Published in final edited form as:

Proteomics. 2012 January ; 12(1): 43–53. doi:10.1002/pmic.201100348.

Proteomic Analysis of the Androgen Receptor via MS-compatible Purification of Biotinylated Protein on Streptavidin Resin

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Abstract

The strength of the streptavidin/biotin interaction poses challenges for the recovery of biotinylated molecules from streptavidin resins. As an alternative to high temperature elution in urea containing buffers, we show mono-biotinylated proteins can be released with relatively gentle heating in the presence of biotin and 2% SDS/Rapigest, avoiding protein carbamylation and minimizing streptavidin dissociation. We demonstrate the utility of this mild elution strategy in two studies of the human androgen receptor (AR). In the first, in which formaldehyde crosslinked complexes are analyzed in yeast, a mass spectrometry-based comparison of the AR complex using SILAC reveals an association between the androgen activated AR and the Hsp90 chaperonin, while Hsp70 chaperonins associate specifically with the unliganded complex. In the second study, the endogenous AR is quantified in the LNCaP cell line by absolute SILAC and MRM-MS showing approximately 127,000 AR copies per cell, substantially more than previously measured using radioligand binding.

Keywords

Absolute SILAC; androgen receptor; prostate cancer; SILAC; streptavidin

Introduction

Some of the strongest protein-ligand interactions yet described occur between the tetrameric protein avidin, isolated from egg whites, or streptavidin, produced by the bacteria *Streptomyces avidinii*, and the water soluble vitamin biotin [1]. The extreme affinity of streptavidin for biotin ($K_D \sim 10^{-15}$ M) has guided the development of a family of essential tools widely used in many fields of biology and chemistry. One such application is the purification of biotinylated molecules on streptavidin derivatized solid phase. The essential

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Conflict of Interest The authors declare no financial or commercial conflict of interest.

Supporting Information Supporting Information includes: MS protein identification and SILAC ratios for AR complex in *S. cerevisiae* (Table S1); absolute SILAC peptide ratios determined by MRM-MS (Table S2); and western blot analysis of LNCaP lysate preparation and AR quantification (Figure S1). Detailed SILAC dataset information and Absolute SILAC measurements are compiled as a spreadsheet (Supplemental Proteomics Data).

irreversibility of the biotin/streptavidin bond can, however, make recovery of biotinylated molecules a major challenge.

In response, various approaches have been employed to induce reversible binding. Some strategies utilize biotin derivatives that either bind with reduced affinity (iminobiotin) [2] or can be cleaved by reducing agents or light [3, 4]. Alternatively, the streptavidin binding site has been engineered to reduce the binding affinity via amino acid modification [5, 6] and by conjugating light, pH, or temperature sensitive polymers to the active site [7-9]. Monomeric avidin supports (available from a number of vendors), consisting of a single subunit of the tetrameric native protein, offer the possibility of gentle elution conditions. Additionally, the streptavidin binding peptide (SBP) and Nano-tag peptide offer alternatives to biotin, which elute from streptavidin under gentle conditions [10, 11]. Finally, introducing an external cleavage site, such as a Tobacco Etch Virus (TEV) protease recognition sequence between the recombinant protein and biotin tag, allows recovery of the purified protein after addition of the appropriate enzyme [12]. Unfortunately, these strategies have limitations and are not applicable in all experimental designs. *In vivo* biotinylation methods preclude using modified biotin [13], and reducing the strength of the streptavidin/biotin interaction can limit the efficiency of capture or preclude capture and washing in the denaturing conditions required to solubilize some proteins and preserve protein modifications susceptible to enzymatic cleavage [14]. Introducing a TEV site may reduce protein solubility [15] and TEV protease can result in unwanted cleavage in some proteins.

For MS-compatible proteomic work-flows, the few existing methods to recover sample after biotin/streptavidin capture are further limited. Direct trypsinization of the bound protein results in significant pollution of the MS sample with streptavidin peptides [14]. Harsh elution conditions (6 M urea, 2 M thiourea, 2% SDS, 30 mM biotin, pH 12 at 96 °C for 15 minutes) can recover 90% of the bound multi-biotinylated protein [16], but heating in the presence of urea causes carbamylation [17] and high temperatures result in dissociation of the streptavidin subunits, again polluting the MS sample. If streptavidin purification is used as the first step in the tandem affinity purification of formaldehyde crosslinked protein complexes, high temperatures can disrupt crosslinking prematurely.

An example of a proteomic strategy that would benefit from a milder MS-compatible elution following streptavidin purification is the hexahistidine-biotin-hexahistidine (HBH) tandem affinity tag, which utilizes an *in vivo* biotinylation signal and a hexahistidine tag [14]. This tag was designed to allow purification of formaldehyde crosslinked proteins under denaturing conditions, in order to preserve modifications in the protein complex of interest and minimize background proteins in subsequent MS-analysis. Here we report a novel and generalizable method to elute monobiotinylated proteins from streptavidin using mild heating in the presence of ionic detergent and biotin resulting in highly purified protein without streptavidin contamination. The practical utility of this method for studies of biomolecules is demonstrated with an MS-based SILAC [18] analysis of the recombinant AR (rAR)- foldosome complex in yeast and an Absolute SILAC [19] quantification of AR in prostate LNCaP cells.

Materials and Methods

Expression of recombinant Androgen Receptor (rAR), LexA and Foamy viral envelope protein for method development

A gene construct coding for full-length AR followed by a carboxy-terminal hexahistidine-biotin-hexahistidine (HBH) tandem affinity tag [14] was cloned into p416TEF plasmid (ATCC) to generate *p416TEF-AR-HBH*. This vector was transformed to *S. cerevisiae* By4741 (Mat A his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) using the uracil selectable marker. 2 L

Complete Minimal Dropout Medium [3.4 g YNB/L, 10 g/L (NH₄)SO₄, 0.77 g CSM-Uracil (MP), 2% dextrose] supplemented with 5 μM d-biotin was inoculated, grown to OD₆₀₀ = 0.8, and harvested by centrifugation. Cell pellets were rinsed in cold PBS, resuspended in a minimal volume of Lysis Buffer (8 M urea, 300 mM NaCl, 0.1% Triton, 50 mM Tris-HCl pH 8, 50 mM sodium phosphate pH 7.4, 1 mM PMSF, protease inhibitors), dripped into liquid nitrogen, and stored at −80 °C for subsequent lysis by mechanical ball mill (*Retsch 100*). Ground cells were resuspended in 10 mL lysis buffer supplemented with 10 mM imidazole. Clarified lysates were incubated with Ni-NTA resin (*Qiagen*) for 1 hour, poured into a column and washed with 10 bed volumes each of Lysis Buffer and Lysis Buffer (pH 6.3), and eluted in imidazole Elution Buffer (0.5 M imidazole, 0.5 M NaCl, 20 mM sodium phosphate pH 7.4). Separately, LexA was cloned in the pJSS3.1 plasmid [20], carboxy-terminal tagged using NheI and HindIII cloning of the HBH epitope [21], and expressed in the BY4705 yeast strain. Cultures were grown to OD₆₀₀ = 0.8, lysed by glass bead beating and purified by tandem affinity as for the rAR. Foamy viral envelope protein carboxy-terminal tagged with the hexahistidine/biotin/TEV (HTB) tandem affinity tag [14] was expressed in 293D mammalian cells.

rAR expression for SILAC crosslinking experiments

pYEX-BX-ARHBH in yDM0001 was cultured separately in isotopically heavy or light Minimal Media [Dropout Medium minus uracil supplemented with 50 mg/L ¹³C¹⁵N arginine, ¹³C¹⁵N lysine (*ISOTEC*), light amino acids, and 5 μM d-biotin]. At OD₆₀₀ = 0.6, 1 L cultures were induced to express rAR with 0.5 mM CuSO₄, and 50 μM d-biotin. After an hour induction, heavy and light cultures were stimulated with 1 μM DHT or ethanol vehicle, respectively and cultured an additional 2 hours. Heavy and light cultures were harvested by centrifugation, quantified by OD₆₀₀, and resuspended in 100 mL PBS as a 1:1 mixture of heavy to light cells. The cell mixture was fixed with 1% formaldehyde for 2 minutes at 30 °C, and quenched with 125 mM glycine for 15 minutes at 30 °C. Fixed cells were spun down, resuspended in a minimal volume of PBS, dripped into liquid nitrogen and stored at −80 °C.

rAR expression for Absolute SILAC

AR-HBH was cloned from *p416TEF-AR-HBH* into a *pYEX-BX* plasmid (*Clontech*) to generate *pYEX-BX-AR-HBH* and expressed in the *S. cerevisiae* yDM0001 (Mat α arg4Δ0 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0). 1 L of Minimal Media [3.4 g/L YNB, 10 g/L (NH₄)SO₄, 2% dextrose] lacking uracil and supplemented with 50 mg/L ¹³C¹⁵N arginine and lysine (*ISOTEC*), light amino acids, and 5 μM d-biotin, was inoculated and cultured to OD₆₀₀ = 0.6. The culture was induced to express protein by adding 0.5 mM CuSO₄, 50 μM d-biotin, and 1 μM dihydrotestosterone (DHT). Cell harvest, lysis, and protein purification followed the optimized 2% SDS mild elution method described. SDS was removed from the elution buffer by SDS-Out (*Pierce*) and purified protein was quantified by BCA at a yield of > 50 μg rAR per liter culture.

Protein purification by reversible biotin-capture on streptavidin

Ni-NTA purified protein was incubated with pre-equilibrated Ultralink Streptavidin Plus beads (*Pierce*) for 1 hr at RT. Beads were washed 3× in batch with 5 column volumes (cv) SA Wash Buffer (200 mM NaCl, 50 mM Tris-HCl pH 8, 0.1% SDS), followed by a stringent batch incubation/wash (60 °C, 10 minute incubation in 5 cv SA Wash Buffer + 2% SDS), and eluted serially with 2× 2.5 cv “SDS/biotin” elution buffer (200 mM NaCl, 50 mM Tris-HCl pH 8, 2.0% SDS, 1 mM d-biotin) batch incubated at 60 °C for 30 min. Optimization of the elution buffer composition and incubation temperature was performed by small volume purifications (20 μl streptavidin beads) with batch elutions incubated on a Eppendorf Mastercycler gradient PCR machine. The “SDS/biotin” elution buffer was

compared against a denaturing “urea/SDS/biotin” buffer [50 mM Na₂HPO₄ pH 12, 6M urea, 2 M thiourea, 2% SDS, 100 mM NaCl, 30 mM biotin] and a “biotin” buffer [50 mM Tris-HCl pH 8, 200 mM NaCl, saturated with excess biotin].

Silver stain and Western analysis

SDS-gly-PAGE gels were run with NuPAGE 4-12% precast gels in MOPS running buffer (*Invitrogen*). Silver gel-staining was performed following the protocols of Shevchenko et. al. [22]. Immunoblot analysis of AR was performed on PVDF (*Millipore*) using rabbit α AR polyclonal (AR-20) or mouse α AR monoclonal (AR-441) primary antibodies (*Santa Cruz Biotechnologies*). Detection by film was performed with goat α -Rabbit IgG HRP (*abcam*) secondary antibody, visualized by ECL detection (*GE Healthcare*). Image densitometry was performed using ImageJ [23].

Preparation and analysis of crosslinked androgen stimulated rAR in *S. cerevisiae*

Crosslinked AR was tandem purified from the pooled SILAC sample using the described lysis and purification methods, modified to include tip sonication of the lysate by 3× 30 second burst at 30% power setting (*Branson Sonifier 250*) prior to tandem affinity purification. Following purification, sample crosslinking was reversed by heating at 95 °C 30 min. Samples were reduced, alkylated, and trypsinized following either SDS/urea-based digestion [24] or in-gel digestion [25] protocols. Detergent was removed from the tryptic digest using an Oasis MCX extraction cartridge (*Waters*) and the sample was subsequently desalted by UltraMicroSpin Vydac C18 silica column (*Nest Group, Inc*) following manufacturer’s specifications. Tryptic peptides were dissolved in Loading Buffer [0.1% formic acid; 1% acetonitrile; 98.9% water;], trapped on a fused silica fritted capillary pre-column packed with 2 cm reverse-phase Magic C18Aq RP spherical silica (75 μ m ID, 5 μ m, 200 Å; *Michrom Bioresources*), and separated over a 15 cm reverse-phase Magic C18Aq RP analytical column (50 μ m ID, 5 μ m, 100 Å). The gradient program was a 60 min linear gradient from 2-35% acetonitrile at a flow rate of 0.35 μ l/min (*Agilent 1100 Series LC* system). Nanospray ESI MS/MS-analysis was performed using a Thermo Scientific LTQ Orbitrap. MS/MS was acquired over a range of 50-2000 m/z using a 60 sec dynamic exclusion time and a 35.0 V collision voltage.

MS spectra were converted to universal mzXML file format by ReAdW version 4.3.1 and searched against protein database FASTA files for *S. cerevisiae* (Uniprot) plus AR and known contaminants. Searches were performed using X!Tandem [26] with the following parameters: tolerable tryptic termini = 1; identifications based on b and y ions; parent mass tolerance = 3.00; daughter ion mass tolerance = 0.50; fixed modifications include carboxyamidomethylation of cysteine (57.02); variable modifications include oxidation of methionine (15.99), SILAC heavy arginine: ¹³C6-¹⁵N4 (10.01), SILAC heavy lysine: ¹³C6-¹⁵N2 8.01. MS/MS peptide assignments were validated by PeptideProphet [27] and protein assignments validated by Protein Prophet [28]. Statistical analysis and error rate information are included in the supplemental materials. Quantitative SILAC ratios for proteins were determined using XPRESS software [29], available in the current TPP distribution (<http://tools.proteomecenter.org/wiki/index.php?title=Software:TPP>). Precursor ion elution profiles of heavy vs. light peptides were determined with a mass tolerance of 0.05. The area under the curve (AUC) was used to determine a SILAC ratio for each peptide. Outlier AUC ratios were identified by RelEx least squares regression fit of AUC profiles [30] and removed to generate a parsimonious dataset. The uncertainties of SILAC ratios were determined for each protein expression level for which multiple peptide measurements were available.

Absolute SILAC of AR in LNCaP

LNCaP cells (ATCC CRL-1740) passaged 8 rounds on RPMI 1640 (- phenol red) were identically plated to two dishes at a density of 3 million cells per 15 cm dish (*Corning*) and grown to 80% confluency. Dish 1 was used for preparing cell lysate and dish 2 used to estimate the number of cells present in dish 1. Cells in dish 1 were harvested by resuspension in 0.4 mL RIPA buffer [10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.2 mM sodium vanadate, 1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS], followed by tip sonication in three 30 second bursts at 30% power setting (*Branson Sonifier 250*). The lysate was cleared of cellular debris by centrifugation (cellular debris showed no detectable AR by immunoblot). Total protein content of LNCaP lysate was quantified at 5.5 ± 0.2 mg in 0.5 mL by BCA assay. The duplicate dish was used to determine the total number of LNCaP cells/dish by cell resuspension and hemacytometer count, which calculated 55.9 ± 2.1 million cells per plate.

AR concentration in the LNCaP lysate was initially determined by quantitative immunoblot using the Odyssey IR Imaging System (*Li-cor Biosciences*). Sample titrations were run on 4-12% precast NuPAGE gel (*Invitrogen*), transferred to Immobilon-FL PVDF (*Millipore*) and probed with AR-N20 or AR-441 followed by detection with goat α -Rabbit(680 nm) or goat α -Mouse(680 nm) per the manufacturer's instructions. Load volumes were controlled for using α -GAPDH(800 nm) (*abcam*). Based on the western analysis, samples were prepared with isotopically heavy rAR spiked in to LNCaP lysate over a 64 fold dynamic range (rAR: 40 ng, 10 ng, 2.5 ng, 0.625 ng; LNCaP: 10 μ g). Samples were gel purified and prepared for MS by in-gel digestion following established protocols [25].

Targeted multiple reaction monitoring (MRM)-MS was performed using a consensus set of proteotypic AR peptides identified from an in-house SpectraST library of LNCaP shotgun data [31]. A putative MRM transition list was generated for 13 consensus peptides using MaRiMba software [32] with a pilot MRM-MS experiment performed on pure rAR standard identifying 7 of the 13 consensus peptides to have robust heavy peptide signal. These 7 peptides were selected as a parsimonious target set for the spike-in samples. A high abundance peptide 'DAVVYPILVEFTR' from hypoxia upregulated protein was monitored as a loading control. Samples were suspended in Loading Buffer, trapped on a Michrom peptide CapTrap (*Applied Biosystems*) and separated by reverse phase over a Dionex Acclaim PepMap100 C18 column (3 μ m, 100 Å) using a 40 minute linear gradient (2-35% acetonitrile) at a flow rate of 300 nl/min (Eksigent Tempo nanoMDLC pump). MRM-MS-analysis was performed on an AB SCIEX QTRAP 5500 (*Applied Biosystems*) targeting 4 MS2 transitions per peptide. Data files (.wiff) were analyzed by Skyline v0.7 [33].

Results and Discussion

MS compatible purification of mono-biotinylated proteins from streptavidin resin

An MS-compatible tandem purification strategy was developed using an HBH-tagged androgen receptor protein, recombinantly expressed in *S. cerevisiae*. This work began with the goal of purifying the rAR, crosslinked by formaldehyde to coactivating proteins, in the context of androgen stimulation or starvation. The HBH is thought to be resistant to covalent destruction by formaldehyde due to the nature of its antigens, the hexa-Histidine tag and biotin. The tag contains a 74 amino acid biotinylation signal sequence, which includes a specific lysine residue that is biotinylated by endogenous biotin ligases present in *S. cerevisiae* and other prokaryotic and eukaryotic cells [14]. Preliminary work was successful in showing that purification of the 110 kD recombinant AR from *S. cerevisiae* lysate using Ni-NTA (QIAGEN) quickly generated an enriched rAR fraction of approximately 10% purity. This product was used for subsequent streptavidin purification tests. In our original

studies, we found that “release” of the rAR protein by trypsin digestion worked, but was severely limited by an excessive amount of streptavidin peptides. These findings motivated the desire to identify a MS compatible release from streptavidin. A separate control purification of rAR directly onto streptavidin beads yielded sample contaminated by yeast proteins acetyl-CoA carboxylase (Acc1p) and pyruvate carboxylase (Pyc1p/2p) consistent with previous reports of these proteins being biotinylated in *S. cerevisiae* [34].

We first sought to confirm that the harsh conditions reported to elute multi-biotinylated protein [16] would result in the recovery of mono-biotinylated rAR, and to test for the necessity of urea, SDS, and biotin in the elution buffer. Nickel column purified rAR was incubated with streptavidin beads for 90 minutes at room temperature to load protein on the column (Fig 1a). AR specific western blot of the nickel purified fraction before and after incubation with streptavidin beads demonstrates that nearly all protein is biotinylated *in vivo* (approximately 95% by image densitometry). Streptavidin beads loaded with rAR were washed with 0.1% SDS and assayed for elution efficiency at 96 °C with three parallel buffers: 1) A denaturing “urea/SDS/biotin” buffer (6 M urea, 2 M thiourea, 2% SDS, 50 mM Na₂HPO₄, 100 mM NaCl, 30 mM biotin, pH 12); 2) an “SDS/biotin” buffer (200 mM NaCl, 50 mM Tris pH 8, 2% SDS saturated with excess biotin); and 3) a “biotin” only buffer (200 mM NaCl, 50 mM Tris pH 8, saturated with excess biotin). Following initial elution (Elate 1), the beads were stripped by a second control elution with “urea/SDS/biotin” buffer at 96 °C to determine remaining protein content (control eluate). Elution efficiencies were assessed by immunoblot with AR-specific antibody (Fig 1a). Results demonstrate that elution with “urea/SDS/biotin” buffer recovers the majority of the rAR in a single elution with a subsequent control elution yielding no additional signal. Slightly less rAR was recovered using the “SDS/Biotin” buffer (lane 2), with subsequent control elution of beads yielding additional rAR signal. Though a previous study has reported elution of biotinylated oligonucleotides from magnetic streptavidin beads (Invitrogen M-280 Dynabeads) with TE buffer (10 mM Tris pH 8, 1 mM EDTA) and biotin alone [35], no elution was achieved from the streptavidin beads in the absence of SDS using “biotin” buffer (lane 3), while subsequent control elution of the beads released the rAR protein. The small loss in recovery observed between the “urea/SDS/biotin” and “SDS/biotin” buffers is likely due to the absence of urea, which alters the streptavidin quaternary structure and acts as a biotin analog at millimolar concentrations [36]. Because carbamylation occurs with the heating of proteins in the presence of urea, we deemed this loss to be acceptable and proceeded with the TBS-based “SDS/biotin” buffer as our standard eluent system.

To investigate the minimal temperature sufficient for elution and to determine the role of biotin in this process, we assayed parallel elutions across a temperature range of 35-96 °C in the presence or absence of biotin. The elution efficiency was determined by a control elution as before, with elutions monitored by western blot and silver staining (Fig 1b). Notably, biotin was found to reduce the rAR elution temperature and stabilize the streptavidin subunits, resulting in less streptavidin dissociation. Western blots demonstrate nearly full recovery of rAR starting at 60 °C in the presence of biotin (+ Biotin) but not until 80 °C in the absence of biotin (- Biotin). The corresponding silver stain shows that at 60 °C there is no streptavidin monomer detectable when biotin is present in the eluent. Without biotin, the temperature required to elute rAR (80 °C) is equivalent to the temperature at which streptavidin subunits begin to dissociate from the solid support despite the stabilizing presence of biotin or biotinylated protein, i.e. protein is released by irreversible destruction of the streptavidin tetramer. Note that the 13 kD streptavidin monomer is eluted from the solid support at 80 °C in the presence of biotin, but at 50 °C in the absence of biotin, consistent with the reduced stability of unliganded streptavidin. This observation agrees with studies showing that in the absence of a ligand, the streptavidin tetramer to monomer transition begins around 50 °C with the midpoint occurring at 61 °C in 0.1% SDS [37]. The

role of biotin in streptavidin stabilization was confirmed by boiling equal amounts of streptavidin beads under a combination of 2% SDS and biotin buffer conditions (Fig 1c). In the absence of a detergent, heat alone is found to cause streptavidin instability, which is mitigated by biotin binding. SDS in the absence of biotin produces the most substantial tetramer dissociation, which is also blunted by bound biotin.

The 60 °C mild elution strategy was tested on additional recombinant proteins to confirm the general applicability of the method for eluting mono-biotinylated targets. LexA expressed in *S. cerevisiae* with a carboxy-terminal HBH tag was tandem purified with parallel streptavidin elutions across a temperature gradient, as for rAR (Fig 2a). Gradient elution of LexA from the streptavidin beads mimics rAR elution with nearly complete recovery of protein achieved at 60 °C in “SDS/biotin” buffer. Separately, we purified human foamy viral envelope protein expressed with a carboxy-terminal hexahistidine/biotin/TEV (HTB) tandem affinity tag in 293D mammalian cells. Elution of the foamy viral protein from streptavidin beads exhibited similar temperature dependence, eluting completely at 65 °C. This is shown in figure 2b where the upper band is the full-length protein and the lower band corresponds to the incorrectly processed transmembrane protein domain.

In addition to testing purification of alternative proteins, we also wished to assess the efficacy of an alternative anionic detergent, acid labile surfactant (ALS), in place of SDS in the elution buffer (Fig 2c). SDS in purified samples can interfere with trypsin digestion and liquid chromatography; it must be removed prior to MS-analysis. ALS offers the advantage that it can be degraded to insoluble components with acid [38]. In figure 2c recovery of rAR begins at 60 °C and peaks at 70 °C with no additional recovery at higher temperatures when eluted in 2% ALS. The control eluate bands indicate that ALS-based elution is slightly less efficient than the SDS-based elution, leaving 25% residual protein on the column versus 12%, respectively.

Having refined an MS-compatible mild elution strategy for purification of biotinylated proteins, we wished to reduce the technique to practice for the proteomic analysis of androgen receptor. Two separate applications were pursued: 1) a purification of formaldehyde crosslinked rAR complexes in *S. cerevisiae* to monitor rAR cofactors in the recombinant system, and 2) application of rAR as a proteomic standard for absolute quantification of endogenous AR in the prostate cell line LNCaP.

SILAC identification of AR cofactors in *S. cerevisiae*

The human AR signaling axis is the central target of drugs used to treat prostate cancer and a number of next generation therapies have been designed to inhibit cofactors of the AR including the chaperonin Hsp90 [39]. The AR cofactor function of Hsp90 and Hsp70 chaperonins was initially detected in *S. cerevisiae* [40] and the organism has frequently been employed for the evaluation of AR-cofactor association by two-hybrid screening [41]. Additionally, *S. cerevisiae* has been used as a model system for the study of AR transactivation [42, 43]. We reasoned that rAR expression in an arg/lys auxotrophic strain of yeast would be useful for quantitative MS-based identification and dynamic monitoring of AR-chaperonins. To this end, we performed a purification of the crosslinked rAR complex using our mild streptavidin elution strategy. To distinguish cofactors associated with the ligand activated and unliganded states of the receptor, a SILAC experiment was performed with two parallel expression cultures. Cells grown on media substituted with isotopically heavy arginine and lysine were stimulated with the AR agonist R1881, while cells grown on light media were treated with ethanol vehicle only. Following a 4 hour induction of rAR, heavy and light cultures were mixed (1:1) and formaldehyde crosslinked using 1% formaldehyde for 2 minutes. Lysed cells were sonicated to solubilize AR complexes crosslinked to DNA; in the absence of sonication, ligand stimulated AR uniformly

fractionated to the DNA pellet. Each sample was subjected to tandem purification by nickel and streptavidin beads as described above, with release performed at 60 °C. Tandem purified samples were trypsinized and analyzed by high mass accuracy MS (*Thermo Orbitrap*).

Thirteen proteins were identified with high confidence in the purified AR complexes (Table S1). Of these proteins, the androgen receptor and chaperonin proteins were the most abundant (Fig 3a). SILAC heavy:light ratios of individual proteins demonstrate the Hsp90 chaperonin (Hsp82) to be associated with the rAR in both the unliganded (light) and liganded (heavy) receptor state (Fig 3b). Separately, Hsp70 chaperonin proteins (SSA1, SSA2, SSA4) are associated with rAR primarily in the unliganded receptor state, exhibiting very low H/L ratios. The structural protein actin (Act1) was also shown to associate with the unliganded receptor in addition to several highly abundant cytoplasmic proteins, while proteins associated exclusively with the androgen liganded receptor were not observed in this analysis.

These findings are consistent with a model of AR chaperonin activity wherein Hsp70 chaperonins bind to the partially folded receptor, recruiting Hsp90 to the unliganded complex. Conformational rearrangement of rAR upon ligand binding coincides with the dissociation of Hsp70 chaperonins, while Hsp90 participates in the trafficking and translocation of AR into the nucleus and subsequent function of the nuclear receptor in yeast. Hsp90 is a potential therapeutic target for prostate cancer treatment with clinical trials employing geldamycin derivatives presently underway [44]. These drugs inhibit Hsp90 function, which is thought to result in the increased degradation of the androgen receptor though the precise mechanism of drug action remains unclear. While Hsp90 association with the unliganded AR is well established, the role of this chaperonin in the activated complex is less well understood.

Our current findings indicate that Hsp90 may play a more substantial role in the activated AR complex than previously understood and suggest that the yeast system may provide a tractable model for pursuing this association. More generally we show the utility of our rapid purification system for proteomic analysis of complexes. It is notable that the stringency of the preparation, which includes two column purifications, the second of which (streptavidin) involves a wash with 2% SDS at 60 °C and a specific elution with biotin, results in an extremely pure and specific output. Our rediscovery of these factors as well as the suggestion of new proteins demonstrates the power of this system for analyzing complexes; especially unstable complexes that require covalent crosslinking to ensure durability through purification. Prior analyses of the AR have been challenging. No studies have been published with pull-down of the endogenous AR, while MS-analysis of a ectopically expressed AR complex in HEK293 cells has identified chaperonins along with cytoskeletal and transport proteins [45]. Importantly, the workflow here could be translated to mammalian systems; our earlier work with foamy viral envelope protein demonstrates the utility of the HTB tag in mammalian cells, which are also easily studied using SILAC.

Quantification of AR in LNCaP using an isotopically heavy protein standard

SILAC-based MS measurements can be extended to absolute quantification of proteins in non-labeled samples through the addition of known amount of isotopically heavy standard. This technique was named Absolute SILAC when first demonstrated using heavy protein produced in *E. coli* [19]. The method involves: 1) titrating a biological sample of interest with a isotopically heavy protein standard across a range of concentrations; 2) processing the sample for targeted MS/MS, potentially enriching for proteins of a given mass; and 3) determining the absolute quantity of endogenous protein by isotopic ratios across available peptides. Absolute SILAC allows for protein quantification in biosamples where isotopic incorporation is not possible (human samples), where antibodies are not available, and offers

the advantage that many proteotypic peptides from an individual protein can be monitored to ensure robust quantification.

We wished to explore the utility of Absolute SILAC for quantifying AR, which is a prognostic biomarker of prostate cancer progression. To this end, we purified isotopically labeled rAR protein from *S. cerevisiae* under mild elution conditions followed by precipitation of SDS to generate a > 95% pure rAR protein standard (as determined by Coomassie stain). The yield of rAR standard was calculated by BCA quantification to be > 50 µg rAR per liter culture with the standard shown to incorporate > 98% ¹³C¹⁵N labeled arginine and lysine by MS-analysis of the trypsinized protein. A dilution series of the rAR standard was measured by quantitative western analysis against a dilution series of protein lysate from a known number of cultured LNCaP prostate cancer cells to estimate the relative concentration (and copy number) of AR in LNCaP cells (Fig 4a). The endogenous AR protein has a predicted mass of 99 kD, but runs slightly larger due to post-translational modification (PTM), while the rAR standard has a predicted mass of 110 kD owing to the 11.5 kD HBH tandem affinity tag. Quantification with the amino-terminal specific antibody AR-N20 (*Santa Cruz Biotech.*) gives an AR concentration of 2.7 ± 0.4 ng protein in 10 µg LNCaP lysate. This was consistent with a second quantification performed using the antibody AR-441, specific for AR residues 299-315 within the receptor amino-terminal domain (*Santa Cruz Biotech.*). Loss of endogenous AR with the cellular debris during preparation of the LNCaP lysate was assayed by western and demonstrated to be less than 0.5% total AR protein (Fig S1).

To perform the quantification of AR, 10 µg LNCaP lysate was doped with a range of heavy rAR protein standard (0.625, 2.5, 10, and 40 ng). These samples were enriched for AR prior to MS-analysis by isolating a 90-125 kD protein fraction by SDS-PAGE purification (Fig 4b). The ratio of heavy rAR standard to light endogenous AR protein in the spike-in fractions was determined by multiple-reaction-monitoring (MRM) targeted MS-analysis of 7 proteotypic peptides, monitoring 4 transitions per peptide. 4 of the 7 targeted peptides gave a high confidence correlation of heavy:light ratios across the spike-in range of 0.625 – 10 ng AR and these standards were used to calibrate the absolute concentration of AR for each peptide measured (Fig 4c). Based on the number of LNCaP cells in the lysate sample, the absolute copy number of AR proteins per LNCaP cell was determined from the MRM-MS data to give an average copy number of $127,000 \pm 61,000$ AR copies per cell (Table 1). Because the 40 ng spike-in sample sat near the limit of linearity for our calibration curves, it was excluded from the absolute quantification model, though incorporation of this standard did not significantly alter the fits (H:L peptide ratios are listed in Table S2).

AR copy values calculated by Absolute SILAC are similar to those determined by quantitative Western ($145,000 \pm 21,000$ cp/cell) demonstrating the consistency of the two approaches and the potential utility of Absolute SILAC quantification where antibodies are not available. An advantage of the Absolute SILAC data is that higher resolution information can be discerned from the multiple peptides targeted. Copy number variability from peptide to peptide can reveal internal PTM sites, mutations, or protein splicing variation that mask the endogenous peptide from detection by targeted MRM-MS. Of the four peptides quantified in Table 1, only the 348-SGALDEAAAYQSR peptide has a reported phosphorylation site Y357 [46], coincident with the low absolute value of this peptide in our spike-in samples (1.52 ng). Our results imply that approximately half of all AR molecules in unstimulated LNCaP cells harbor a phosphorylation at Y357 (a phosphopeptide standard would be required for definitive measurement). Separately, the 32-EVIQNPGPR peptide also shows a comparatively low absolute value (1.34 ng), but this peptide contains no previously reported modifications and lacks S,T,Y, or K residues which typically participate in PTM's. While potential splice variants should have been filtered by

the 90-120 kD enrichment step, it is possible that differential degradation at the protein amino-terminus during handling contributed to reduced signal from the endogenous 32-EVIQNPGRP peptide. Peptides near the amino-terminus of proteins may be suboptimal for MS-based quantification due to processing. Significant degradation products were not observed by western using either the amino-terminal AR-N20 or internal AR-441 antibody probes, though partial loss of a short AR fragment cannot be reliably assessed by western blot. Additionally, MRM-MS was unable to measure an absolute calibration curve for 3 of the 7 peptides targeted. This was partially a consequence of poor signal to noise in these targets, although PTM's may have been a compounding factor as all three peptides have reported modifications (phosphorylation sites: Y223 [46] S300, T301, Y307 [47]; sumoylation site: K520 [48]).

To our knowledge, this is the first reported quantification of the AR in LNCaP cells by western and quantitative MS. Notably, the AR copy number determined by these methods is significantly higher than quantities previously reported by radioligand binding assay, which place the value between 10,000 - 15,000 AR copies per cell [49, 50]. While these assays measure the specific binding of tritiated hormone to the AR, the protein immunoblot and MS-based methods employed here do not measure AR functional activity, but rather quantify gross protein content. Thus, discrepancy between the different measurements is not necessarily inconsistent and may be explained by the presence of a large fraction of unfolded or otherwise non-ligand binding AR in the cell. The possibility that the radioligand binding assay underestimates the functional AR content of the cell has previously been raised and is based on the observation that the release of DHT ligand from the receptor is too slow to effectively exchange ligand in these assays [51]. Short equilibrium-based assays may therefore fail to detect ligand bound AR, which can represent the predominant receptor state depending on the cell preparation and the method of lysis [52]. The possibility that there are 150,000 functional AR copies in the cell may resolve a paradox that the number of AR-binding sites in the genome vastly outnumber the reported copies of AR in the cell. For instance, a recent ChIP-Seq analysis of AR DNA-binding sites has reported more than 37,000 discrete AR-dimer binding sites in LNCaP [53]. This number of occupied DNA-binding sites is in agreement with other studies of the AR cistrome and intuitively consistent with our measurement of 150,000 AR molecules per cell. Additional measurements to determine the number of functional AR proteins in the cell and the ligand-binding status of these receptors will be valuable in understanding the mechanisms of prostate cancer therapies that target the AR [44].

The Absolute SILAC method, employed in the current study, has multiple advantages over the addition of a synthetic peptide. The first is that multiple peptides can be targeted. The second is that sensitivity can be improved by co-purification at the protein level, such as the gel purification employed here. Further, co-digestion of proteins avoids artifactual mis-measurement due to partial cleavage and entirely sidesteps any problems that could occur with peptide precipitation or loss due to adsorbance on surfaces. Finally, as we demonstrate, the fraction of a particular site that is phosphorylated can be inferred from measurements referenced against peptides where modifications are unlikely. A potential concern with our strategy is that *S. cerevisiae* is capable of making PTM's, which could lead to a reduction in the apparent standard, resulting in an overestimation of endogenous protein. Alternative expression in *E. coli*, which does not decorate recombinant proteins (and typically produces a larger yield) may ultimately provide a better baseline standard for evaluating maximum protein concentration by MRM-MS. However, the production of full-length proteins in *E. coli*, especially large proteins, can prove challenging, and the yeast system may be useful in cases such as the one described.

Concluding Remarks

We have established that mono-biotinylated proteins can be conveniently released from streptavidin beads with relatively gentle heating in the presence of 2% SDS and biotin. This mild elution strategy is advantageous in studies of protein complexes preserved by *in vivo* crosslinking because it avoids carbamylation associated with heating in the presence of urea, minimizes streptavidin dissociation, and preserves formaldehyde crosslinks. Alternatively, TBS/2% ALS, biotin solutions can be used to prepare samples that are to proceed directly to trypsin digestion and MS-analysis with only slight decreases in recovery. The system is functional in yeast and mammalian cells and can be adapted for absolute quantification of protein using mass spectrometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Cancer Institute K08 CA097282 and PNW Prostate SPORE P50 CA097186 grants to Daniel Martin; CDMRP training grant W81XWH-10-1-0220 to Ryan Austin; and NIH/NIGMS center grant P50 GM076547 to the Institute for Systems Biology.

Abbreviations

ALS	acid labile surfactant
AR	androgen receptor
DHT	dihydrotestosterone
HBH	hexahistidine-biotin-hexahistidine
HTB	hexahistidine-TEV-biotin
rAR	recombinant androgen receptor
SA	streptavidin
TEV	tobacco etch virus

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Figure 1. Optimization of mild streptavidin elution with androgen receptor

(A) Western blot of rAR purification on streptavidin. The efficiency of 3 elution buffers was compared, heating beads for 15 minutes at 96 °C (eluate 1). Residual streptavidin-bound rAR was assessed with a second “control eluate” in urea/SDS/biotin buffer. Pre and Post lanes show nickel column purified rAR before and after incubation with streptavidin beads. (B) Western blot and silver stain of a temperature gradient elution of AR from streptavidin beads with 2% SDS solution $\pm$ biotin for 30 min (eluate 1) followed by control elution. (C) Silver stain of streptavidin monomers present after boiling streptavidin beads $\pm$ 2% SDS $\pm$ biotin.

Figure 2. Mild streptavidin elution of biotinylated proteins

(**A,B**) Western blot for temperature gradient elution of LexA or Foamy Viral Envelope protein off of streptavidin beads in 2% SDS/biotin buffer for 30 minutes (eluate 1) followed by control elution. Double bands in the LexA eluates correspond to full-length protein and a smaller transmembrane protein domain, which has been incorrectly processed (**C**) Western blot for temperature gradient elution of rAR off of streptavidin beads in 2% ALS/biotin for 30 minutes (eluate 1) followed by control elution.

Figure 3. SILAC measurement of AR-chaperonin dynamics

(A) Silver stain of rAR crosslinked complex purified from yeast lysate and heat denatured for SDS-PAGE. (B) Heavy:Light SILAC ratios of eluted proteins. The H/L ratio of rAR and Hsp82p are similar, indicating that the two proteins are associated in both the unliganded (light) and liganded (heavy) receptor state. Separately, Hsp70 chaperonins (SSA1, SSA2, SSA4) specifically associate with the unliganded rAR as indicated by very low H/L ratios. Error bars are based on the distribution of constituent peptide H/L ratios.

Figure 4. Absolute quantification of AR in LNCaP

(A) Quantitative AR western. Two dilution series of the rAR standard [40 – 0.625 ng] and the LNCaP lysate [10 – 0.1 µg] were probed with AR specific antibody (AR-N20) to quantify endogenous AR in the LNCaP lysate. The endogenous concentration of AR is calculated to be 2.7 ± 0.4 ng AR per 10 µg LNCaP lysate, corresponding to an average concentration of $145,000 \pm 21,000$ AR copies per LNCaP cell. (B) Coomassie SDS-PAGE purification of spike-in samples. Heavy rAR standard was titrated into LNCaP samples and mass purified by SDS-PAGE. Dashed lines indicate the excised mass range. (C) AR quantitation by MS. Log/Log plots of Heavy:Light ratios for 4 proteotypic AR peptides quantified by MRM-MS.

Table 1**Androgen Receptor Quantification**

MRM-MS (peptide)	Androgen Receptor	
	ng ^a	cp/cell ^b
32-EVIQNPGPR	1.34 ± 0.01	72,000
128-GCVPEPGAAVAASK	3.73 ± 0.02	201,000
221-DNYLGGTSTISDNAK		
300-STEDTAEYSPFK		
348-SGALDEAAAYQSR	1.52 ± 0.04	82,000
511-VPYPSPTCVK		
862-LLDSVQPIAR	2.86 ± 0.01	154,000
<u>Quantitative Western (antibody)</u>		
AR-N20	2.7 ± 0.4	145,000
AR-441	2.5 ± 0.5	134,000

^a Absolute concentration of AR [ng] per 10 µg LNCaP lysate was calculated from linear regression fit of the titrated rAR standard protein.

^b AR copy number per cell of the 99 kD endogenous protein was determined from replica plate cell counting which estimated 55.9 ± 2.1 million cells per 5.5 ± 0.2 mg protein lysate.