Identification of the estrogen receptor Cd-binding sites by chemical modification

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Received 24th January 2005, Accepted 16th May 2005

First published as an Advance Article on the web

DOI: 10.1039/b501192b

The widely reported interactions of the estrogen receptor (ER) with endocrine disrupting chemicals (EDCs) present in the environment gave rise to public concern and led to a number of screening and testing initiatives on the international level. Recent studies indicated that certain heavy metals, including cadmium, can mimic the effects of the endogenous estrogen receptor agonist 17 β estradiol, and lead to estrogen receptor activation. Previous studies of the chimeric proteins, which incorporate the ligand binding domain of the human ER, identified Cys 381, Cys 447, Glu 523, His 524 and Asp 538 as possible sites of interactions with cadmium. In the present study we utilized the rainbow trout ER ligand binding domain fused to glutathione S transferase, and used Cd shielding against various types of chemical modification of the fusion protein to study non covalent interactions between the ER and Cd. The distribution of exposed and shielded residues allowed to identify amino acid residues involved in the interaction. Our data indicated preferential protection of Cys groups by cadmium, suggesting their involvement in the interaction. This supports data found in the literature on the strong binding affinity of the thiol group towards metals. However, not all Cys in the fusion protein sequence were protected against chemical modification, illustrating the importance of their chemical environment. In general, the location of *rt*ER LBD Cys residues implicated in Cd interactions did not confirm assignments made by alanine scanning mutagenesis for the *h*ER, probably due to differences in experimental setup and fusion proteins used. The involvement of other functional groups such as carboxylic acids in the Cd interactions, though not confirmed, can not be completely ruled out due to the general limitations of the chemical modification approach discussed in detail. Suggestions for an improved experimental setup were made.

Introduction

The discovery of the fact that certain environmental chemicals are capable of mimicking steroidal sex hormones or interfering with their metabolism, thus affecting the endocrine system, raised a lot of concern in the scientific community.^{1,2} It also opened up an entirely new field of research in environmental science.^{3,4}

Endocrine disrupting chemicals (EDCs) include natural and synthetic hormones, phytoestrogens, pesticides and a variety of industrial chemicals and by products. EDCs have already been implicated in the disruption of the reproductive system in wild animals.^{5–9} They are also thought to affect sexual development, adult fertility, the nervous system, and lead to behavioral impairments, immune dysfunction and hormone related cancers in Western populations.^{10,11} While research on these compounds is advancing, most environmental compounds including heavy metals have yet to be studied for endocrine disrupting effects. A variety of EDCs present in the environment exert their physiological effect through the estrogen receptor (ER), a typical member of the nuclear receptor (NR) transcription factors family. The C terminal ligand binding domain (LBD) of the ER recognizes a variety of structurally distinct compounds that bind to the ER with

different degrees of affinity and potency and contains important regions for hormone and DNA binding, dimerization, and transactivation.^{12–14} The ability of the ER to interact with a wide repertoire of compounds can be attributed to both the size and shape of the hydrophobic hormone binding cavity within the core of the receptor, which permits a variety of ligand binding modes.¹⁵ Some of these compounds such as the ER's natural ligand 17 β estradiol (E2) and other steroidal estrogens act as activators of the receptor's biological function influencing the development and functioning of multiple tissues.¹⁶ Recent studies indicated that certain heavy metals, including cadmium, can mimic the effects of estradiol.^{17,18} The biological mechanisms of heavy metal estrogenicity are coming to light only now, but it appears that heavy metals may block the binding of estradiol and lead to estrogen receptor activation,¹⁷ although data indicating that estradiol binding to the receptor was not affected by cadmium was also presented.¹⁹

With 17 000 tonnes of cadmium produced annually, mainly intended to satisfy ever growing needs of industrial societies, this heavy metal is a major environmental pollutant, present in soil and therefore in many foods.²⁰ Once taken up by an organism Cd tends to accumulate in kidneys, liver, lungs, and prostate and with a half life of 10 to 30 years is slow to excrete. Cd exposure is linked not only to kidney damage and bone disease, but also to lung, breast and prostate cancers.^{17,21} Early

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studies at Georgetown University demonstrated that cadmium is a potent activator of both estrogen receptors and the androgen receptor that play a critical role in the pathways of sex hormone signalling and cancer proliferation.¹⁷ It was observed that cadmium can mimic the effects of estradiol and suggested that its estrogenic properties are due to the formation of a high affinity complex with the receptor hormone binding domain which blocks subsequent access of its natural ligands. However, very limited substantial information is available on the specifics of Cd interactions with the ER active sites. To our knowledge, the only attempt to identify specific amino acid residues involved in Cd interaction was done by alanine scanning mutagenesis,¹⁷ a well established but quite laborious technique used to study protein interaction surfaces.²² Examination of the cadmium binding and subsequent activation of the wild type and mutants of ER α , identified cysteines C381 and C447, glutamic acid E523, histidine H524, and aspartic acid D538 as potential sites of interaction in the hormone binding domain of the *h*ER α .¹⁷ Though site directed mutagenesis remains the molecular biologists' method of choice for investigating the functional roles of amino acids in proteins, possible disruption of binding caused by the alanine substitution is only an indirect proof for molecular interactions that still requires validation by structural analysis of the complex.²² Chemical modification of amino acids during ligand binding, on the other hand, is a very attractive alternative for characterizing residues involved in non covalent interactions.²³ Reactions can be carried out either in solution or on a MS target, providing a convenient method for determining the relative importance of various functional groups in the active site for ligand binding. This approach can provide detailed information on the active site of an enzyme, when combined with modern mass spectrometric methods.^{23–25}

The purpose of the work presented here was to determine the amino acid residues of the *h*ER α involved in specific Cd binding, by using chemical derivatization and mass spectrometry. In this report we utilized reagents for the specific modification of Cys, His and carboxylic acid groups implicated in Cd binding by previous studies.¹⁷ Our results in general show active participation of the Cys residues in Cd interaction, while a participation of His and carboxylic acid residues, though not confirmed by our data, can still not be ruled out completely.

Materials and methods

Chemicals

Inorganic compounds used for buffer solutions, trifluoroacetic acid (TFA), α cyano 4 hydroxycinnamic acid, sinapic acid, sodium *N* lauroylsarcosinate, dithiothreitol (DTT) and water soluble 1 ethyl 3 (3 dimethylaminopropyl)carbodiimide (WSC) were from Fluka Chemie AG, Buchs, Switzerland. Organic solvents (methanol, acetonitrile, acetone, ethanol) were from Merck KGaA, Darmstadt, Germany. Trimethyloxonium tetrafluoroborate (TMO) was from Acros Organics, NJ, USA. The sodium salt of 2 (*N,N* diethylamino) diazenolate 2 oxide (DEANONOate) was from Alexis Biochemicals, Lausen, Switzerland. Diethylpyrocarbonate (DEPC),

S nitrosoglutathione (GSNO), glycine ethyl ester (GEE), conalbumin, pepsin and glutathione immobilized agarose beads were from Sigma Aldrich Chemie GmbH, Steinheim, Germany. Trypsin (recombinant, proteomics grade), Glu C and chymotrypsin (sequencing grade), were from Roche Diagnostics GmbH, Mannheim, Germany.

Chemical modification of the *rt*ER-LBD GST fusion protein

The GST fusion protein containing the ligand binding domain of the *rt*ER α was over expressed *E coli* and purified by glutathione agarose beads as described elsewhere.^{26,27} The biological activity of the over expressed fusion protein was verified using radio labelled E2 ligand binding assays which also indicated specificity of the observed interactions. The resulting recombinant protein contains 571 amino acid (aa) residues including 218 from Glutathione *S* transferase (GST) and 353 from the D, E and F ligand binding domains of the *rt*ER (Fig. 1). To allow comparison with related research, the numbering of the aa residues in the *rt*ER LBD, part of the fusion protein was done according to the original wild type sequence. Chemical modification of the agarose bound protein preparation was performed by incubation of fusion protein aliquots with different modifying agents. In all cases experimental conditions were designed to maximize the modification of the target amino acid residue, while preserving peak intensity of the modified protein. To optimize the protein modification, the protein MW and peak intensity were monitored after derivatizing under different conditions. The final conditions were chosen based on the best ratio between extent of protein modification, based on the shift in molecular weight, and the peak intensity and shape of the modified protein as detected by MALDI TOF MS. The effects of Cd protection against chemical modification were studied by pre incubation of the PBS buffered agarose bound protein preparation in the absence and presence of a fivefold molar ligand excess for 2 h at RT. Following Cd pre equilibration, samples were treated with chemical modification reagents using established experimental protocols. These results were compared against data derived from the control samples left without chemical modification and samples pre incubated with E2.

Sulfhydryl modification of Cys residues was performed using DEPC, GSNO and DEANONOate, as described previously.^{28–30} DEPC was used for *S* carbethoxylation, while both DEANONOate and GSNO were used for *S* nitrosylation. The latter reagent was also used for *S* glutathionylation. In addition to modification of Cys residues, DEPC could also be used for derivatization of the His, Tyr and Lys residues. DEPC solutions for protein modification were freshly prepared by diluting the original stock with cold absolute ethanol and further diluting with PBS buffer (pH 7.4) to the desired concentration range. The reaction was initiated by the addition of 50 μ l of buffered DEPC solution to aliquots of fusion protein in the same buffer to obtain the final DEPC concentration ranging from 0.04 to 10 mM. The mixture was incubated for 30 min at 0 °C. The reaction was stopped by removal of the supernatant and subsequent washing of the agarose beads. The imidazole group of His was modified with

Fig. 1 Amino acid sequence and tryptic map of cleavage sites for the GST *rtER* LBD fusion protein realigned with *hER* LBD. A sequence alignment of the LBD part of the *hER* is presented below that of *rtER*. The numbering of the fusion protein corresponding residue in *h* and *rtER* wild type sequences is given in curly brackets. Residues in bold were identified as being involved in Cd binding by alanine scanning mutagenesis. Cys residues are underlined in both protein sequences.

freshly prepared buffered DEPC ethanol solution using the protocol described above. In addition to the modification of Cys and His residues, DEPC could also be used for carbethoxylation of the phenolic hydroxyl group of Tyr and the ϵ amino group of Lys. The effect of Cd and E2 protection against both S nitrosylation and S glutathionylation by GSNO was determined by incubation of the pre equilibrated agarose bound protein preparations (final concentration ranging between 1 and 5 μ M) with GSNO, at pH 7.4 (25 mM PBS)

for 30 min at RT. The final concentration of GSNO ranged between 0.04 and 10 mM. Specific S nitrosylation of Cys residues alone was carried out using DEANONOate. Cadmium and E2 pre equilibrated preparations of the fusion protein and samples left without pre equilibration were incubated in PBS buffer with freshly prepared DEANONOate solution for 20 min at RT. The reaction was quenched by the removal of the supernatant, samples were then washed and taken for subsequent MS analysis.

The side chain carboxylic acids of Asp and Glu were modified either by esterification with TMO or coupled with glycine ethyl ester in the presence of water soluble 1 ethyl 3 (3 dimethylaminopropyl)carbodiimide (WSC), as described previously.^{31,32} For the latter, solutions of the fusion protein already containing glycine ethyl ester were mixed with WSC to a final carbodiimide concentration 0.1–100 mM and stirred for 2 h at RT. Esterification with TMO was done by adding the protein preparation slurry with or without ligand to a weighted amount of TMO crystals to yield a final reagent concentration of 20–30 mM. Due to the rapid hydrolytic breakdown of the reagent, the concentration of the TMO reaching the protein preparation is unknown. Agarose bound protein preparation was buffered by 10 times the volume of PBS buffer (pH 7.6), which was enough to maintain the reaction pH without significant changes. The reaction mixture was incubated at RT for 10 min after the addition of TMO. At the end of the incubation, the supernatant from the reaction mixture was removed, samples were then washed and analyzed by MS. In all cases control samples, left without pre equilibration and chemical modification, were incubated under the same experimental conditions.

MS analysis

Molecular weights (MW) of the intact proteins and peptide mass profiles were analyzed using MALDI MS. To prepare fusion proteins with and without chemical modification for further MS analysis they were removed from the agarose support by incubation in a saturated 0.1% TFA solution of sinapic acid in MeOH/ACN/acetone. After a 10 min incubation, 1 µl aliquots were deposited on the target plate, allowed to dry at room temperature, and analyzed without further purification on a Bruker Reflex III MALDI TOF MS equipped with a nitrogen laser (Bruker Daltonics GmbH, Faellanden, Switzerland). Three hundred laser shots were summed per sample spectrum and on average three spectra were used. Alternatively, proteins allowed to remain on the agarose support were digested and their peptide mass profiles were obtained. Sequencing grade modified trypsin (enzyme : substrate ratio of 1 : 20) was used to digest either intact or chemically modified proteins. Aliquots containing protein samples were diluted in 10 mM Tris buffer, pH 7.4 and incubated with trypsin for 2 h at 37 °C. The tryptic peptides were separated from agarose by centrifugation. The samples were analyzed by MALDI TOF MS without further purification. 1 µl aliquots of digested sample were mixed with 9 µl matrix solution (a saturated solution of either α cyano 4 hydroxycinnamic acid, or sinapic acid in MeOH/ACN/acetone, 0.1% TFA), then spotted onto the target plate and allowed to dry at room temperature. Three hundred laser shots were summed per sample spectrum and on average five spectra were used. The masses obtained were compared against theoretical digestion of the known sequence of the fusion protein. MW measurements of the intact and chemically modified proteins were carried out in a linear mode on samples internally calibrated with conalbumin, while peptide mass fingerprinting experiments (PMF) were carried out in reflectron mode.

Results and discussion

The goal of the work presented here was to clarify the role of the amino acids identified in previously published work as being involved in Cd binding. For this we compared results obtained with the *rtER* pre equilibrated with Cd and then derivatized with various reagents, with a Cd free control. These experiments were carried out with reagents specific for different functional groups of the *rtER* LBD. Derivatization of the fusion protein Cys residues was done using DEANONOate, GSNO and DEPC, which was also used to cross check modification of His residues. Modification of Asp and Glu residues was done using TMO and WSC together with GEE.

Cys and His residues

Derivatization using diethylpyrocarbonate (DEPC). Preliminary experiments with DEPC showed that treatment of the intact protein with rising DEPC concentrations lead to increasing MW, indicating extensive protein modification, combined with loss of signal intensity (Fig. 2A). It appears that a 2 mM DEPC concentration gives the best ratio between extent of modification and signal intensity, and for this reason was chosen for subsequent experiments. Data presented in Fig. 2B showed that Cd pre equilibration resulted in the partial protection of the *rtER* LBD against modifications by 2 mM DEPC. For example, treatment of the *rtER* LBD with 2 mM DEPC solution resulted in the increase of the protein MW from 63935 to 65322, an increase in 1387 Da, which corresponds roughly to 19 modified His, Tyr, Cys, Lys residues. The mean standard deviation of the molecular weight determination for *rtER* LBD by MALDI MS using internal calibrants was obtained by analyzing seven different spots

Fig. 2 Effect of DEPC concentration and ligand pre equilibration on the extent of fusion protein modification. (A) MALDI mass spectrum of the fusion protein incubated for 30 min at 4 °C with increasing concentrations of DEPC, washed and removed from the agarose support by a saturated solution of sinapic acid. (B) MALDI mass spectrum of the fusion protein pre equilibrated with fivefold molar ligand excess, incubated with 2 mM DEPC for 30 min at 4 °C and removed from the agarose support by a saturated solution of sinapic acid.

seven times and was 41.4 Da. Any shift in MW of less than 42 Da was not considered significant.

Pre equilibration in 20 μ M Cd solution prior to the 2 mM DEPC treatment showed a smaller increase in MW (1102 Da), corresponding to 15 modified aa residues and protection of another 4. Interestingly, pre equilibration of the *rrER* LBD with E2 did not offer any protection against DEPC modification. As a matter of fact, DEPC modification of the E2 pre equilibrated sample resulted in a MW shift of 1518 Da, which is even bigger than that for the not pre equilibrated DEPC treated sample, indicating modification of 2 more residues. This result suggests that binding of E2 to the *rrER* LBD leads to a more open conformation, susceptible to more extensive derivatization.

Identification of the amino acid residues involved in the interaction with Cd was done by comparing peptide profiles of chemically modified samples in the protected and unprotected state. Table 1 lists 7 Cys containing peptides belonging to the *rrER* LBD sequence which were the subject of further investigation. MALDI spectra of the tryptic digest of the *rrER* LBD control sample left without pre equilibration and chemical modification showed prominent appearance of the T36, T42, T51 and T56 tryptic peptides. Further examination also revealed 2 weak signals corresponding to the T55 fragment with and without missed cleavage (Table 1), while the T49 and T61 fragments were not detected all, either due to signal suppression or matrix interference. Thus, analysis of the interaction of Cd with the *rrER* LBD was limited to the 4 tryptic fragments giving strong MALDI signals.

The MALDI peptide mass fingerprints (PMF) presented in Fig. 3 show that 2 mM DEPC treatment of the *rrER* LBD leads to the modification of the 4 tryptic peptides T36, T42, T51, and T56. However, Cd pre equilibration of the *rrER* LBD prior to the 2 mM DEPC treatment prevented modification of the T51 fragment, while T56 remained unprotected (data not shown) and the extent of the modification for T36, T42 decreased (Fig. 3). The decrease was determined by normalizing the ratios to adjacent peaks. Pre equilibration of the *rrER* LBD with E2 on the other hand resulted in the modification of all four tryptic peptides, with relative intensities similar to the 2 mM DEPC treated control, thus confirming the results with the intact protein (Fig. 2B). This suggests that Cd is coordinated with the cysteines of T36, T42 and T51, with Cys430 being most strongly protected. Cys475 was not derivatized at all, suggesting no substantial Cd binding activity.

It is mentioned in the literature that DEPC can also modify His, Tyr, Lys residues³³ and possibly Arg as well.³⁴ It was also

reported that His is directly involved in the interaction with Cd, though with lesser affinity than Cys.¹⁷ Evaluation of the Cd interaction with His is complicated by the fact that some of the His containing peptides also include Tyr and Lys in their sequence, thus potentially leading to multiple modifications. The MALDI spectra showed that Cd did not offer any protection against DEPC modification and that multiple modifications occurred for peptides carrying multiple reaction groups (His, Tyr). In contrast to these observations no additional modifications were detected for the peptides, which in addition to Lys or Arg, also included either Cys, His, or Tyr residues in their sequence. Results obtained here are consistent with the recent findings from a theoretical study investigating interactions of Cys with a variety of metal cations, including Cd, indicating the possibility of establishing a strong mostly ionic cadmium sulfur bond with a small covalent character.³⁵

Derivatization using S-nitrosoglutathione (GSNO). The protective properties of Cd and E2 against nitrosylation were assessed using GSNO and DEANONOate. GSNO concentrations between 0.5 and 2 mM resulted in an only marginal increase of the protein MW from 63856 to 64059 (+203 Da) which could correspond either to the attachment on average of less than one glutathione molecule or several molecules of NO, both possible derivatization products (data not shown). However, increasing the GSNO concentration to 10 mM produced a substantial MW shift (+1147 Da), possibly corresponding to the attachment of four glutathione molecules (data not shown). The better reaction yields resulting in a single albeit broader peak, suggesting similar degrees of derivatization in the resulting conjugate mixture, made us choose the 10 mM GSNO concentration for further experiments.

GSNO treated protein samples showed a molecular weight shift of 1200 1500 Da compared to the unreacted control sample (data not shown). This increase corresponds to the glutathionylation of 4 to 5 Cys. Pre equilibration of the *rrER* LBD with Cd and E2 prior to GSNO exposure did not protect any Cys against derivatization with 10 mM GSNO, but actually resulted in more extensive modification compared with the GSNO treated sample left without pre equilibration.

The peptide patterns derived from the trypsin digestion of the *rrER* LBD, did not show significant differences between GSNO treated protein samples with and without Cd/E2 pre equilibration (data not shown). The 4 Cys containing peptides detected by MALDI MS were successfully glutathionylated, resulting in a mass shift of +305 Da for each peptide. Glutathionylation for Cd and E2 pre equilibrated samples appeared to be more extensive than for the sample without

Table 1 Tryptic map of the cys containing peptides from the *rrER* LBD

#	m/z	Start	End	Cys#	Sequence
T36	838.4	227	234	279	YCGPAGDR
T42	2510.3	270	292	341	ITMPPEQVLFLQLQAEPALCSR
T49	741.4	357	363	411	SIHCPGK
T51	2373.1	375	396	430	SEGDCVEGMAEIFDMLLATVSR
T55	964.5	404	411	460	PEEFVCLK
	1205.6	402	411		LKPEEFVCLK
T56	5632.8	412	464	475	AIILLNSGAFSFCNSVESLHNSSAVESMLDNITDALIHHSISGASVQQQPR
T61	250.1	492	493	543	CK

Fig. 3 Selected tryptic maps of the DEPC treated samples with and without ligand pre equilibration. Aliquots of fusion protein were pre equilibrated with fivefold molar ligand excess, incubated for 30 min at 4 °C with 2 mM DEPC, the reagent excess removed after centrifugation and the precipitate subjected to proteolytic digestion for 2 h at 37 °C. (A) Control, (B) DEPC treated sample without ligand pre equilibration, (C) and (D) DEPC treated samples pre equilibrated with E2 and Cd, respectively.

pre equilibration, as determined by comparing signal ratios of the glutathione conjugated peptides to the adjacent peaks (data not shown). This observation is consistent with data obtained for the intact protein, which also showed more extensive modification for pre equilibrated samples. However, unlike with the DEPC results, Cd pre equilibration did not protect the Cys in the 4 detectable peptides against derivatization with GSNO modification. The reason for this is that GSNO competes with the cysteines for Cd binding, and hence is inadequate for investigating the interaction of Cd with the ER.

Derivatization using 2-(N,N-diethylamino)-diazene-2-oxide (DEANONOate). The use of the S nitrosylating reagent DEANONOate (DEANO) was investigated in order to avoid the problems found with GSNO. Fig. 4 shows that DEANO concentrations up to 10 mM primarily lead to loss of absolute signal intensity and a trend to higher MW. It appears that the signal intensity directly relates to the extent of DEANO modification and it was decided to do subsequent experiments at 2 and 10 mM solutions of DEANO.

The MALDI spectra of the *rr*ER LBD samples with and without pre equilibration treated with 2 mM DEANO (Fig. 4B) shows comparable signal intensities for the Cd pre equilibrated preparation and the control sample, while the

Fig. 4 Effect of DEANO concentrations and ligand pre equilibration on the extent of fusion protein modification. (A) MALDI mass spectrum of fusion protein samples incubated with increasing DEANO concentrations for 10 min at 25 °C, washed and removed from the agarose support by a saturated solution of sinapic acid. (B) and (C) MALDI mass spectra of fusion protein samples pre equilibrated with fivefold molar ligand excess, incubated with 2 and 10 mM DEANO, respectively, and removed from the agarose support by a saturated solution of sinapic acid.

signals of the DEANO treated samples with and without E2 pre equilibration were reduced by half. This suggests that Cd protects the Cys against chemical modification. Signal reduction was even more pronounced for the 10 mM DEANO treatment (Fig. 4C). Treatment of the *rtER* LBD preparation with 2 and 10 mM DEANO resulted in a slight increase in MW (74 and 157 Da, respectively), comparable to that measured for E2 pre equilibration (20 and 179 Da), while the MW shift for the Cd pre equilibration was not significant.

Analysis of the peptide patterns for the 2 mM DEANO treated samples with and without Cd/E2 pre equilibration did not show significant differences from that of the control sample except for the significant decrease in the intensity of the T56 fragment (data not shown). Most importantly, no S nitrosylation of the Cys containing peptides T36, T42, T51 and T56 was observed.

Increasing the DEANO concentration to 10 mM did not affect the signal intensities of the tryptic peptides when pre equilibrated with Cd, while significantly decreasing signal intensities for the other DEANO treated samples (Fig. 5). In particular, T36, T42 and T51 were reduced in the E2 pre equilibrated sample as well as in the DEANO treated sample without ligand, indicating derivatization of the corresponding

Cys residues for these cases, while Cd prevents reaction with the thiol groups. Cd pre equilibration of the protein preparation did not protect the cysteine in T56, as was already seen for the DEPC treatment. It appears that C475 does not interact with Cd, possibly due to restricted accessibility or distance.

Consistent with the results obtained with GSNO no S nitrosylation (+29 Da) was detected. Previous work carried out by Wada *et al.* revealed significantly different ion yields for S nitrosylated peptides depending on the desorption/ionization technique.³⁶ For instance, though S nitrosylated peptides were abundant in the ESI mass spectra, they were not detected with MALDI. Possible reasons are photo induced dissociation of the S NO bond by the UV laser, or reduction of the S nitrosylated species through ion molecule reactions taking place in the desorption plume. This argument is supported by the fact that also no S nitrosylated peptides were observed when using a 2.94 μm IR laser.

Digestion with other proteases. As has been shown earlier, analysis of the tryptic fragments from the *rtER* LBD was limited to T36, T42, T51 and T56, because T49 and T61 were completely suppressed and T55 with Cys 460 only produced a very weak, not easily reproduced signal in the MALDI

Fig. 5 Selected tryptic maps of the DEANO treated samples with and without ligand pre equilibration. Aliquots of fusion protein were pre equilibrated with fivefold molar ligand excess, incubated for 10 min at 25 °C with 10 mM DEANO, the reagent excess removed after centrifugation and the precipitate subjected to proteolytic digestion for 2 h at 37 °C. (A) Control, (B) DEANO treated sample without ligand pre equilibration, (C) and (D) DEANO treated samples pre equilibrated with E2 and Cd, respectively.

spectrum. For this reason, other proteases were evaluated, in order to improve the ion yield for Cys 411, Cys 460, and Cys 543 containing peptides. Pepsin, chymotrypsin and Glu C endoprotease are all likely candidates for producing sizeable fragments containing these cysteines. However, only pepsin was successful in producing the desired peptides. Analysis of the peptide maps after derivatization with both DEPC and DEANO and digestion showed that both Cys 411 and Cys 543 were partially protected by Cd against chemical modification (data not shown). Cys 460, on the other hand, did not produce conclusive results because the fragment VCL only gave a very weak and irregular signal at 334.2 Da.

Asp/Glu residues

Work published by Stoica *et al.* indicated a possible involvement of Asp and Glu in the interaction with cadmium.¹⁷ However, modification of carboxylic groups in proteins has always been challenging.²³ Derivatization using water soluble 1 ethyl 3 (3 dimethylaminopropyl) carbodiimide (WSC), has become by far the most widely used method for studying the role of carboxylic groups in chemical interactions.^{37–39} Unfortunately, free sulfhydryl and phenolic groups can also react with the carbodiimide.^{40–42} An additional complication is that relatively high carbodiimide concentrations are required (0.1 M), leading to competition for Cd binding through the available nitrogen atoms. Trimethyloxonium tetrafluoroborate (TMO) is another reagent widely used since the seventies for the specific esterification of carboxylates in biological macromolecules.³¹

Derivatization using water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (WSC) with glycine ethyl ester (GEE). Relatively low concentrations of carbodiimide (3.6 mM) resulted in a drastic decrease in the protein signal intensity and only a moderate increase of up to 260 Da in MW (data not shown). Based on this observation, 1 and 5 mM concentrations of WSC were used to assess the participation of carboxylic acid residues in the interaction with Cd. It turned out that neither Cd nor E2 protected against a chemical modification with WSC, and that pre equilibration with Cd preserved signal intensity (data not shown). Similar effects and MW shifts were observed with the 5 mM WSC treatment (data not shown). The peptide mass fingerprints did not show significant differences for WSC treated samples with and without Cd/E2 pre equilibration (data not shown). No mass increments of 85 Da, corresponding to derivatized carboxylic acids were observed in the 1 mM WSC treatment.

However, at least 4 peaks were observed representing a mass loss of 34 Da from T36, T42, T51, and T56 (data not shown). All these peaks correspond to peptides known to contain Cys, and T36, T42, T51 have already been implicated in the interaction with Cd. This is further supported by the fact that Cd pre equilibration partially protected the T36, T42, and T51 fragments against loss of 34 Da, which is evident from the changes in the ratio of the modified to original peak intensities. The observed 34 Da weight loss for Cys containing peptides is most probably caused by SH₂ loss from the free Cys residue through beta elimination. The resulting formation of

dehydroalanine has already been reported in the literature, but not yet observed in connection with WSC.^{43–45} Increase of the carbodiimide concentrations to 5 mM did not significantly change the appearance of the PMF mass spectra for *rr*ER samples with and without ligand pre equilibration. Further increase in reagent concentrations to 0.1 M, widely used in the literature, leads to drastic changes in the appearance of the PMF mass spectra similar for all samples. New signals appeared, spaced 85 Da apart, that generally correlated with the number of carboxylic acid residues in the peptide sequence (data not shown). For example, derivatization of the T15 fragment carrying 3 carboxylic residues (1516.77 Da) resulted in the appearance of 3 corresponding signals 85 Da apart. Interestingly, some of the Cys containing peptides that experienced 34 Da loss during treatment with 1 mM WSC, were successfully derivatized under these conditions. For T36 carrying one carboxylic acid this led to the appearance of one signal at 889 Da, while for T42 this led to two signals at 2562 (T42 – 34 + 85) and 2647 (2562 + 85) corresponding to the two glutamic acid residues. The observed lack of protection against modification by WSC could be attributed to the high concentration of the modifying reagent, possibly causing protein unfolding and simultaneous competition for the Cd attached to the protein surface.

Derivatization using trimethyloxonium tetrafluoroborate (TMO). The TMO ion is a highly reactive methylating agent that transforms carboxylic acids to neutral methyl esters under mild aqueous conditions. Minimal interference with neighboring groups, small mass shift (+14 Da) and the absence of atoms competing for Cd binding, makes it an ideal reagent for the study of Cd interactions. Relatively high concentrations of TMO (20–30 mM) however had to be used in order to compensate for its high hydrolysis rate (estimated to be 0.14 s⁻¹).^{46,47} Also, in order to prevent unwanted decrease of the Cd binding caused by dropping pH due to released oxonium ions, the solution was buffered with 200 mM PBS at pH 7.6.

TMO treated proteins with and without ligand pre equilibration showed a mass increase of 80–100 Da suggesting modification of 6 to 7 carboxylic acids (data not shown), but no significant difference between the various treatments and no signal loss were observed, indicating no shielding of Glu and Asp by the ligands. The spectra of the tryptic peptides from the different treatments confirmed this conclusion as there were no visible differences, further indication that Cd has a very weak interaction with carboxylic acid residues if any (data not shown).

The observed lack of shielding of Asp and Glu by Cd is understandably due to reported low cadmium affinities towards carboxylic acid residues and high reagent concentrations used in the WSC and TMO experiments. Competition for Cd binding as expected for WSC however is less important, since the non competing TMO did not indicate any shielding.

Correlation of the current data with previous results

To the best of our knowledge the investigation of Cd binding to the *rr*ER LBD by chemical modification presented here is

the first of its kind. Nevertheless, it is interesting to compare the results of the current study with the work done by Stoica *et al* on binding Cd to the human ER, using molecular biology techniques.¹⁷ Both proteins belong to the same nuclear receptor family, with 62% sequence homology and conserved number of residues in the domains responsible for DNA and ligand binding (Fig. 1). As mentioned earlier, alanine scanning mutagenesis using transfection and binding assays identified Cys 381, Cys 447, Glu 523, His 524 and Asp 538 in the *hER* α as possibly involved in Cd binding.¹⁷ Realignment of the *h* and *rtER* receptors showed conservation of 4 out of the 5 residues (except for Cys 381 in *hER*) implicated as potential cadmium interaction sites in the *hER* (Cys 460, Glu 536, His 537, Asp 551 in *rtER*). Hereafter, the numbering of the *hER* sequence will be given in curly brackets.

Cross examination of the results obtained for the *rtER* by chemical modification and MALDI MS against assignments made by alanine scanning mutagenesis for the *hER* showed no agreement in what amino acids are involved in Cd binding, a fact that could be attributed to different experimental conditions, the differences in protein sequence, or a combination of these factors (Table 2). Among the 4 conserved amino acids in the *rtER* sequence that potentially bind Cd, only binding of Cd to Cys 460^{32,48} can not be ruled out based on the results obtained with the chemical modification experiments, because of sensitivity problems. Much better agreement on the reactivity of the *rtER* Cys residues was obtained with two previously published reports investigating S carboxymethylation of *hER* α cysteines.^{48,49} In both cases {Cys 530} and {Cys 417} were derivatized, {Cys 381} only in one,⁴⁸ while {Cys 447} was not modified in both cases since, as the crystal structure shows, it is sufficiently buried for chemical derivatization to be highly unlikely. The results from these investigations are consistent with the data obtained here, which also clearly showed modification of Cys 543 {Cys 530} and Cys 430 {Cys 417} in the absence of Cd shielding. No conclusion could be reached with regard to the role and reactivity of Cys 460 {Cys 447} due to sensitivity problems, and {Cys 381}, because it is replaced by a serine in the *rtER* sequence.

Table 2 Comparison of the *h* and *rtER* α Cd interaction sites

Estrogen receptor		Cd interactions				
Human	Trout	<i>hER</i> ^a	<i>rtER</i> ^b			
			DEPC	DEANO	WSC	TMO
Leu 265	Cys 279		+	+		
Tyr 328	Cys 341		+	+		
Cys 381	Ser 394	+				
His 398	Cys 411		+	+		
Cys 417	Cys 430		+	+		
Cys 447	Cys 460	+	?	?		
Leu 462	Cys 475					
Glu 523	Glu 536	+				
His 524	His 537	+				
Cys 530	Cys 543		+	+		
Asp 538	Asp 551	+				

^a Results obtained with alanine scanning mutagenesis, co transfection and binding studies.¹⁷ ^b Results obtained using chemical modification and MALDI MS (this work).

No definite conclusion regarding the involvement in Cd binding could be reached for E₁Glu 536, His 537, and Asp 551 in the *rtER* sequence, since no protection against derivatization was observed. This could be interpreted as lack of interaction between these residues and cadmium. Alternatively, it could also be explained by an interaction so weak that it can not be detected by the chemical modification technique. These results are consistent with previous reports on Cd binding to Glu, His and Asp residues in phytochelatin related peptides and synthases, all of which either indicated a weak, or not decisively provable interaction with Cd.^{50–52} Participation of {Glu 523}, {His 524} and {Asp 538} in Cd binding was derived solely from transfection experiments that showed that Asp, His and Glu mutants completely lost the ability to bind cadmium.¹⁷ This is surprising because all mutants contain free Cys groups in their sequences, which have the strongest affinity for cadmium.^{51,52} One possible explanation is a conformational change in the mutant that hides the originally available Cys groups. A further explanation could be that Cd binding experiments with the *hER* mutants was done with transfected cells, while the wild type control samples were based on the commercially available full length recombinant human ER α , and MCF 7 cell extracts, introducing further variability.¹⁷

Additional verification of the results obtained was done by comparing them with the crystallographic structure of the ER ligand binding domain structures.^{53–55} It is known that all ligands bind to the ER across a sizeable ligand binding cavity which is buried deep within the hydrophobic core and located between helices H3 and H12.^{53,54} Consistent with this data, residues of the *hER* involved in Cd binding are positioned between H3 and H12, and located on the protein surface. Providing that the conformations of the LBDs in the chimeric receptors and the ER used in the crystallographic studies are similar, one can see that except for C279 {Leu 265} all cysteine residues within the *rtER* structure implicated in Cd binding are also located within the H3 to H12 range. Extending this similarity of the receptor folds to the spatial distribution of *rtER* Cys residues, one can see that the majority are located on the receptor surface and easily approachable by cadmium and modification reagents, except for Cys 460 {Cys 447}, which is deeply buried within the structure. No conclusion could be reached with regards to the spatial location of Cys 279 because the corresponding residue in the *hER* sequence, {Leu 265}, was outside the regions used to obtain the crystal structures.

At the same time it appears that sheer accessibility of Cys residues is no pre requisite for Cd binding since Cys 475, located on the protein surface could be modified even when pre equilibrated with Cd. This could also indicate that shielding against chemical modification is maximal when Cd is coordinated by a number of nearby residues, and not just Cys alone.

The possible involvement of buried {Cys 447} in Cd binding as proposed by Stoica *et al* and lack of shielding against chemical modification in the case of {Cys 530} that is located on the protein surface supports the same conclusion.

A discussion of different investigations of the ER Cd interaction by chemical and biological approaches should also mention that neither study was using the full sequences of the

respective ERs. Both investigations used ER hormone binding domains fused to parts of either the GST or DNA binding domain of the yeast transcription factor GAL 4.¹⁷ These differences in the fusion protein sequence will introduce a variability in Cd binding, not only when comparing the fusion proteins but, most importantly, also when comparing to the wild type protein, the ultimate goal of both studies. Furthermore, since the fusion proteins contained the ligand binding domain only, no information could be obtained regarding a potential participation in Cd binding of cysteines located in other domains. Another complication in comparing the results obtained by the site directed mutagenesis and chemical modification approach is that replacement with an alanine introduces a methyl group at that position, while chemical modification adds a chemical group to an already existing side chain, which may also be a reason for differences in results.

Limitation of the chemical modification approach to study protein interactions

Study of non covalent protein interactions by chemical modification is an attractive approach applicable to a variety of protein systems that avoids the need for time consuming and quite laborious cloning and over expression of the multiple mutants used in alanine scanning mutagenesis.

However, experimental conditions have to be well worked out in order to control stability and reactivity of the reagents involved. Analysis of the results should take into account the possibility of side reactions and competition for the attached ligand between a protein and reagent. Considerable caution should also be exercised when assessing non covalent interaction based on shielding against chemical modification. While the protection against chemical modification strongly suggests shielding by, e.g., a ligand, the opposite is not always correct. It is quite possible that the strength of the interaction between ligands and certain amino acid residues in the active site is not enough to prevent their derivatization which in this case, however, wrongly suggests the lack of ligand binding. The extent of amino acid modification derived from a shift in molecular weight has to be confirmed by a detailed peptide mass fingerprinting, because of the decreased accuracy at high mass. Chemical modification can result in the appearance of multiple protein conjugates, which can reduce sensitivity up to the point where the protein signal disappears entirely. One should also be aware that suppression effects, signal overlap and interferences from low molecular weight peptides and matrix can reduce sequence coverage obtained with peptide mass fingerprinting. Most of the time, however, these problems can be addressed by using different matrices or other digestion procedures.

Conclusion

The partial and full Cd blockades of the *rt*ER amino acid residues against their chemical modification allowed to identify residues involved in the Cd interactions.

The observed preferential protection of the 279, 341, 411, 430, 543 Cys *rt*ER groups by Cd, suggests their involvement in metal interactions, and is consistent with the strong binding

affinity of the thiol group towards metals, capable of establishing strong ionic bonds with a certain degree of covalent character.³⁵ These results were cross checked against available data from molecular biology, crystallography and residues chemical reactivity data. The location of the Cys residues within the *rt*ER LBD suggested that involvement in Cd interactions was consistent with the data from the known crystallographic structures of *h*ER complexes. Though the assignment of the *rt* and *h*ER LBD amino acid residues implicated in Cd interactions by chemical modification and alanine scanning mutagenesis studies showed considerable differences, even among conserved Cys residues. In addition, participation of the His, Asp and Glu residues, shown to be involved in the Cd interactions by Stoica's molecular biology study, still remains to be clarified. The observed lack of the Cd protection against chemical modification of these residues is possibly an indication of the general limitation of the chemical modification approach for studying weak non covalent interactions. Overall, the observed disagreement with previous molecular biology observations could be rationalized by taking into account the use of differently constructed fusion proteins, their different sequences and, most importantly, the different experimental approaches used. Furthermore, both difficulties with the over expression of the full length receptor and unsuitability of the preparations available commercially, limited investigations of the *rt* and *h*ER binding properties to the LBD alone, introducing some restrictions in the experimental setup which may not allow to draw a direct comparison with the original receptor properties. Thus, it seems beneficial to expand investigation of the ER non covalent interactions with a variety of ligands to the full receptor sequences when they become available.

Acknowledgements

VJN is thankful to Dr Adrian Ammann for fruitful discussions during experimental work and manuscript preparation. Support for this work by both the VELUX Foundation and the Swiss National Science Foundations is very much appreciated. Novartis is greatly acknowledged for their loan of the Bruker Reflex III TOF.

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