, 12% glycerol and 40 mM Tris, pH 6.8. Samples were analyzed on linear 3−10% acrylamide gradient gels containing 0.1% SDS (*24*). Gels were calibrated with prestained high molecular weight markers (Bio-Rad) and a high molecular weight calibration kit (Pharmacia). Immunoblots were performed with AR52 or AR-M1 antibody.

DNA Mobility Shift Assays. Unless otherwise indicated, DNA mobility shift assays were performed in the absence of dI-dC nonspecific DNA with 25 ng/20 μL (0.0125 μM) up to 1.5 μg/10 μL (1.5 μM) purified AR, 0.2 ng (0.5 nM) of 20 000 cpm ^{32}P-labeled androgen response element oligonucleotides in DNA binding buffer containing 20 mM KCl, 10% glycerol, 0.2 mM EDTA, 0.02% NP-40 (Sigma), 1 mM DTT, 0.2 μM dihydrotestosterone, and 10 mM Tris-HCl, pH 7.5, with or without 4 mg/mL BSA and 1 μg of AR antibody AR52 (*25*) or AR-M1. Oligonucleotides were labeled with [α-^{32}P]dCTP, 3000 Ci/mmol (NEN Dupont) and dNTPs (Pharmacia) and the Klenow fragment of DNA polymerase (Promega). DNA sequence derived from the first intron of the rat C3 prostatein gene (androgen response element underlined) (*26*) was prepared by annealing oligo 5′ CGACCAGAGTACGTGATGTTCTCAGG 3′ with 5′ *Acc*I compatible end and oligo 5′ GATCCCTGAGAACATCACGTACTCTGGT 3′ with 3′ *Bam*HI compatible end. In some experiments, purified AR (0.025 μM) in the presence of BSA was incubated before or after the addition of androgen response element DNA with 1 mM diamide for 5 min at 4 °C, followed by exposure to increasing concentrations of DTT. Reactions were incubated on ice for 40 min and complexes were separated at 4 °C for 4 h at 150 V on 4% acrylamide gels containing 2 mM EDTA, 90 mM boric acid, and 90 mM Tris, pH 7.5. Gels were dried and exposed to X-ray film. For DNA binding at high AR concentrations, binding reactions were performed as above except that 10 μL reactions were used and purified AR was dialyzed against DNA binding buffer. Unless otherwise indicated, each reaction contained 1.0−1.3 μg of AR (1−1.3 μM), and 1.5 ng (7.5 nM) of ^{32}P-labeled (140 000 cpm) and 16 ng (80 nM) of unlabeled annealed androgen response element DNA (88 nM total DNA). Films were exposed for 4 h or overnight.

Other Procedures. Protein concentration was determined by the Bradford (*27*) or Lowry et al. (*28*) method with BSA as standard. Protein staining in SDS polyacrylamide gels made use of the Bio-Rad silver staining kit.

RESULTS

AR Purification and Antibody Production. Initial purification of recombinant, baculovirus-expressed human AR complexed with dihydrotestosterone (AR) made use of an NH_2-terminal six-histidine tag AR construct. The addition of saturating concentrations of dihydrotestosterone (1 μM) during all purification steps was required to stabilize AR against degradation (*4*). Attempts to purify the AR in the absence of androgen were unsuccessful according to the protocol described below. A 266-fold purification of the AR−dihydrotestosterone complex was achieved by cobalt chelating chromatography (Table 1). Contaminating proteins were hydrophobic and were not separated by the anion resins Mono-S HR5/5 and Mono-Q HR5/5 (Pharmacia). Isoelectric focusing chromatography was also ineffective because AR tended to aggregate in low-salt buffers. Sequential chromatography on phenyl-Sepharose (elution in 25 mM CHAPS, Figure 1A) and heparin−Sepharose (elution in 0.3 M NaCl, Figure 1B) resulted in the isolation of the 58 Å, 114 kDa AR monomer (*29*) by gel filtration on Superdex 200pg (Figure 1C) with a yield of approximately 0.7 mg of AR from 10^9 infected Sf9 insect cells (Table 1). Silver staining of cytosol and column fractions (Figure 2A) reflected an overall 475-fold purification with ∼95% purity and 6.5% recovery (Table 1). Immunoblotting with AR52 antipeptide antibody confirmed the predominant 114 kDa band as AR, with AR degradation fragments evident in earlier steps during purification (Figure 2B). Minor silver stained bands at 80−90 kDa reflected partial AR degradation detected by immunoblot with AR antibody. AR concentration was estimated during purification by ELISA with purified AR as standard and the AR-M1 monoclonal antibody described below.

Table 1: Purification of Histidine-Tagged, Baculovirus-Expressed Human AR from Sf9 Insect Cells[a]

column	[protein] (μg/mL)	total protein (mg)	[AR] (μg/mL)	total AR (mg)	purity (%)	*x*-fold	recovery (%)
cytosol	53 180	5318	105	10.5	0.2	1	100
metal	369	14.7	195	7.8	53.1	266	74
phenyl	188	7.5	117	4.6	61.2	306	44
heparin	138	2.5	120	2.2	88.7	444	21
Superdex	30	0.7		0.7	95.0	475	6.5

[a] Quantitation is shown for AR purification following a typical isolation from (1.6−2.8) × 10^9 infected Sf9 cells as described in Experimental Procedures. Protein concentration was determined by Lowry and Bradford methods (*27*, *28*) with BSA as standard. AR was quantitated by ELISA with AR-M1 monoclonal antibody. A final AR purity of 95% was estimated on the basis of the apparent single band on Coomassie blue staining and a standard curve with AR from the final purification step. Optical absorption at 495 nm in ELISA was compared to the standard curve.

The epitope of an IgG-1 AR-M1 monoclonal antibody raised against purified AR in mice was mapped by immunoblot analysis to residues 360−422 in the human AR NH_2-terminal domain because of reactivity with deletion mutant AR1−442 but not AR1−359, where full-length AR is 1−919. Of three 12-amino acid residue peptides created on the basis of antigenic index (*30*), only ^{357}TyrGlnSerArgAspTyrTyrAsnPheProLeuAla^{368} inhibited immunoreactivity by ELISA and immunoblot analysis (data not shown). Cross-reactivity with rat AR on immunoblots (Figure 3A) despite one amino acid difference suggested the epitope was limited to ^{360}ArgAspTyrTyrAsnPhePro^{366} in human AR (rat AR residues 358−366). Specificity for AR was demonstrated by lack of immunoreactivity with human progesterone or glucocorticoid receptors (Figure 3A). Reactivity with native, ligand-free AR was demonstrated by immunoprecipitation of the AR double bands evident on SDS−polyacrylamide gels, where the upper band results from serine 94 phosphorylation (*12*) (Figure 3B). Reactivity of AR-M1 with native AR was also indicated by the slowed migration of the AR−DNA complex in DNA mobility shift assays (see Figure 5A).

AR Solution Dimerization and the Effect of DNA Binding. Because mobility shift assays used in previous studies did not distinguish AR dimerization from DNA binding (*7*, *13*), it had not been established whether AR dimerizes in solution or requires DNA binding for dimerization. Purified AR was

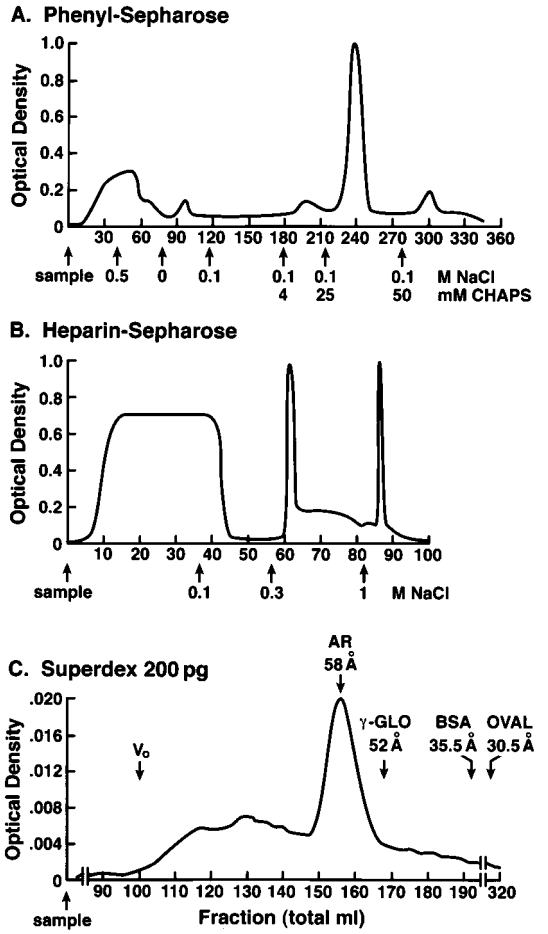

FIGURE 1: Chromatographic profiles of human AR purification from Sf9 cells. Typical chromatographic scans are shown following chromatography of the metal affinity elution sample onto (A) phenyl-Sepharose, (B) heparin−Sepharose, and (C) Superdex 200pg. Sf9 cell extracts expressed AR from baculovirus with an NH_2-terminal six-residue histidine tag. Elution patterns are shown relative to the total volume in milliliters (*x* axis). The position of buffer changes (panels A and B) and approximate Stokes radius relative to the void volume V_0 (panel C) are indicated. Purification was performed as described in Experimental Procedures. AR was detected by immunoblot and quantitated by ELISA of peak fractions from phenyl-Sepharose (240 mL), heparin−Sepharose (67 mL), and Superdex 200pg (156 mL). Optical density was determined at 280 nm.

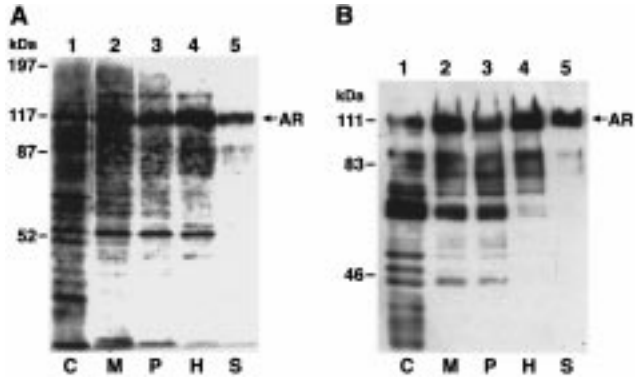

FIGURE 2: Purification of baculovirus-expressed AR. AR was purified as described in Experimental Procedures and as shown in Figure 1. (A) Silver staining of the following column peak fractions: Sf9 cell cytosol (lane 1, C, 10 μg of protein) expressing the histidine-tagged, human AR was chromatographed on a cobalt metal affinity Talon column (Clontech) and peak fractions eluting in buffer A containing 0.5 M NaCl and 100 mM imidazole were analyzed (lane 2, M, 5.5 μg of protein). The metal column peak fractions were applied to phenyl-Sepharose and the AR-containing fraction at 240 mL is shown (lane 3, P, 3.5 μg of protein). Phenyl-Sepharose AR fractions were chromatographed on heparin−Sepharose and column fraction at 67 mL containing AR is shown (lane 4, H, 3.5 μg of protein). Heparin−Sepharose fractions were separated by Superdex 200pg gel-filtration chromatography and the column fraction at 156 mL elution volume is shown (lane 5, S, 1 μg of protein). Silver staining was performed with Silver Stain Plus from Bio-Rad. (B) Immunoblot of column peak fractions including cytosol (lane 1, C, 100 μg of protein); metal affinity chromatography (lane 2, M, 5.5 μg of protein); phenyl-Sepharose peak fraction 4 (lane 3, P, 3.5 μg of protein); heparin−Sepharose peak fraction 67 (lane 4, H, 3.5 μg of protein); Superdex 200pg peak fraction 156 (lane 5, S, 1.0 μg of protein). The immunoblot was analyzed with 1 μg/mL AR52 antipeptide antibody.

therefore analyzed in the presence and absence of unlabeled androgen response element DNA on 3−10% linear acrylamide gradient gels and detected by immunoblot analysis. Samples were treated with 2% SDS prior to electrophoresis. In some experiments the cross-linking reagent diamide or glutaraldehyde was used to stabilize dimers before exposure to SDS. Diamide reversibly oxidizes vicinal free sulfhydryl groups to form disulfide bonds (*31*, *32*).

Purified AR at $\geq$0.2 μM was required to detect a 220-kDa AR dimer in 0.1 mM DTT in the absence of DNA (Figure 4A, lanes 3, 5, and 7). The dimer represented up to 12% of the 114 kDa monomer at 1.3 μM AR (Figure 4A, lane 7). Immunoreactive bands smaller than the 114 kDa AR monomer resulted from AR proteolytic degradation. At $\geq$0.2 μM AR, dimer intensity increased 3−5-fold with the addition of androgen response element DNA (Figure 4A, lanes 4, 6, and 8). Dimers were almost undetectable after sulfhydryl reduction with 10 mM DTT in the absence or presence of DNA (Figure 4C, lanes 5 and 6). The AR dimer therefore appeared to be disulfide-linked under these low reducing, partially denaturing conditions.

Because the extent of AR solution dimerization is underestimated under the denaturing conditions of SDS gel electrophoresis, glutaraldehyde and diamide cross-linking reagents were used to stabilize preexisting dimers. At 0.1 μM AR in the presence of 0.1 mM DTT, dimers were detected only in the presence of androgen response element DNA after glutaraldehyde treatment (Figure 4B, lanes 1 and 2), demonstrating a striking dependence of dimerization on the presence of DNA. As the AR concentration increased, dimerization in the absence of DNA became detectable after glutaraldehyde treatment at 0.2 μM AR and increased to 20% of the total monomer at 1.3 μM AR (Figure 4B, lanes 3, 5, and 7). In the absence of DNA at 1 μM AR and 1 mM DTT, the dimer represented ~25% of the monomer after oxidation with 5 mM diamide (Figure 4C, lane 3). The dimer remained about 25% of the monomer when 1 μM AR was first reduced with 10 mM DTT (10 min at 4 °C) and then oxidized with 20 mM diamide (Figure 4C, lane 7), suggesting that intermolecular disulfide bonds were not required for solution dimerization. In experiments not shown, formation of the dimer with diamide treatment reached 50% or greater of the monomer with 0.2 μM AR in the presence of DNA. At 0.02 μM AR in the absence or presence of 4 mg/mL BSA, the dimer was also detected after diamide treatment.

The striking ~30-fold increase in dimerization with DNA binding at 0.1−0.4 μM AR indicated that essentially all the AR was a dimer bound to DNA (Figure 4B, lanes 1−6).

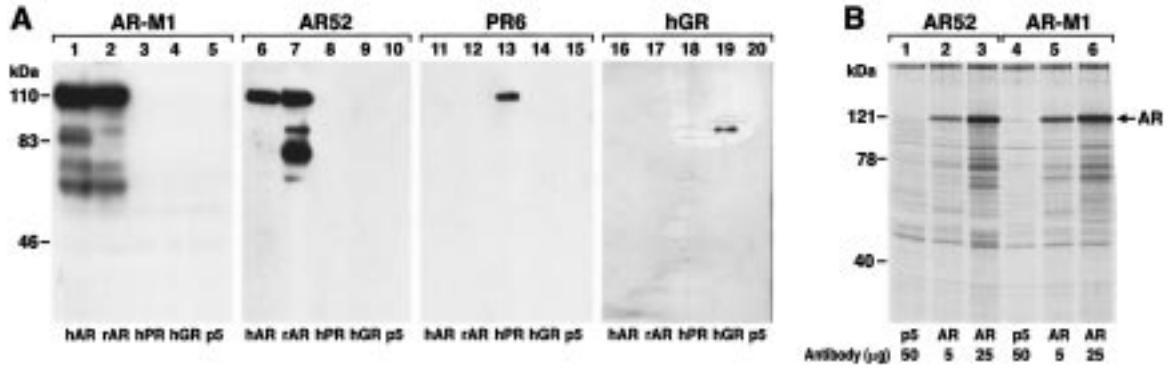

FIGURE 3: Specificity of monoclonal antibody AR-M1. Full-length AR and other steroid receptors were expressed from pCMV5 in COS cells lacking the NH_2-terminal histidine tag as described in Experimental Procedures. (A) Specificity of AR-M1 immunoreactivity. Human (hAR) and rat (rAR) AR, human glucocorticoid (hGR), and progesterone (hPR) receptors were expressed in COS cells from the parent plasmid pCMV5 (p5) as described in Experimental Procedures and analyzed by immunoblot. Rabbit polyclonal anti-human GR antibody AhuGR 150−175 (Affinity BioReagents) and PR6 mouse monoclonal anti-human PR antibody (David Toft, Mayo Clinic) were used at 1 μg/mL. (B) Immunoprecipitation of [^{35}S]methionine/cysteine-labeled recombinant AR expressed in COS cells. COS cells plated at 1.4×10^6/10 dish were transfected using DEAE dextran with 10 μg of pCMVhAR or 10 μg of the parent vector pCMV5 (p5). Cells were incubated with 100 μCi of [^{35}S]methionine/cysteine (New England Nuclear, 1175 Ci/mmol, Easy Tag Express labeling mix) in the absence of androgen, harvested in RIPA buffer as previously described (*4*), and immunoprecipitated with 5, 25, or 50 μg of AR52 or AR-M1 antibody as indicated.

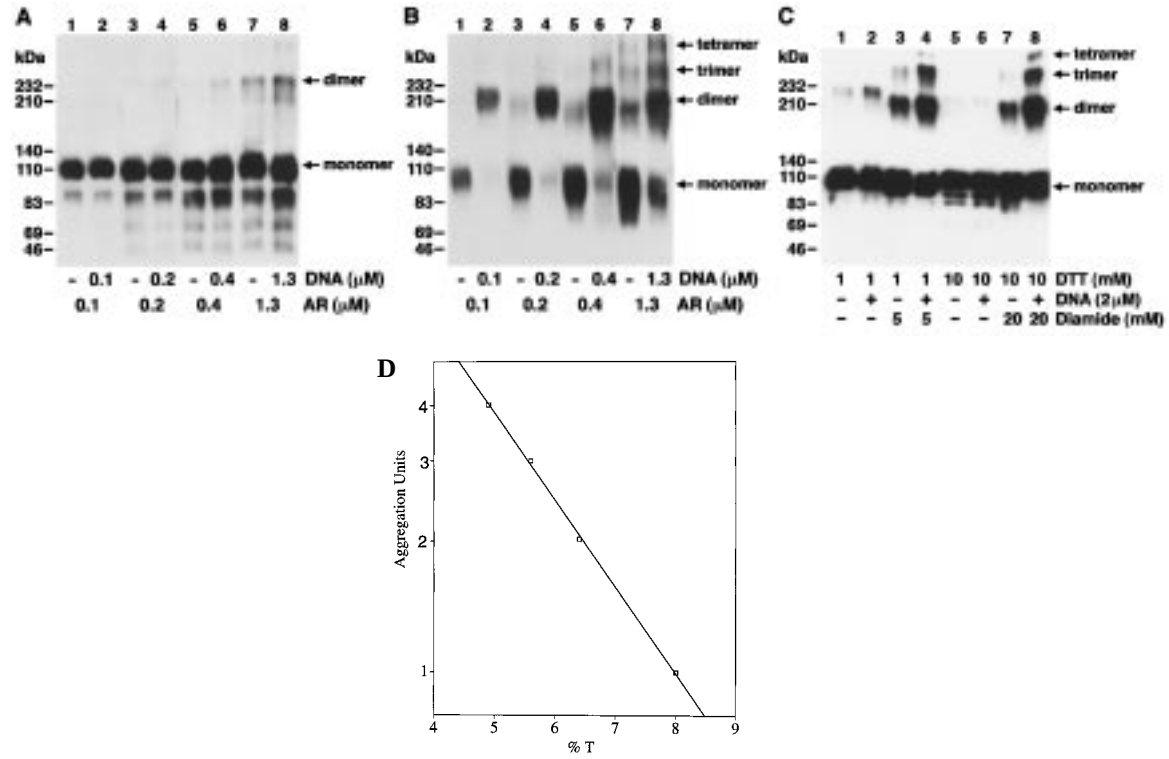

FIGURE 4: Immunoblot of AR dimerization in the presence and absence of androgen response element DNA and cross-linking reagents. DNA binding reactions contained 20 mM KCl without carrier protein. (A) Purified AR at increasing concentrations from 0.1 to 1.3 μM was incubated for 40 min at 4 °C in the presence of 0.1 mM DTT and in the absence or presence of equimolar unlabeled androgen response element DNA (0.1−1.3 μM) as indicated. (B) Samples were prepared as in panel A and cross-linked with 10 mM glutaraldehyde for 30 min at room temperature and quenched with 2% hydrazine. (C) Purified AR at 1.3 μM was incubated with 1 (lanes 1−4) or 10 (lanes 5−8) mM DTT for 10 min at 4 °C. Unlabeled androgen response element DNA (2 μM) was added (lanes 2, 4, 6, and 8) and incubated for 40 min at 4 °C. Samples were treated with 5 (lanes 3 and 4) or 20 (lanes 7 and 8) mM diamide for 5 min at 4 °C. In panels A−C, samples were analyzed on 3−10% gradient acrylamide gels containing 0.1% SDS. Samples were not heated or reduced but were partially denatured with 2% SDS. Gels were processed for immunoblots as described in Experimental Procedures with AR52 antibody (1 μg/mL). Migration of the predicted monomer, dimer, trimer, and tetramer is indicated by arrows on the right and molecular weight markers on the left. Samples shown in panels A and B were from the same gel but the order of the lanes was changed for clarity. (D) Modified Ferguson plot (*24*, *60*, *61*) where the band migration in panel B, lane 8 expressed as percent total acrylamide concentration (% T) was plotted against the assumed aggregation state. % T on a logarithmic scale reflects the linear 3−10% acrylamide gel and was calculated on the basis of relative mobility (r_f). Correlation coefficient $r = 1.00$.

When the AR concentration increased to 1.3 μM, the increase in dimerization with DNA binding was only 3−5-fold (Figure 4B, lanes 7 and 8). The effect of AR DNA binding on dimerization was therefore less evident at the higher AR concentrations that promote AR solution dimerization. The 3−5-fold increase in dimerization with DNA binding at the

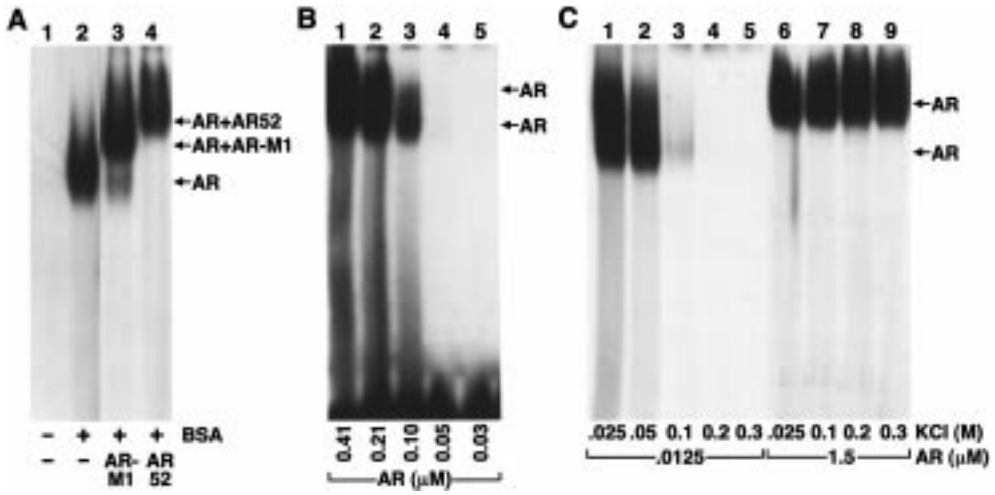

FIGURE 5: DNA mobility shift assays with purified AR. (A) Effect of carrier protein and antibody. Purified AR (0.0125 μM; 25 ng/20 μL reaction) was incubated with 0.2 nM ^{32}P-labeled androgen response element DNA in 20 mM KCl and 1 mM DTT in the absence (lane 1) or presence (lanes 2−4) of 4 mg/mL BSA. The AR monoclonal antibody AR-M1 (lane 3, 0.25 μg of AR-M1) or rabbit antipeptide antibody AR52 (lane 4, 1 μg of AR52) was added. Indicated at the right is the migration of AR−DNA complexes (AR) with or without antibody addition. (B) Concentration dependence of purified AR DNA binding in the absence of carrier protein. Increasing concentrations of purified AR (0.03−0.41 μM; 3−41 μg/mL; 30−410 ng of AR/10 μL reaction) were incubated with 7.5 nM ^{32}P-labeled and 80 nM unlabeled androgen response element DNA in the absence of carrier protein and in the presence of 20 mM KCl and 1 mM DTT. Migration of the AR-DNA complex (AR) is indicated at the right. (C) Effect of ionic strength on AR-DNA complexes at low and high AR concentrations. Increasing salt concentration from 0.025 to 0.3 M KCl was included in the DNA mobility shift reaction buffer with 7.5 nM ^{32}P-labeled and 80 nM unlabeled androgen response element DNA and 1 mM DTT with 0.0125 (left panel) or 1.5 (right panel) μM purified AR in the presence (lanes 1−5) or absence (lanes 6−9) of 4 mg/mL BSA. Migration of the two major AR bands is indicated by the arrows on the right.

higher AR concentrations with glutaraldehyde treatment (1.3 μM AR, Figure 4B, lanes 7 and 8) parallels the increase seen without cross-linking under low reducing conditions at 1.3 μM AR (Figure 4A, lanes 7 and 8; Figure 4C, lanes 1 and 2). The results suggest a correlation between solution dimerization and DNA binding. That the AR concentration required to detect solution dimerization ($\geq$0.2 μM) was similar to that required to detect DNA binding (0.1 μM) in mobility shift assays (see below) supports solution dimerization contributing to DNA binding.

Complexes with molecular masses greater than the 242 kDa dimer were also evident at 1 μM AR after cross-linking with glutaraldehyde or diamide (Figure 4B,C). The correlation between aggregation units and AR migration with increasing acrylamide concentration based on Ferguson plot analysis (Figure 4D) was highly significant ($r = 1.00$), indicating the multimers represent a 323 kDa AR trimer and a 462 kDa tetramer. The amount of these AR multimers increased with the addition of DNA.

DNA Binding Properties of Purified AR. Binding of purified AR to ^{32}P-labeled 28-bp androgen response element DNA at $\leq$0.015 μM AR was detected in the presence but not the absence of carrier protein (Figure 5A, lanes 1 and 2) with 4 mg/mL BSA. At this low AR concentration, carrier protein of $\geq$0.1 mg/mL was required and optimal DNA binding was observed at 0.5 mg/mL BSA. Ovalbumin or γ-globulin also enhanced DNA binding at low AR concentrations, suggesting a nonspecific protein stabilizing effect on the AR−DNA complex. The apparent equilibrium binding affinity for binding androgen response element DNA was 2.0 $\pm$ 0.4 nM, determined with increasing amounts of AR and 0.5 nM ^{32}P-labeled DNA. A Hill coefficient of 2.1 $\pm$ 0.1 suggested strong cooperative binding of two AR monomers to DNA (data not shown).

The addition of BSA as carrier protein was also required in previous DNA mobility shift assays with crude AR expressed from baculovirus (*7*), suggesting that factors other than elevated protein concentration increased AR DNA binding. A number of reagents were tested for their ability to increase DNA binding at $\leq$0.015 μM AR in the absence of carrier protein; however, none of the following reagents stabilized the complex: 0.1−2% glucopyranoside, 0.05−2% Tween 20, 5−30 mM CHAPS, 0.002−1 mM Zn^{2+}, 0.002−1 mM Mg^{2+}, 10−30% glycerol, 10−20% Ficoll, 10−30% poly(ethylene glycol) 8000, 0.5−1 M sucrose, 0.1−0.5 M urea, 0.1−2% trifluoroethanol, or 10−20 mM sodium cacodylate. Short peptides (0.3−4 mg/mL), small proteins such as insulin (0.01−1 mg/mL), high androgen concentrations (0.05−1 μM dihydrotestosterone or R1881), polyamines (0.1−2 mM spermine), pH 6−8, or AR52 antibody (1 μg/reaction) did not replace the carrier protein requirement for AR DNA binding. Addition of monoclonal antibody AR-M1 (0.24 μg) or antipeptide antibody AR52 promoted AR−DNA complex formation and caused a supershifted band (Figure 5A, lanes 3 and 4). However, the unshifted AR−DNA complex was also observed with control hybridoma supernatant, indicating a nonspecific protein stabilization effect.

Because <0.1 μM AR required carrier protein to bind ^{32}P-labeled androgen response element DNA, we investigated whether carrier protein increased DNA binding by promoting AR solution dimerization. However, the addition of 4 mg/mL BSA to 0.02 or 0.2 μM AR had no effect on AR solution dimerization in the absence or presence of diamide, using the denaturing gradient gels described in Figure 4. In data not shown, immunoblot analysis of 0.02 μM AR indicated that BSA increased the amount of AR monomer 2-fold without changing the percentage of AR bound to DNA after treatment with diamide. Thus the use of BSA as carrier protein appears to stabilize DNA binding at low AR concentrations by preventing nonspecific loss of the AR.

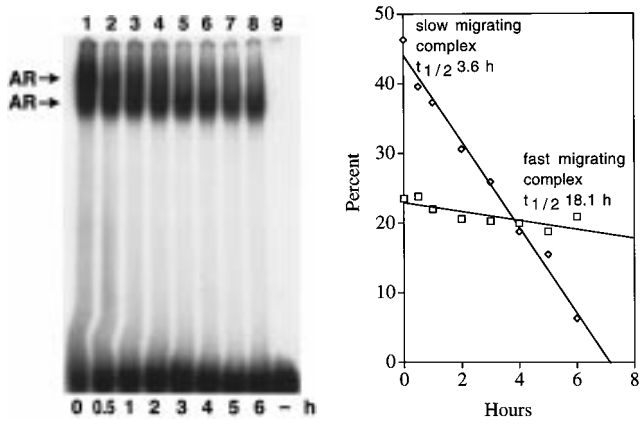

FIGURE 6: Dissociation rate of purified DHT-bound AR from androgen response element DNA. Purified AR (0.015 μM, 20 ng/20 μL reaction) was incubated with ^{32}P-labeled androgen response element DNA (0.2 ng/20 μL reaction, 20 000 cpm; 0.5 nM) in the presence of 20 mM KCl, 1 mM DTT, and 4 mg/mL BSA on ice for 40 min as described in Experimental Procedures. Unlabeled DNA (200 ng/reaction, 1000-fold excess) was added and incubated for 0−6 h before loading on the gel. Intensity of the ^{32}P-AR−DNA complexes in the upper and lower bands (lanes 1−8) and of the free ^{32}P-DNA (lane 9) shown in the autoradiogram (left panel) was determined with an LKB densitometer. The data were plotted as the percent of AR-DNA complex relative to the total complexes plus the free ^{32}P-labeled oligonucleotide relative to the dissociation time in hours (right panel).

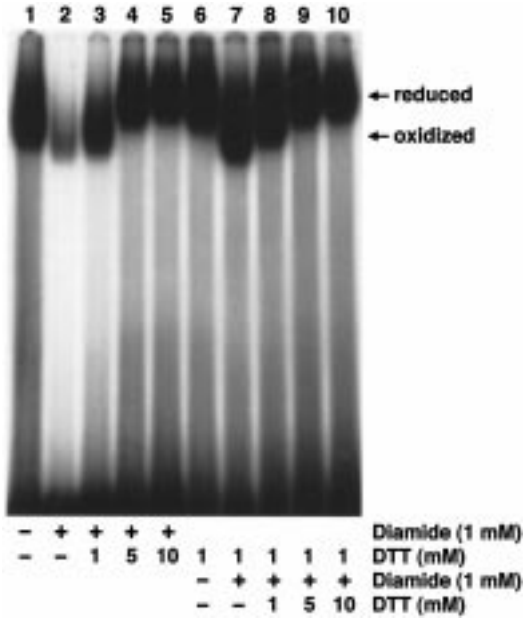

FIGURE 7: Oxidation/reduction properties of AR DNA binding. Purified AR (0.025 μM; 50 ng/20 μL reaction) was added to DNA binding buffer lacking DTT (final DTT concentration 0.05 mM) and containing 4 mg/mL BSA and 20 mM KCl. Samples were not further treated (lane 1) or in lanes 2−5, samples were treated with 1 mM diamide for 5 min at 4 °C followed by incubation for 40 min at 4 °C with 20 000 cpm (0.5 nM) ^{32}P-labeled androgen response element and the indicated concentration of DTT. In lanes 6−10, DTT was added to 1 mM and samples were incubated with 20 000 cpm (0.5 nM) ^{32}P-labeled androgen response element for 40 min at 4 °C. In lanes 7−10, samples were subsequently incubated with 1 mM diamide for 5 min at 4 °C. The reactions were not further treated (lanes 2 and 7) or were incubated with 1 (lanes 3 and 8), 5 (lanes 4 and 9), or 10 mM (lanes 5 and 10) DTT for 40 min at 4 °C and analyzed by DNA mobility shift assay as described in Experimental Procedures. Indicated on the right by arrows is the migration of the reduced and oxidized forms of the AR DNA complexes.

To determine the effect of increasing AR concentration on DNA binding in the absence of carrier protein, purified AR was concentrated by chromatography on heparin−Sepharose. At ≤0.05 μM AR, AR DNA binding remained undetectable (Figure 5B, lanes 4 and 5) but at ≥0.1 μM AR, AR DNA complexes were evident with increasing intensity. In addition, a second slower migrating complex was observed with increasing AR concentration (Figure 5B, lanes 1−3). AR−DNA complexes formed at 0.0125 μM AR in the presence of carrier protein were unstable in 0.1 M KCl, whereas those formed at 1.5 μM AR were stable in 0.3 M KCl (Figure 5C). Migration of the AR−DNA complex at 1.5 μM AR was slow like a portion of the complexes detected at 0.0125 μM AR at low salt concentrations (Figure 5C). With increasing DTT concentrations to 10 mM (30 min, 4 °C) and 0.0125 μM AR, 4 mg/mL BSA, and 20 mM KCl, there was a shift of the faster migrating band to the slower migrating form (data not shown), suggesting the fast-migrating complex represents a partially oxidized form of AR. Thus two AR DNA binding forms were observed, a fast-migrating complex predominant at low AR concentrations and a slower migrating form observed at higher AR concentrations and under increased reducing conditions. Both were observed in the presence of 1 mM DTT and shifted to the slower migrating band in 10 mM DTT. The half-time of dissociation of AR from ^{32}P-labeled DNA at 4 °C was 3.6 ± 0.4 h for the slower migrating complex and 18.1 ± 1.6 h for the faster migrating form (Figure 6).

Oxidation/Reduction Requirements for AR DNA Binding. We investigated further the structural basis for the two AR DNA binding forms by testing the influence of oxidation and reduction on purified AR androgen response element DNA complexes in mobility shift assays. AR initially treated with 1 mM diamide in the presence of 0.05 mM DTT (5 min at 4 °C) decreased DNA binding without significantly affecting migration of the remaining fast-migrating AR−DNA complex (Figure 7, lane 2). Subsequent reduction with 1 mM DTT partially restored DNA binding but did not alter migration of the complex (Figure 7, lane 3). Reduction with 5 or 10 mM DTT (10 min at 4 °C) fully restored AR DNA binding and shifted the AR−DNA complex to its slower migrating form (Figure 7, lanes 4 and 5). When AR−DNA complexes were formed prior to oxidation with diamide under the same conditions, there was no loss in DNA binding activity but complex migration increased (Figure 7, lane 7). Subsequent reduction with 1 mM DTT partially restored, and 5 or 10 mM DTT fully restored, the slow-migrating form (Figure 7, lanes 8−10). Essentially identical results were obtained with crude Sf9 cell extracts overexpressing the AR. Diamide treatment of crude AR after DNA binding revealed the fast-migrating AR−DNA complex, which shifted to the slower migrating form after subsequent reduction with DTT. The results suggest that the two AR−DNA complexes often observed in DNA mobility shift assays result from reduced and oxidized forms of AR, the latter being a fast-migrating, disulfide-linked AR dimer−DNA complex. The different stabilities of the two complexes (Figure 6) raise the possibility of redox control of AR DNA binding.

DISCUSSION

Recombinant full-length histidine-tagged, dihydrotestosterone-bound human AR was purified and a monoclonal antibody was raised that selectively recognizes the NH_2-terminal domain epitope ArgAspTyrTyrAsnPhePro (human AR residues 360−366; rat AR residues 358−364) in native and denatured rat and human AR. In addition, two forms of the AR were identified that bind androgen response element DNA. On the basis of studies using high concentrations of DTT and diamide, a reagent that reversibly cross-links closely positioned free sulfhydryls to disulfide bonds (*31, 32*), these forms represent oxidized and reduced AR dimers bound to DNA. The two forms differed in dissociation rate from DNA, where the slower migrating, reduced form dissociated 5-fold faster than the faster migrating form that appeared to be linked by disulfide bonds. The binding of oxidized and reduced forms of AR to androgen response element DNA raises the possibility of redox control in androgen-mediated gene activation. The stability of the AR−DNA complex in its oxidized state is striking and could reflect a physiologically important complex.

The propensity of AR to form intermolecular disulfide bonds suggests that reactive sulfhydryl groups in the AR dimer are sensitive to redox conditions and that disulfide bond formation promotes dimerization in vivo and stabilizes AR dimers bound to DNA. Under relatively low reducing conditions (1 mM DTT at 4 °C) there was evidence for intermolecular disulfide-linked AR dimers in the absence of DNA, and with cross-linking reagents, the dimer represented up to 50% of the monomer. Precedence for disulfide-linked dimers bound to DNA comes from the E2A DNA binding protein, which like AR forms an intermolecular disulfide bond when bound to DNA (*33*). Also like AR, E2A has two amphipathic α-helices in its DNA binding domain and does not bind DNA as a monomer. E2A binding as a disulfide-linked dimer was cell type-specific, suggesting that redox conditions influence its activity and could be important as well in AR activation.

The higher molecular weight AR trimers and tetramers that reversibly convert to the monomer after reduction with a high concentration of DTT (10 mM at 4 °C) suggest that additional sulfhydryl groups are available for intermolecular reactions. However, pretreatment with 10 mM DTT did not prevent dimer and multimer formation by subsequent exposure to excess diamide, indicating that dimers were present that were not disulfide-linked. The reversible formation of oxidized and reduced AR−DNA complexes may reflect conformational changes in AR dimerization and DNA binding. Redox mechanisms could influence the activation state of AR, thereby altering the transcription of AR-regulated genes. It is of interest, therefore, that nuclear factor κ-B (NFκB) is a redox-sensitive transcription factor and also migrates as two bands in DNA mobility shift assays (*34*).

Steroid receptors bind response element DNA as homodimers (*35, 36*) through two dimerization regions. A short 5 amino acid sequence in the DNA binding domain (*37*−*39*) promotes weak dimerization since DNA binding domain fragments fail to dimerize in solution (*37*) even at millimolar concentrations (*40*). A less well characterized ligand binding domain region (*41*) contributes to solution dimerization of nuclear receptors, which is strengthened through DNA binding domain interactions in association with specific DNA binding (*42*). A conserved hydrophobic heptad repeat was implicated in ligand binding and dimerization of the estrogen receptor (*41*). Experiments with estrogen, glucocorticoid, and progesterone receptors suggest that full-length receptors dimerize in solution prior to binding DNA (*40*). Coimmunoprecipitation of progesterone receptor fragments or immobilization of fragments by affinity chromatography supported solution dimerization independent of DNA binding (*43*). Solution dimerization of the purified glucocorticoid receptor was rate-limiting for high-affinity binding to glucocorticoid response element DNA (*44*), as was solution dimerization for the estrogen receptor (*45*). Dimerization via the ligand binding domain stabilized solution homodimerization of other nuclear factors such as hepatocyte nuclear factor 4, facilitating DNA binding and stabilizing protein−DNA complexes (*46*). Steroid receptors may nevertheless form less stable dimers in the absence of DNA (*47*).

Relative to other steroid receptors, solution dimerization of AR requires high receptor concentrations (0.2 μM), with dimerization greatly increased by DNA binding. Because AR DNA binding is observed at low AR concentrations in the presence of carrier protein, and because in data not shown the addition of albumin did not enhance AR dimerization, solution dimerization is not likely required for AR dimerization in association with DNA binding. However, the similar concentration dependence of solution dimerization and DNA binding in the absence of carrier protein suggests that prior dimerization facilitates DNA binding. The lack of stable dimers at low AR concentrations in the absence of DNA and the absence of AR monomer binding to androgen response element DNA (*7*) indicate a critical association between DNA binding and AR dimerization.

The activities of several transcription factors are sensitive to redox conditions (*48*). The DNA binding and activation state of NFκB is increased by reactive oxygen species and H_2O_2 (*49, 50*). Activator protein 1 (AP-1) composed of Fos and Jun monomers forms heterodimers after treatment with diamide, creating a slower migrating form with decreased DNA binding activity (*34*). A cysteine residue in the DNA binding domain of the Fos and Jun transcription factor family is positioned in a conserved lysine-cysteine-arginine motif that contributes to redox-dependent DNA binding (*34, 51*). Other transcription factors susceptible to redox control include Sp1 (52) and the glucocorticoid receptor (*53*). An increase in the intracellular reducing conditions after treatment of cells with dithiothreitol (DTT) enhanced specific gene expression in response to glucocorticoids (*53*). An association of protein disulfide isomerase with estrogen receptor DNA binding further supports redox regulation of steroid receptors (*54*). More recently it was shown that H_2O_2 inhibition of estrogen receptor-mediated gene transcription was reversed by overexpression of thioredoxin (*55*).

There are nine conserved cysteine residues in the AR DNA binding domain, eight of which comprise the zinc finger region required for DNA binding. Loss of DNA binding activity with diamide treatment prior to AR DNA binding likely results from cross-linking of cysteine residues in the DNA binding domain. This may be reflected in the observations that crude or purified AR preparations can lose DNA binding activity despite high AR expression levels due to oxidation of critical sulfhydryl groups in the DNA binding

domain. Oxidation of the glucocorticoid receptor prior to DNA binding also interfered with DNA binding (*56*−*58*). It is therefore important to distinguish that dimer formation by diamide was most pronounced after AR binding to DNA. Under these conditions, zinc finger cysteines are likely buried and less affected by disulfide cross-linking reagents, whereas other sulfhydryl groups may be correctly aligned to form disulfide bonds. Cysteine 619 in human AR, the ninth conserved cysteine of the DNA binding domain, does not chelate zinc (*10*) and is preceded by arginine 617 and lysine 618 that form part of the bipartite nuclear targeting signal (*5*). These positively charged residues provide a basic environment that could increase the exchange rate of cysteine sulfhydryl groups with disulfide bonds (*59, 34*), making cysteine 619 a candidate for redox control. Crystal structures of other steroid receptor DNA binding domains indicate, however, that the ninth cysteine side chain is buried (*40*). More likely candidates involved in disulfide-linked AR dimers bound to DNA may be among the five cysteine residues in the steroid binding domain, one in the hinge region, and the 10 residues in the NH_2-terminal domain.

The 5-fold slower dissociation rate of the oxidized, disulfide-linked AR from DNA compared to the reduced form suggests that cellular redox state may influence androgen-dependent gene activation. This may be particularly important in certain disease states such as prostate cancer since reactive oxygen radicals are known to accumulate with increasing age. Although it remains to be demonstrated whether disulfide-linked AR dimers bound to DNA occur in vivo and whether they influence androgen-induced gene activity, precedence for redox-regulated and/or disulfide-linked transcription factors makes it conceivable that changing redox states influence AR transcriptional activity.

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