Reactivity of Zinc Finger Cysteines: Chemical Modifications Within Labile Zinc Fingers in Estrogen Receptor

Christian Atsriku,[†] Gary K. Scott, Christopher C. Benz,[*] and Michael A. Baldwin[†]

Buck Institute for Age Research, Novato, California, USA

Estrogen receptor (ER, alpha isoform) is a 67 kDa zinc finger transcription factor that plays a fundamental role in both normal reproductive gland development and breast carcinogenesis, and also represents a critical molecular target for breast cancer therapy. We are investigating the structural consequences of chemical exposures thought to modify essential zinc finger cysteine residues in human ER. The current study employs mass spectrometry to probe ER zinc finger structural changes induced by a redox-reactive vitamin K3 analog, menadione; a commonly used cysteine alkylator, iodoacetic acid; and a thiol alkylating fluorophore, monobromobimane. Although they are slower to react, the sterically bulkier reagents, monobromobimane and menadione, effectively alkylate the most susceptible ER zinc finger cysteine sulfhydryl groups. Menadione arylation results first in Michael addition of the hydroquinone followed by rapid oxidation to the corresponding quinone, evidenced by a 2 Da mass loss per cysteine residue. Mass spectrometric analysis performed under MALDI conditions reveals both hydroquinone and quinone forms of arylated menadione, whereas only the quinone product is detectable under ESI conditions. Tandem mass spectrometry of a synthetic peptide encompassing the C-terminal half of the structurally more labile second zinc finger of ER (ZnF2B) demonstrates that the two nucleophilic thiols in ZnF2B (Cys-237, Cys-240) are not chemically equivalent in their reactivity to bromobimane or menadione, consistent with their unequal positioning near basic amino acids that affect thiol pKa, thereby rendering Cys-240 more reactive than Cys-237. These findings demonstrate important differential susceptibility of ER zinc finger cysteine residues to thiol reactions. (J Am Soc Mass Spectrom 2005, 16, 2017–2026) © 2005 American Society for Mass Spectrometry

The 67 kDa human estrogen receptor (ER, alpha isoform) represents a critical transcription factor protein that mediates estrogen induced gene expression essential for normal reproductive gland development, the overexpression of which also drives the development of most human breast cancers and serves as the molecular target for all forms of breast cancer endocrine therapy [1, 2]. Oxidative stress or chemical interference with either of the two Cys_4-type zinc finger structures located within the ER DNA-binding domain (ER-DBD) may prevent its ability to directly bind DNA in a sequence-specific manner without impairing its ability to modulate gene expression by protein–protein interactions with other DNA-bound transcription factors [3, 4]. Given the susceptibility of all zinc finger transcription factors to oxidative stress, recent studies have focused on the reactivity of zinc finger structures to various electrophilic compounds that selectively target nucleophilic residues, such as the zinc coordinating cysteine thiols found in the ER-DBD [4–7].

Evidence from computational and tertiary structural analysis by nuclear magnetic resonance (NMR) indicates that the second (C-terminal) zinc finger of ER (ZnF2) is loosely structured and, therefore, likely more labile [7–9], potentially explaining why the cysteine residues of ER ZnF2 appear more susceptible to oxidative stress and chemical attack than those in the more N-terminal zinc finger of ER [10]. While it has been proposed that chemical modification of ER zinc fingers may inhibit the growth of some ER expressing cancers by preventing ER dimerization and transactivation [5, 6], it has also been shown that exposure to electrophilic quinones such as those generated during estrogen metabolism can induce tumorigenesis via their redox cycling or direct structural attack of nucleic acids and proteins, including ER [11–15]. Of particular interest, protein-quinone conjugates can theoretically become molecular platforms for continued intracellular quinone-hydroquinone cycling, local generation of reactive oxy-

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Address reprint requests to Dr. M. A. Baldwin, Buck Institute for Age Research, 8001 Redwood Blvd., Novato, CA 94945, USA. E-mail: mikeab@itsa.ucsf.edu

[*] Also at the Comprehensive Cancer Center and Division of Oncology-Hematology, University of California, San Francisco, CA 94143-1770.

[†] Also at the Mass Spectrometry Facility, Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446.

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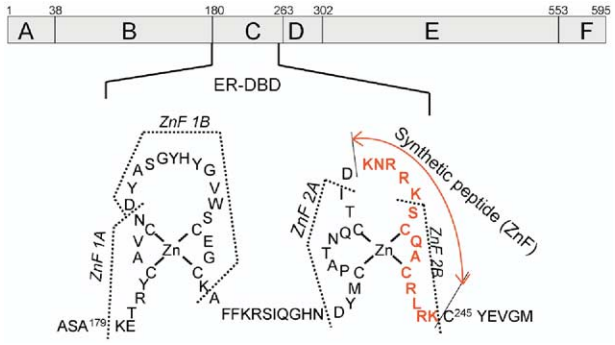


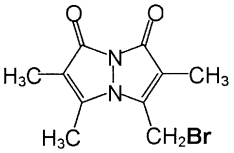
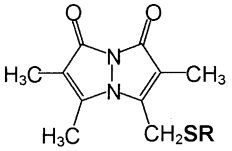
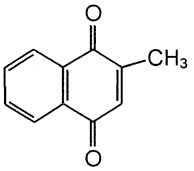
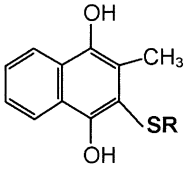
Figure 1. ER-DBD showing the N-terminal and C-terminal zinc fingers. Double protease digestion with endoproteinase Lys-C and Asp-N yields four diagnostic zinc finger peptides: ZnF1A, 1B, 2A, and 2B. The synthetic peptide ZnF corresponds to residues 231–244.

gen species (ROS), and chronic propagation of oxidative damage to surrounding macromolecules.

The vitamin K analog menadione (K3), capable of both redox cycling and arylating nucleophilic substrates by Michael addition, has been extensively studied as a model stress inducing quinone [15]. In addition to activating intracellular oxidant stress pathways, mass spectrometry (MS) analysis has recently revealed that brief exposure to K3 can result in arylation of the singular cysteine residue (Cys110) within histone H3.3/H3 [16]. Earlier studies had shown that brief treatment of ER-expressing breast cancer cells in culture with K3 also results in irreversible functional loss of ER-DNA binding [3], presumably due to the Michael addition of either the quinone or hydroquinone form of K3 to one or more nucleophilic groups within the ER-DBD [17, 18]. However, analytical and structural studies of chemical modifications within the ER-DBD remain challenging, given the much lower abundance of endogenous ER relative to proteins like histone H3.3/H3, even when ER is markedly overexpressed in human breast cancer cells. Biochemical methods such as the zinc ejection assay have been used to indirectly assess the lability of ER zinc fingers to electrophilic attack [5]; but we believe that direct MS assessment will ultimately be required to measure and precisely identify chemical modifications within the DBD of endogenously expressed ER.

As an important step towards this aim, we have sought to develop and optimize a mass spectrometry based technique to fingerprint in vitro covalent changes occurring in the zinc fingers of human ER protein. The current study employed human recombinant ER protein and synthetic ER zinc finger peptides to develop MS and tandem MS (MS/MS) methods capable of probing the ER-DBD for structural changes induced by various thiol-reactive agents. To specifically assess arylation products formed by quinones like K3, we extended our previously described protocol involving double enzymatic digestion of ER (Figure 1), followed by liquid chromatography-electrospray ionization (LC-ESI) MS [19]. As shown in Table 1, in addition to K3, other thiol-reactive agents used to treat ER protein or synthetic peptides included iodoacetic acid (IAA) and monobromobimane (BrB). IAA is typical of several standard alkylating reagents widely used in protein chemistry and MS studies to protect cysteine thiols against oxidation or uncontrolled alkylation, as seen during polyacrylamide gel electrophoresis [20]. The fluorescent agent BrB is less well known in MS studies; similar in size to K3, its steric bulkiness has been used to map drug binding sites in proteins [21]. While BrB fluorescence is a valuable property for monitoring overall thiol oxidation status in proteins, the existence of four cysteine residues outside the ER-DBD (which itself contains nine cysteines, as shown in Figure 1), renders total BrB fluorescence noninformative with respect to ER-DBD thiol status. To probe for position-specific cysteine reactivity within an ER-DBD zinc finger, we

Table 1. Chemical structures of alkylating/arylating reagents used in this study

Reagent	Structure	Peptide conjugate	Nominal mass change per Cys
Iodoacetic acid (IAA)	ICH_2COOH	**RS**-CH_2COOH	58 Da
Bromobimane (BrB)		 *(Fluorescent)*	190 Da
Menadione (k3)			172 Da