Published in final edited form as:

Int J Mass Spectrom. 2012 December 15; 330-332: 302–309. doi:10.1016/j.ijms.2012.10.013.

Hydrogen/Deuterium Exchange Reflects Binding of Human Centrin 2 to Ca²⁺ and Xeroderma Pigmentosum Group C Peptide: An Example of EX1 Kinetics

Justin B. Sperry^{1,2}, Zachary C. Ryan³, Rajiv Kumar³, and Michael L. Gross²

¹Analytical Research and Development, Pfizer Inc., Chesterfield, MO 63017

²Department of Chemistry, Washington University in St. Louis, St. Louis, MO 63130

³Departments of Medicine and Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905

Abstract

Xeroderma pigmentosum (XP) is a genetic disease affecting 1 in 10,000-100,000 and predisposes people to early-age skin cancer, a disease that is increasing. Those with XP have decreased ability to repair UV-induced DNA damage, leading to increased susceptibility of cancerous non-melanomas and melanomas. A vital, heterotrimeric protein complex is linked to the nucleotide excision repair pathway for the damaged DNA. The complex consists of XPC protein, human centrin 2, and RAD23B. One of the members, human centrin 2, is a ubiquitous, acidic, Ca²⁺-binding protein belonging to the calmodulin superfamily. The XPC protein contains a sequence motif specific for binding to human centrin 2. We report here the Ca²⁺-binding properties of human centrin 2 and its interaction with the XPC peptide motif. We utilized a region-specific H/D exchange protocol to localize the interaction of the XPC peptide with the C-terminal domain of centrin, the binding of which is different than that of calmodulin complexes. The binding dynamics of human centrin 2 to the XPC peptide in the absence and presence of Ca²⁺ are revealed by the observation of EX1 H/D exchange regime, indicating that a locally unfolded population exists in solution and undergoes fast H/D exchange.

Keywords

hydrogen/deuterium exchange; EX1 kinetics; protein; protein/peptide interaction; centrin 2; xeroderma pigmentosum

1. Introduction

The incidence of skin cancer is doubling every decade owing to the aging population and increased UV exposure, caused inter alia by ozone depletion and personal habits of excessive tanning (1, 2). Approximately 1 in 10,000-100,000 people suffer from the genetic disease xeroderma pigmentosum (XP). The XP patients have a deficiency in the ability to repair damage by ultraviolet (UV) light, thus making them predisposed to skin cancer. In fact, those with XP have a 4800-fold and 2000-fold higher probability in developing non-

© 2012 Elsevier B.V. All rights reserved.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

melanoma and melanoma skin cancers, respectively (3). The inability of XP patients to repair UV damage has been linked to the nucleotide excision repair (NER) pathway (4). This pathway repairs DNA damage, including UV-induced photoproducts that disrupt normal transcription and replication processes (5). The repair by the NER pathway occurs by two sub-pathways: (1) induction by RNA polymerase to remove DNA adducts by transcription and (2) global genome (GG) repair initiated by a protein complex including xeroderma pigmentosum group C protein (XPC), human centrin 2 (HC2), and RAD23B (6-8).

Human centrin 2 is a member of the calmodulin (CaM) superfamily of calcium-binding proteins and highly conserved in eukaryotes (9-11). The centrin family plays numerous roles in algae, yeast, and humans and has been extensively studied (12-19). Human centrin 2 is 50% sequence identical and nearly 85% sequence similar to calmodulin; nevertheless, their calcium-binding properties are different. Calmodulin undergoes a significant conformational change upon binding calcium (Ca^{2+}) (20) whereas human centrin 2 apparently does not (21, 22). In fact, full-length human centrin 2 can self-assemble in a Ca^{2+} -saturated solution, but the protein with an N-terminal truncation of the first 25 amino acid residues ($\Delta 25\text{-HC2}$) does not assemble (23).

The recognition activity of XPC to damaged DNA increases in the presence of human centrin 2; thus, the characterization of the complex is of importance in biomedicine and biophysics (24). Human centrin 2 binds on the C-terminus of XPC between amino acids 847 and 863 (8), which has the sequence NWKLLAKGLLIRERLKR. The affinity of centrin to the XPC-peptide, as determined by isothermal titration calorimetry (8), is high ($K_a = 2.1 \times 10^8 \text{ M}^{-1}$) in the presence of saturating Ca^{2+} , and decreases in the absence of Ca^{2+} ($K_a = 7.8 \times 10^6 \text{ M}^{-1}$). More details are available in recent reviews on the subject of centrin 2 and XPC interactions (25, 26).

We applied a region-specific H/D exchange (H/DX) protocol to probe the binding of Ca^{2+} and XPC-peptide to human centrin 2. The protocol should reveal deuterium uptake changes in human centrin 2 upon binding Ca^{2+} . We also tested the interaction of human centrin 2 to the XPC peptide in the presence and absence of Ca^{2+} . The affinity of human centrin 2 to Ca^{2+} in the presence of XPC has not been previously reported.

This report also continues our interest in the biophysics of Ca^{2+} -binding proteins and the development and application of mass spectral methods for investigating them (27-31). We are pleased to contribute this article in honor of Peter Armentrout who has contributed significantly and productively to physical chemistry and mass spectrometry.

2. Experimental

2.1. Materials

The $\Delta 25$ -human centrin 2 ($\Delta 25\text{-HC2}$) protein (N-terminal truncation of the first 25 amino acids) was expressed in *E. coli* at the Mayo Clinic (Rochester, MN). A sequence comparison of full length versus the $\Delta 25\text{-HC2}$ protein is provided in **Figure 1**. The residue numbering in this report refers to the full length protein sequence. Deuterium oxide, potassium chloride, formic acid, calcium chloride, acetonitrile, EGTA (ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetra-acetic acid), HEPES [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)], and HEPES sodium salt were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity available. The XPC peptide (Sequence: NWKLLAKGLLIRERLKR, 9 mg, 92.6% pure) was purchased from GenScript (Piscataway, NJ). Immobilized pepsin on agarose was purchased from Pierce (Rockford, IL). The molecular masses of $\Delta 25\text{-HC2}$ and the XPC peptide, as determined by electrospray ionization (ESI) mass spectrometry, were

17085.1 Da (theoretical average mass of 17085.4 Da) and 2106.3 Da (theoretical monoisotopic mass of 2106.3 Da), respectively.

2.2. H/D Exchange Kinetics

A stock solution of centrin 2 (119 ! M) was prepared from by diluting a 7.31 mM solution of centrin 2 (previously prepared at Mayo Clinic) with 10 mM HEPES (pH = 7.4) and 150 mM KCl. A stock solution of a 3:1 protein:peptide complex was made by mixing 3.25 ! L of the master human centrin 2 solution (7.31 mM) with 6.75 ! L XPC peptide (10.7 mM) and diluted to a final centrin 2 concentration of 119 uM using 10 mM HEPES and 150 mM KCl. H/D exchange kinetics experiments were conducted with four samples: Ca²⁺-free human centrin 2 (1 mM EGTA), Ca²⁺-bound human centrin 2 (2 mM Ca²⁺), Ca²⁺-free XPC:human centrin 2 (3:1 ratio, 1 mM EGTA), and Ca²⁺-bound XPC:human centrin 2 (3:1 ratio, 2 mM Ca²⁺). To initiate exchange, 0.5 ! L of the protein stock solutions were diluted with 20 ! L of D₂O containing 10 mM HEPES and 150 mM KCl at 25 °C to give a solution that was > 97% D₂O. After the specified times, the exchange was quenched with ice cold 1.0 M HCl to give a final pH of 2.0.

To examine deuterium uptake for regions of the protein, 5 ! L of immobilized pepsin on agarose was added to the quenched solution. The digestion was allowed to take place for 3 min at 0 °C, accompanied by a quick vortexing pulse every 15 sec. After digestion, the mixture containing the beads was briefly centrifuged (2 – 3 seconds) so that they congregated at the bottom of the sample tube. The supernatant protein digest, including some undigested protein, was loaded on a C₁₈ column (LC Packings, 1×15 mm, PepMap cartridge, Dionex Corp., Sunnyvale, CA) that was pre-equilibrated with 100 ! L of 0.2% formic acid in water (0 °C). The column was washed with 300 ! L of 0.2% formic acid in water (0 °C), back exchanging the labile sites of the peptides and the undigested protein. The peptides were separated with a LC gradient (5% B to 40% B in 6 min, 40% B to 75% B in 2 min, 75% B to 5% B in 0.5 min, 5% B to 40% B in 0.5 min, then back to 5% B for equilibration) at a flow rate of 40 ! L/min (Solvent A: 95% water, 5% acetonitrile containing 0.3% formic acid; Solvent B: 5% water, 95% acetonitrile containing 0.3% formic acid). To minimize back exchange, the incoming/outgoing LC solvent line, injection valve, and sample loop were submersed in ice (0 °C).

2.3. LC-ESI/MS Analysis with a Q-TOF Mass Spectrometer

All ESI mass spectra monitoring the H/DX were acquired in the positive-ion mode with a Waters (Micromass) Q-TOF Ultima (Manchester, U.K.) equipped with a Z-spray ESI source. The capillary voltage was 3.2 kV, cone voltage readback of 100 V, and the source and desolvation temperatures were 80 and 180 °C. The cone and desolvation gas flows were 40 and 400 L/h. The MS profile used for quadrupole transmission was from *m/z* 500, dwell for 5% of the scan time, ramped to *m/z* 1000 for 45% of the scan time, and then dwelled at *m/z* 1000 for 50% of the scan time. The total scan time was 2 sec for ions of *m/z* 300-2000.

2.4. LC-ESI/MS-MS Analysis of Protein Digest

A standard proteomic protocol was used to identify accurately the peptides produced during the pepsin digest. The results of this experiment verified the peptide masses observed in the H/DX protocol above. After 3 min of pepsin digestion, following the protocol above, the solution containing the protein digest, including some undigested protein, was loaded onto a C₁₈ custom-packed column (75 µm i.d., 10 cm length). The peptides were separated over 70 min using an Eksigent NanoLC-1D (Dublin, CA) with an LC gradient from 3-97% acetonitrile containing 0.1% formic acid at a flow rate of 260 nL/min. The solution was sprayed directly from the column into the mass spectrometer using a PicoView PV-500 nanospray source (New Objective, Woburn, MA) attached to the LTQ-FTMS (Thermo, San

Jose, CA), which afforded accurate mass measurements and product-ion sequencing by MS/MS. A full mass spectrum was recorded in the FT part of the instrument, operating at 100,000 mass resolving power at m/z 400, while data-dependent product-ion spectra of precursors selected by the FTMS were collected by the ion trap. The MS/MS experiments carried out in the LTQ utilized wide-band activation and dynamic exclusion for a duration of 30 sec and a repeat number of two. The peptides were identified based on their accurate m/z values and their product-ion spectra and by searching against a protein database using Mascot (Matrix Science, Oxford, UK). The spectra of peptides utilized in this research were manually verified by *de novo* sequencing.

2.5. Data Analysis

For H/D exchange data collected on the Q-TOF, the protein mass spectrum at each H/D exchange time point was deconvoluted using the MaxEnt1 algorithm (MassLynx 4.0). The deuterium level at each time point was determined by subtracting the average mass of the undeuterated protein from the average mass of the deuterated protein. The rate of back exchange was measured to be 1 deuterium loss per min. Ion signals for the deuterated peptides were smoothed twice in MassLynx with a Savitsky-Golay algorithm and imported into Microsoft Excel as an x,y pair (mass, intensity). The centroid and width of the deuterium distribution for each peptide was analyzed using HX-Express software (32). The back exchange occurs at the same rate as in the experiment in which exchange at the global level was examined; however, no corrections were made for this deuterium loss because only relative (not absolute) deuterium levels were compared in all experiments. All experiments were conducted in triplicate.

2.6. Kinetic Modeling

The H/D exchange data were fit with a fixed rate-constant binning model in which all exchangeable hydrogens were separated into four fixed rate-constant bins, as described previously (28). The bins were chosen to span four orders of magnitude and represent rate constants that cover the experimental time scale of the experiment. The four rate-constant bins were 10, 1, 0.1, and 0.01 min⁻¹. The forward deuterium exchange reaction follows pseudo first-order kinetics because the concentration of D₂O (>95%) was large compared to the concentration of H₂O and the protein. This model was applied globally to the protein as well as to individual component peptides seen in a protein digest. The number of exchangeable hydrogens was optimized by using the “Minimize” function in MathCAD to minimize the root mean square (RMS) of the residuals. Each trial (typically a total of three) was fit separately, and the results were averaged and reported with $\pm$ one standard deviation. The detailed outcome of the binning are not reported because the only point of the kinetic analysis was to demonstrate that the experimental points can be fit with four rate constants for the H/DX.

3. Results and Discussion

We begin the study of Δ 25-human centrin 2 and XPC by determining the time-dependent deuterium uptake on four samples (described in 2.2). The kinetics experiments afford information regarding the number of amide sites affected by Ca²⁺ and/or XPC binding. To locate the sites that change their H/DX kinetics (take up lower amounts of deuterium), we utilized pepsin digestion under quench conditions followed by fast HPLC/MS analysis of the peptides produced in the digestion.

3.1. H/D Exchanges Kinetics of Δ 25-HC2

As found for another calcium-binding protein, calmodulin (28), we observed that the pepsin digestion was incomplete (30–50% protein remained undigested) probably a result of the

low temperature ($\sim 0^{\circ}\text{C}$). We took advantage of the incomplete digestion to obtain in the same experiment the D uptake data for the whole protein and for its constituent peptides. We conducted forward H/D exchange experiments on four samples over a 60 min time course: (1) $\Delta 25\text{-HC2}$ with 1 mM EGTA, (2) $\Delta 25\text{-HC2}$ with 2 mM Ca^{2+} , (3) 3:1 XPC: $\Delta 25\text{-HC2}$ with 1 mM EGTA, and (4) 3:1 XPC: $\Delta 25\text{-HC2}$ with 2 mM Ca^{2+} . The centrin 2 concentration during H/D exchange was 3.0 μM for all experiments (see Experimental section). The addition of EGTA to give a 1 mM solution ensures that the apo state can be formed completely. To some samples, we added Ca^{2+} to make its concentration 2 mM to insure that the protein is nearly completely Ca^{2+} -bound. Based on the literature affinity constants for centrin 2 to the XPC peptide, the percents bound during H/D exchange in the absence and presence of Ca^{2+} are 98.6 and 100.0, respectively.

The N-terminal truncation of human centrin 2 has 149 amino acids and contains two prolines, which do not have exchangeable hydrogens. Therefore, the total number of exchangeable H's is 146 because the N-terminus is considered a fast-exchanging site. Given that the percent D_2O in all H/DX experiments with $\Delta 25\text{-HC2}$ was 97%, the maximum number of observable exchange events is 142. A plot of deuterium uptake versus H/D exchange time affords the extent of deuteration for $\Delta 25\text{-HC2}$ in the four samples (**Figure 2**). At all H/D exchange times, the deuterium uptakes of Ca^{2+} -free and Ca^{2+} -bound $\Delta 25\text{-HC2}$ are equivalent, except for a small, yet statistically significant difference at 15 sec. The extent of deuteration at all H/DX times has a range of 119-127, indicating that $\Delta 25\text{-HC2}$ becomes 84-89% deuterated. The lack of any differences in deuterium uptake indicates that $\Delta 25\text{-HC2}$ undergoes minimal conformational change upon binding Ca^{2+} , a property observed by nuclear magnetic resonance (14) and circular dichroism (21, 33).

The XPC: $\Delta 25\text{-HC2}$ complex (3:1) exhibits a different deuterium uptake pattern in the absence and presence of Ca^{2+} . In the absence of Ca^{2+} and at short times of exchange (15 and 45 sec), $\Delta 25\text{-HC2}$ undergoes protection at ~ 40 amide sites upon binding the XPC peptide. At 10 and 60 min of exchange, the protection disappears, and the extent of deuterium uptake is similar to that of $\Delta 25\text{-HC2}$ itself. At 3 min of H/D exchange, we observe two populations of protein, suggesting EX1 exchange kinetics (see **Figure 3**). This type of exchange is rarely observed under physiological conditions and is typically induced with chemical denaturants or pH/temperature changes (34-38). At 3 min of H/DX, one population remains folded in the presence of XPC, whereas another population unfolds and undergoes nearly complete exchange with deuterium similar to that at 10 and 60 min for the protein itself.

In the presence of saturating Ca^{2+} levels, the protein:peptide complex exhibits a gradual increase in deuterium uptake. The $\Delta 25\text{-human centrin 2}$ is protected at short exchange times in the presence of XPC, but the deuterium uptake begins approaching that of the free protein at longer times (e.g., 60 min) of exchange. Any local unfolding observed in the absence of Ca^{2+} is, therefore, stabilized when Ca^{2+} is present. The high extents of deuteration of human centrin 2 in the absence and presence of Ca^{2+} do not preclude it from having secondary and tertiary structure; rather they point to high dynamics in the N terminal region. Prior circular dichroism and thermal denaturation revealed secondary structure and enhanced thermal stability (decrease in backbone and side chain dynamics) in the presence and absence of XPC(8).

The results of the H/DX studies indicate that $\Delta 25\text{-HC2}$ undergoes minimal conformational change upon binding Ca^{2+} , as was seen in other studies. In the presence of XPC, the Ca^{2+} -free protein exhibits a bimodal population at 3 min of exchange, one population that unfolds and exchanges rapidly while the other folded population exchanges more slowly. Because both states exist at 3 min of exchange and there is excess peptide present in solution, we approximate the half-life for bound XPC to be approximately 3 min. To explore the region

of $\Delta 25$ -HC2 responsible for this interesting binding behavior, we digested the protein following H/DX to determine the sites of protection upon Ca^{2+} binding in the complex.

3.2. The N-terminus of $\Delta 25$ -HC2 Shows No Change in Deuterium Uptake

Digestion of the deuterated protein in four different samples affords region-specific information regarding extents of deuteration. There are six peptides that cover the first 96 amino acids of the protein and represent 95% sequence coverage of that domain (peptides containing residues 53-57 were not observed). Each peptic peptide shows that the N-terminus is not affected by the presence of Ca^{2+} or XPC (**Figure 4**). Peptide 40-52, part of EF-hand 1, does show a small difference at early exchange times (15 and 45 sec), possibly due to a weak affinity for Ca^{2+} . In fact, Craescu and coworkers (39) determined the Ca^{2+} affinity for an isolated N-terminal domain fragment to be 10^2 – 10^3 M^{-1} and remarked that the biological role of the N-terminus is not calcium-dependent. The extents of H/DX for the constituent peptides at the N-terminal side are consistent with that of the protein, between 80-90% at all H/DX times. No regions of the N-terminus yield any information regarding the EX1 kinetics observed in the protein H/D exchange studies. B factor data, which are an indirect measure of thermal dynamics, from the X-ray structure (2GGM) also corroborate that regions representing residues from the N-terminus, 45-55 and 60-100, in the presence of Ca^{2+} and XPC, are more dynamic than the C-terminal region of human centrin 2.

The central linker, represented by peptide 77-119, connects the N-terminal domain to the C-terminal domain. This peptide also shows little change in deuterium uptake for all four samples. Other Ca^{2+} -binding proteins, such as calmodulin, produce a hydrophobic channel by wrapping their N- and C-terminal domains around the binding peptide (40). Sequence similarity suggests that centrins should do the same. The H/DX results show that these regions are highly flexible and show high extents of H/DX at 1 min and longer of exchange. The results also show that XPC binding does not occur at the N-terminal domain.

3.3. The Binding of XPC-peptide Occurs at the C-terminal Domain

Peptides from regions of the C-terminal domain of $\Delta 25$ -HC2 in the presence of XPC show differences in deuterium uptake with and without Ca^{2+} (**Figure 5**). A peptide representing residues 110-136 displays bimodal kinetics in the Ca^{2+} -free protein-peptide complex, as indicated by two discrete isotopic distributions in the mass spectra (**Figure 6**). At 3 min of exchange, the existence of two populations is pronounced, with one centered at m/z 788.3 (4+ ion) and the other at m/z 791.2 (4+ ion). The ions representing the low mass distribution take up deuterium in a manner that follows the trend set by those at 15 and 45 sec of exchange, whereas the deuterium distribution at higher mass is similar to that seen at 10 and 60 min of exchange. The region containing this peptide includes EF-hand 3 and must be involved in the binding of XPC.

Two other peptides show differences in deuterium uptake upon complex formation with and without Ca^{2+} present. Peptide 146-162 contains EF-hand 4, which was determined to be the highest affinity site for Ca^{2+} (14, 21). There is no apparent bimodal H/DX distribution; however, the peptide is affected by XPC binding at early exchange times. The C-terminal peptide containing residues 163-172 exhibits a decrease in extent of deuteration upon $\Delta 25$ -HC2:XPC complex formation and also does not reveal any bimodal D distributions. Interestingly, the extent of deuterium uptake for these peptides converges at 10 min to that of the protein free of XPC. This exchange behavior follows that of the full-length protein.

The peptides in the C-terminal domain are not affected by Ca^{2+} binding in the absence of XPC, the results of which are consistent with those of the N-terminal domain and the protein as a whole. The behavior of EF-hand 3 mimics that of the whole protein in the presence of

XPC and the absence of Ca^{2+} . The interaction of XPC is near EF-hand 3 and also affects the deuterium uptake in EF-hand 4.

We attribute the bimodal distribution of exchange to EX1 kinetics. The kinetics that are seen at the protein level are localized in part to the region represented by peptide 110-136. The difference in the two distributions for peptide 110-135 at 3 min (~ 12 D) only accounts for part of the difference in the distributions (26 amides) seen for the whole protein (**Figure 3**). We suggest the difference is in region corresponding to peptide 137-145, which is also likely to exhibit EX1 behavior, but unfortunately we could not detect any peptides representing this region. Both regions include EF hands 3 and 4. The EX1 behavior in the presence of Ca^{2+} is shifted to 10 min (**Figure 6**, Panel 4).

We suggest that the cause for the EX1 behavior is that the XPC/centrin complex undergoes slow local unfolding in the region 110-145 to give a state that undergoes fast H/DX. This local unfolding occurs at or near the binding site for XPC (green region in **Figure 7**) and is faster than the XPC off rate. Addition of Ca^{2+} to centrin 2 stabilizes the protein and shifts the EX1 kinetic behavior to 10 min (**Figure 6**, Panel 4).

An alternative explanation, suggested by one of the reviewers, takes as a starting point the observation that there is slow exchange by centrin 2 in complex with XPC peptide and fast exchange when it is free (panel 1 of Fig 6). Upon XPC dissociation, the free centrin undergoes full deuteration. Thus, the bimodal behavior at 3 min would not be indicative of EX1 exchange kinetics but rather depend on the off rate of the peptide ligand. If this explanation were correct, then other regions of centrin 2 that are involved in XPC binding (e.g., 146-162 and particularly 163-172) should show similar behavior, but they do not. Thus, the region containing EF hand 3 and represented by peptide 110-136 (and possibly the region 137-145) undergoes local unfolding to permit fast H/DX, whereas the adjoining region (represented by C-terminal peptides 146-162 and 163-172) does not.

The X-ray crystal structures of $\Delta 25\text{-HC2}$ in the presence of the XPC peptide and Ca^{2+} (41, 42) are consistent with the region-specific H/D exchange data. They show that the XPC peptide is bound to $\Delta 25\text{-HC2}$ near EF hand 3 (**Figure 7**). The structures also reveal the stoichiometry of Ca^{2+} binding to be 2:1, with both Ca^{2+} ions binding in the C-terminal domain. The interface of $\Delta 25\text{-HC2}$ and XPC is described as hydrophobic; XPC has a 1,4,8 binding motif with interactions of tryptophan, leucine, and leucine occurring in those positions.

4. Conclusion

The $\Delta 25$ -construct of human centrin 2 does not change conformation when saturating amounts of Ca^{2+} are added in solution, as revealed by no detectable difference in H/DX of the protein or constituent peptides. Binding to the XPC binding fragment, in the form of a peptide model, induces an interesting binding dynamic in $\Delta 25\text{-HC2}$ when no Ca^{2+} is present. The XPC ligand binds and causes the protein to undergo local unfolding, as seen at 3 min of H/DX. The unfolded state undergoes rapid H/DX consistent with EX1 kinetics. The region-specific data reveal that it is EF-hand 3 that is responsible for binding the XPC fragment and exhibits similar behavior in the presence or absence of Ca^{2+} , in agreement with published X-ray structures. In the presence of saturating Ca^{2+} levels, centrin undergoes less H/DX at short times, but its protection largely disappears after longer times and many cycles of on-and-off interactions of the protein with XPC peptide.

Acknowledgments

This research was supported by the NIH (Grant No. 8 P41 GM103422-35). We thank one of the reviewers for an alternative explanation for the H/DX kinetics.

REFERENCES

1. Gruijl, F. R. d.; Longstreth, J.; Norval, M.; Cullen, AP.; Slaper, H.; Kripke, ML.; Takizawa, Y.; Leun, J. C. v. d. Health effects from stratospheric ozone depletion and interactions with climate change. *Photochemical & Photobiological Sciences*. 2003; 2:16–28. [PubMed: 12659536]
2. Daya-Grosjean L, Sarasin A. The role of UV induced lesions in skin carcinogenesis: an overview of oncogene and tumor suppressor gene modifications in xeroderma pigmentosum skin tumors. *Mutation Research*. 2005; 571:43–56. [PubMed: 15748637]
3. Kraemer KH, Lee MM, Scotto J. Xeroderma pigmentosum. Cutaneous, ocular, and neurologic abnormalities in 830 published cases. *Archives of Dermatology*. 1987; 123:241–250. [PubMed: 3545087]
4. Friedberg, EC.; Walker, GC.; Siede, W.; Wood, RD.; Schultz, RA.; Ellenberger, T. *DNA Repair and Mutagenesis*. ASM Press; 2005.
5. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. *Nature*. 2001; 411:366–374. [PubMed: 11357144]
6. Laat, W. L. d.; Jaspers, NGJ.; Hoeijmakers, JHJ. Molecular mechanism of nucleotide excision repair. *Genes and Development*. 1999; 13:768–785. [PubMed: 10197977]
7. Araki M, Masutani C, Takemura M, Uchida A, Sugawara K, Kondoh J, Ohkuma Y, Hanaoka F. Centrosome protein centrin 2 / caltractin 1 is part of the xeroderma pigmentosum group C complex that initiates global genome nucleotide excision repair. *Journal of Biological Chemistry*. 2001; 276:18665–18672. [PubMed: 11279143]
8. Popescu A, Miron S, Blouquit Y, Duchambon P, Christova P, Craescu CT. Xeroderma pigmentosum group C protein possesses a high affinity binding site to human centrin 2 and calmodulin. *Journal of Biological Chemistry*. 2003; 278:40252–40261. [PubMed: 12890685]
9. Salisbury JL, Baron A, Surek B, Melkonian M. Striated flagellar roots: isolation and partial characterization of a calcium-modulated contractile organelle. *Journal of Cell Biology*. 1984; 99:962–970. [PubMed: 6381510]
10. Salisbury JL. Centrin, centrosomes, and mitotic spindle poles. *Current Opinion in Cell Biology*. 1995; 7:39–45. [PubMed: 7755988]
11. Schiebel E, Bornens M. In search of a function for centrins. *Trends in Cell Biology*. 1995; 5:197–201. [PubMed: 14731449]
12. Hu H, Chazin WJ. Unique features in the C-terminal domain provide caltractin with target specificity. *Journal of Molecular Biology*. 2003; 330:473–484. [PubMed: 12842464]
13. Salisbury JL, Suino KM, Busby R, Springett M. Centrin-2 is required for centriole duplication in mammalian cells. *Current Biology*. 2002; 12:1287–1292. [PubMed: 12176356]
14. Matei E, Miron S, Blouquit Y, Duchambon P, Durussel I, Cox JA, Craescu CT. C-terminal half of human centrin 2 behaves like a regulatory EF-hand domain. *Biochemistry*. 2003; 42:1439–1450. [PubMed: 12578356]
15. Kilmartin JV. Sfi1p has conserved centrin-binding sites and an essential function in budding yeast spindle pole body duplication. *Journal of Cell Biology*. 2003; 162:1211–1221. [PubMed: 14504268]
16. Hu H, Sheehan JH, Chazin WJ. The mode of action of centrin. *Journal of Biological Chemistry*. 2004; 279:50895–50903. [PubMed: 15452116]
17. Salisbury JL. Centrosomes: Sfi1p and centrin unravel a structural riddle. *Current Biology*. 2004; 14:R27–R29. [PubMed: 14711432]
18. Sheehan JH, Bunick CG, Hu H, Fagan PA, Meyn SM, Chazin WJ. Structure of the N-terminal calcium sensor domain of centrin reveals the biochemical basis for domain-specific function. *Journal of Biological Chemistry*. 2006; 281:2876–2881. [PubMed: 16317001]

19. Li S, Sandercock AM, Conduit P, Robinson CV, Williams RL, Kilmartin JV. Structural role of Sfi1p-centrin filaments in budding yeast spindle pole body duplication. *Journal of Cell Biology*. 2006; 173:867–877. [PubMed: 16785321]
20. Klee CB, Crouch TH, Richman PG. Calmodulin. *Annual Review of Biochemistry*. 1980; 49:489–515.
21. Durussel I, Blouquit Y, Middendorp S, Craescu CT, Cox JA. Cation and peptide-binding properties of human centrin 2. *FEBS Letters*. 2000; 472:208–212. [PubMed: 10788612]
22. Craig TA, Benson LM, Bergen HR, Venyaminov SY, Salisbury JL, Ryan ZC, Thompson JR, Sperry J, Gross ML, Kumar R. Metal-binding properties of human centrin-2 determined by micro-electrospray ionization mass spectrometry and UV spectroscopy. *Journal of the American Society for Mass Spectrometry*. 2006; 17:1158–1171. [PubMed: 16750384]
23. Tourbez M, Firanescu C, Yang A, Unipan L, Duchambon P, Blouquit Y, Craescu CT. Calcium-dependent self-assembly of human centrin 2. *Journal of Biological Chemistry*. 2004; 279:47672–47680. [PubMed: 15356003]
24. Nishi R, Okuda Y, Watanabe E, Mori T, Iwai S, Masutani C, Sugawara K, Hanaoka F. Centrin 2 stimulates nucleotide excision repair by interacting with xeroderma pigmentosum group c protein. *Molecular and Cellular Biology*. 2005; 25:5664–5674. [PubMed: 15964821]
25. Clement FC, Camenisch U, Fei J, Kaczmarek N, Mathieu N, Naegeli H. Dynamic two-stage mechanism of versatile DNA damage recognition by xeroderma pigmentosum group C protein. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*. 2010; 685:21–28. [PubMed: 19686765]
26. Sugawara, K. XPC: Its product and biological roles. 2008. p. 47-56.
27. Zhang H, Gau BC, Jones LM, Vidavsky I, Gross ML. Fast photochemical oxidation of proteins for comparing structures of protein-ligand complexes: The calmodulin-peptide model system. *Analytical Chemistry*. 2011; 83:311–318. [PubMed: 21142124]
28. Sperry JB, Huang RY, Zhu MM, Rempel DL, Gross ML. Hydrophobic Peptides Affect Binding of Calmodulin and Ca^{2+} as Explored by H/D Amide Exchange and Mass Spectrometry. *Int J Mass Spectrom*. 2011; 302:85–92. [PubMed: 21765646]
29. Zhu MM, Rempel DL, Zhao J, Giblin DE, Gross ML. Probing Ca^{2+} -induced conformational changes in porcine calmodulin by H/D exchange and ESI-MS: Effect of cations and ionic strength. *Biochemistry*. 2003; 42:15388–15397. [PubMed: 14690449]
30. Nemirovskiy O, Giblin DE, Gross ML. Electrospray Ionization Mass Spectrometry and Hydrogen/Deuterium Exchange for Probing the Interaction of Calmodulin with Calcium. *J. Am. Soc. Mass Spectrom*. 1999; 10:711–718. [PubMed: 10439509]
31. Huang RYC, Rempel DL, Gross ML. HD exchange and PLIMSTEX determine the affinities and order of binding of Ca^{2+} with troponin C. *Biochemistry*. 2011; 50:5426–5435. [PubMed: 21574565]
32. Weis DD, Engen JR, Kass IJ. Semi-automated data processing of hydrogen exchange mass spectra using *HX-Express*. *Journal of the American Society for Mass Spectrometry*. 2006; 17:1700–1703. [PubMed: 16931036]
33. Wiech H, Geier BM, Paschke T, Spang A, Grein K, Steinkotter J, Melkonian M, Schiebel E. Characterization of green alga, yeast, and human centrins. *Journal of Biological Chemistry*. 1996; 271:22453–22461. [PubMed: 8798410]
34. Sivaraman T, Robertson AD. Kinetics of conformational fluctuations by EX1 hydrogen exchange in native proteins. *Methods in Molecular Biology*. 2001; 168:193–214. [PubMed: 11357626]
35. Ferraro DM, Lazo ND, Robertson AD. EX1 hydrogen exchange and protein folding. *Biochemistry*. 2004; 43:587–594. [PubMed: 14730962]
36. Roder H, Wagner G, Wuthrich K. Amide proton exchange in proteins by EX1 kinetics: studies of the basic pancreatic trypsin inhibitor at variable pH2 and temperature. *Biochemistry*. 1985; 24:7396–7407. [PubMed: 2417625]
37. Arrington CB, Robertson AD. Microsecond to minute dynamics revealed by EX1-type hydrogen exchange at nearly every backbone hydrogen bond in a native protein. *Journal of Molecular Biology*. 2000; 296:1307–1317. [PubMed: 10698635]

38. Weis DD, Wales TE, Engen JR, Hotchko M, Eyck LFT. Identification and characterization of EX1 kinetics in H/D exchange mass spectrometry by peak width analysis. *Journal of the American Society for Mass Spectrometry*. 2006; 17:1498–1509. [PubMed: 16875839]
39. Yang A, Miron S, Duchambon P, Assairi L, Blouquit Y, Craescu CT. The N-terminal domain of human centrin 2 has a closed structure, binding calcium with a very low affinity, and plays a role in the protein self-assembly. *Biochemistry*. 2006; 45:880–889. [PubMed: 16411764]
40. Crivici A, Ikura M. Molecular and structural basis of target recognition by calmodulin. *Annual Reviews of Biophysics and Biomolecular Structure*. 1995; 24:85–116.
41. Thompson JR, Ryan ZC, Salisbury JL, Kumar R. The structure of the human centrin 2-Xeroderma pigmentosum group C protein complex. *Journal of Biological Chemistry*. 2006; 281:18746–18752. [PubMed: 16627479]
42. Charbonnier J-B, Renaud E, Miron S, Du MHL, Blouquit Y, Duchambon P, Christova P, Shosheva A, Rose T, Angulo JF, Craescu CT. Structural, thermodynamic, and cellular characterization of human centrin 2 interaction with xeroderma pigmentosum group C protein. *Journal of Molecular Biology*. 2007; 373:1032–1046. [PubMed: 17897675]

Highlights

Kinetics of H/DX of centrin 2 was measured by proteolysis and mass spectrometry.

Kinetics shows the site of interaction of the protein with the XPC peptide.

The kinetics constitutes an unusual example of EX1 kinetics of H/DX.

Local unfolding of centrin/XPC gives a species that undergoes fast H/DX.

⁻⁵GPLGS¹MASNFKKANMASSSQRKRMSPKPEL²⁶TEEQKQEIREAFDLFDADGT 45
 GP²⁶TEEQKQEIREAFDLFDADGT

 GTIDVKELKVAMRALGFEPKKEEIKKMISEIDKEGTGKMNF GDFLTVM TQ 95
 GTIDVKELKVAMRALGFEPKKEEIKKMISEIDKEGTGKMNF GDFLTVM TQ

 KMSEKDTKEEILKAFKLFDDDETGKISFKNLKRVAKELGENLTDEELQEM 145
 KMSEKDTKEEILKAFKLFDDDETGKISFKNLKRVAKELGENLTDEELQEM

 IDEADRDGDGEVSEQEFLRIMKKTSLY 172
 IDEADRDGDGEVSEQEFLRIMKKTSLY

Figure 1.

The full length human centrin 2 sequence (top) compared to the delta-25 sequence (bottom). The amino acids in red were part of the start sequence in the expression vector. The amino acids in blue are those 25 which were deleted to make delta-25 human centrin 2. The numbering scheme refers to the full length human centrin 2 sequence.

Figure 2.

Global H/D exchange kinetics experiments: $\Delta 25$ -HC2 with no Ca^{2+} (1 mM EGTA) (squares), $\Delta 25$ -HC2 with 2 mM Ca^{2+} (diamonds), 3:1 XPC: $\Delta 25$ -HC2 with no Ca^{2+} (1 mM EGTA) (triangles), and 3:1 XPC: $\Delta 25$ -HC2 with 2 mM Ca^{2+} (circles). H/D exchange was conducted over a 60 min time course with 97% D_2O , 10 mM HEPES (pH 7.4), 150 mM KCl, and 2.9 ! M $\Delta 25$ -HC2. There is no fit curve at 3 min of exchange for the 3:1 complex in the absence of Ca^{2+} because there are two protein populations (see **Figure 3**). Error bars represent one standard deviation in the triplicate experiments.

Figure 3.

The presence of two distinct protein populations at 3 min of H/D exchange. The lower mass species, representing bound protein, has an average deuterium uptake of 95 ± 3 Da. The higher mass species, representing unbound protein, has an average deuterium uptake of 122 ± 2 . The spectrum is from one trial.

Figure 4.

Region-specific H/D exchange kinetics of the N-terminus of $\Delta 25$ -HC2, showing no differences in deuterium uptake and indicating that the differences observed for the intact protein occur in the C-terminal domain. Four states of $\Delta 25$ -HC2 are shown: with no Ca^{2+} (squares), with 2 mM Ca^{2+} (diamonds), at 3:1 XPC: $\Delta 25$ -HC2 and no Ca^{2+} (triangles), and at 3:1 XPC: $\Delta 25$ -HC2 with 2 mM Ca^{2+} (circles).

Figure 5.

Region-specific H/D exchange kinetics experiments of the C-terminus of $\Delta 25$ -HC2. Peptide 110-136, part of EF-hand 3, exhibits EX1 exchange, revealed by two deuterium distributions at 3 min of exchange (see **Figure 6**). Peptide 146-162, part of EF-hand 4, becomes protected upon binding XPC in the absence and presence of Ca^{2+} . There are small differences in deuterium uptake for the final C-terminal peptide, 163-172. Four states of $\Delta 25$ -HC2 are shown: no Ca^{2+} (squares), with 2 mM Ca^{2+} (diamonds), 3:1 XPC: $\Delta 25$ -HC2 and no Ca^{2+} (triangles), and 3:1 XPC: $\Delta 25$ -HC2 with 2 mM Ca^{2+} (circles).

Figure 6.

ESI mass spectra of peptide 110-136 ($[M + 4H]^+$ monoisotopic ion of m/z 786.19) at all H/D exchange times and in four binding states. The EX1 exchange regime is seen in panel 3, where low and high mass populations exist. Four states of $\Delta 25$ -HC2 are shown: no Ca^{2+} (Panel 1), with 2 mM Ca^{2+} (Panel 2), 3:1 XPC: $\Delta 25$ -HC2 and no Ca^{2+} (Panel 3), and 3:1 XPC: $\Delta 25$ -HC2 with 2 mM Ca^{2+} (Panel 4). The dashed lines are for visual reference.

Figure 7. X-ray crystal structure (PDB: 2GGM) of human centrin 2 (red) in complex with Ca^{2+} (yellow) and the XPC peptide (blue). The XPC peptide interacts on the C-terminal tail of centrin, in agreement with the region-specific H/D exchange results. Peptide 110-136 of human centrin 2 is highlighted in green, 146-162 in orange, and 163-172 in purple.