An NMR based model of the ubiquitin-bound human ubiquitin conjugation complex Mms2/Ubc13: The structural basis for lysine 63 chain catalysis.

SEAN MCKENNA¹, TREVOR MORAES¹, LANDON PASTUSHOK³, CHRISTOPHER PTAK¹, WEI XIAO³, LEO SPYRACOPOULOS¹,²*, and MICHAEL J. ELLISON¹,²*
¹Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada.
²Institute for Biomolecular Design, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada.
e-mail: mike.ellison@ualberta.ca
Tel.: 780-492-6352
Fax.: 780-492-0886

Email: leo.spyracopoulos@ualberta.ca
Tel.: 780-492-2417
Fax.: 780-492-0886
* Corresponding authors

³Department of Microbiology and Immunology, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5E5, Canada.
e-mail: xiaow@sask.usask.ca
Tel.: 306-966-4308
Fax.: 306-966-4311

Running Title: The structural basis for lysine 63 chain catalysis.
Summary

A heterodimer composed of the catalytically active ubiquitin conjugating enzyme hUbc13 and its catalytically inactive paralogue, hMms2, forms the catalytic core for the synthesis of an alternative type of multi-ubiquitin chain where ubiquitin molecules are tandemly linked to one another through a K63 isopeptide bond. This type of linkage, as opposed to the more typical K48-linked chains, serves as a non-proteolytic marker of protein targets involved in error-free post-replicative DNA repair and NF-κB signal transduction. Using a two dimensional $^1$H-$^{15}$N nuclear magnetic resonance approach, we have mapped: 1) the interaction between the subunits of the human Ubc13/Mms2 heterodimer and 2) the interactions between each of the subunits or heterodimer with a non-covalently bound acceptor ubiquitin or a thiolester-linked donor ubiquitin. Using these NMR-derived constraints and an unbiased docking approach, we have assembled the four components of this catalytic complex into a three dimensional model that agrees well with its catalytic function.

Introduction

The post-translational modification of intracellular proteins by ubiquitination fulfills an important regulatory function in many cellular pathways. Protein ubiquitination involves a cascade of enzymatic steps where ubiquitin (Ub) is passed sequentially as an activated thiolester from a Ub activating enzyme (E1) to a Ub conjugating enzyme (E2), and finally to the protein target with the help of a Ub protein ligase (E3) [1,2].

The assembly of poly-Ub chains onto a targeted protein has proven to be a hallmark of a variety of processes, such as cell cycle control [3], DNA repair [4], ribosome biogenesis [5], the inflammatory response [6], endocytosis of cell surface proteins [7], and NF-κB-dependent signal transduction [8]. These chains are synthesized in an E2 dependent reaction where each Ub
within the chain is covalently bound to its neighbor by an isopeptide bond that links the c-terminus to a surface lysine of its target-proximal Ub partner. Previous observations have demonstrated that these chains can exist in different configurations that are defined by the specific lysine residue that links each Ub molecule within the chain [9-14].

The most prevalent and best-documented examples of protein ubiquitination use the K48-linked chain configuration to target proteins for degradation by the 26S proteasome [2]. Recently, however, a non-proteolytic ubiquitination pathway has come to light that results in the substrate-tethered assembly of multi-Ub chains where Ub molecules are tandemly linked to one another through K63 [12,15-19]. This pathway plays a key role in error-free DNA post-replicative repair [20-22] endocytosis [15], polosome stability [17], and is an important component of NF-κB signal transduction [18,19].

The error-free repair and NF-κB pathways both catalyze the assembly of K63 chains using a conserved E2 heterodimer, composed of a catalytically active hUbc13 subunit and a catalytically inactive E2-like subunit termed Ubiquitin Enzyme Variant (UEV). UEV proteins share significant sequence similarity with other E2s, but lack the characteristic active-site cysteine residue required for thiolester formation. In the error-free repair pathway of S. cerevisiae, two chromatin-associated RING finger proteins, Rad5 and Rad18, recruit the hUbc13/hMms2 heterodimer and Ubc2 (Rad6) to DNA [23]. In very recent work, Hoege et al. [24] have demonstrated that a target of this pathway is the yeast proliferating cell nuclear antigen (PCNA), which is first mono-ubiquitinated through Rad6 and Rad18 and then poly-ubiquitinated by hUbc13/hMms2 in conjunction with Rad5. In NF-κB signal transduction, Traf6, a RING domain E3 protein, functions together with the hUbc13/UEV heterodimer (containing either hUEV1a or the functionally equivalent hMms2) in the formation of K63-linked poly-Ub chains.
that are required for the activation of IκB kinase (IKK), a key signal transducer in the NF-κB pathway [18,19].

Insight into the mechanism of K63 chain assembly and its relationship to structure recently became possible with the simultaneous determination of both *S. cerevisiae* [25] and human [26] Ubc13/Mms2 heterodimer structures by X-ray crystallography, and an NMR-based approach for mapping the protein-protein interactions within the Ub bound complex [27]. Using the previously determined assignments for Ub in 1H-15N HSQC NMR experiments, we were able to footprint the surface of Ub that interacted with the human hUbc13/hMms2 heterodimer and each of its subunits in either the thiolester-linked or unlinked forms [27]. The results of this study were consistent with a two binding site model in which an “acceptor” molecule of Ub bound non-covalently to hMms2 was positioned in an orientation such that a second Ub molecule that was linked to hUbc13 as a thiolester could be transferred to K63 of the accepting Ub molecule. The NMR assignments of both hMms2 and hUbc13 is an obvious prerequisite for footprinting the surfaces of the heterodimer that interact with both the covalently linked and unlinked forms of Ub. In the present work we have determined the footprint that both Ub molecules make on the surface of the hUbc13/hMms2 heterodimer. Taken together with our previous work, a compelling model is presented for the tetrameric structure that places K63 of the accepting Ub molecule in catalytic proximity of the c-terminus of the donor Ub molecule.

**Experimental Procedures**

*Protein expression*

hUbc13 and hMms2 were expressed and purified as described previously [27] with the following exceptions. Proteins were expressed in the *Escherichia coli* strain BL21(DE3)-RP (Stratagene),
and 2 L cultures were grown at 25 C to OD$_{590}$ = 0.3 in minimal media containing $^{15}$NH$_4$Cl as the sole nitrogen source and induced with isopropyl β-D-thiogalactopyranoside (0.4 mM) for an additional 24 hours at 25 C. *S. cerevisiae* UbK48R, UbK63R, and Uba1 (E1) were expressed and purified as described previously [27].

**NMR spectroscopy**

All NMR spectra were obtained using a Varian Unity INOVA 600 MHz spectrometer at 30C. The 2D $^1$H-$^{15}$N-HSQC NMR spectra were acquired using the sensitivity-enhanced gradient pulse scheme developed by Kay and co-workers [45,46]. The $^1$H and $^{15}$N sweep widths were 8000 and 2200 Hz, respectively. A minimum of 64 transients were collected for each spectrum. All NMR samples were prepared to include HEPES (50 mM, pH 7.5), NaCl (75 mM), EDTA (1 mM), DTT (1 mM), and DSS (1 mM) in the presence of 9:1 D$_2$O:H$_2$O.

Spectral processing was accomplished with the NMRPipe program [47]. The NMRview program [48] was employed in the assignment of all 2D $^1$H-$^{15}$N-HSQC NMR crosspeaks. To calculate the total average change in backbone amide $^1$HN and $^{15}$N chemical shifts for each resonance, the following equation was applied [49]:

$$\Delta\delta_{\text{total}} = \sqrt{(\Delta\delta_{^{15}\text{N}})^2 + (\Delta\delta_{^1\text{HN}})^2}$$

where $\Delta\delta_{^{15}\text{N}}$ and $\Delta\delta_{^1\text{HN}}$ are the chemical shift changes in Hertz. The average change in total chemical shift was then calculated for each identified residue, with the exception of those whose resonances had broadened past detectability in the 2D $^1$H-$^{15}$N-HSQC NMR spectra. The standard deviation associated with each dataset was also calculated.

$^{15}$N-hMms2 chemical shift perturbation experiments
An initial 2D $^1$H-$^{15}$N-HSQC spectrum was acquired for $^{15}$N-hMms2 (250 M) as a point of reference for subsequent chemical shift perturbation experiments. The spectrum also served to confirm the proper folding and lack of aggregation of $^{15}$N-hMms2.

The interactions between $^{15}$N-hMms2 and hUbc13 were examined by inclusion of a slight excess of unlabeled hUbc13 (300 M) to the sample described above for $^{15}$N-hMms2 alone. The NMR tube was allowed to equilibrate for 1 hour at 30°C to insure heterodimerization would proceed to completion. A 2D $^1$H-$^{15}$N-HSQC spectrum was then acquired for the sample.

Non-covalent interactions between $^{15}$N-hMms2 (250 M) and Ub were examined by including unlabeled UbK48R (600 M) into NMR samples in the presence or absence of unlabeled hUbc13 (300 M). A 2D $^1$H-$^{15}$N-HSQC spectrum was then acquired for each sample. Chemical shift assignments in the 2D $^1$H-$^{15}$N-HSQC spectra were again completed assuming that the closest cross peak represented the correct change in chemical shift. The 2D $^1$H-$^{15}$N-HSQC NMR reference spectrum used when calculating changes caused by Ub were either (i) $^{15}$N-hMms2 alone to examine the changes cause in hMms2 by itself or (ii) $^{15}$N-hMms2/hUbc13 in order to probe the changes in chemical shift in hMms2 in the context of the heterodimer.

$^{15}$N-hUbc13 chemical shift perturbation experiments.

As in the case of hMms2, an initial 2D $^1$H-$^{15}$N-HSQC NMR spectrum was acquired as a point of reference, and confirmed the proper folding and lack of aggregation of $^{15}$N-hUbc13 (305 M).

The interactions between $^{15}$N-hUbc13 and hMms2 were examined by inclusion of a slight excess of unlabeled hMms2 (330 M) to the sample described above for $^{15}$N-hUbc13 alone. Sample equilibration and acquisition were performed as described for the $^{15}$N-hMms2 samples.
Thiolester-linked interactions between $^{15}$N-hUbc13 (305M) and Ub (330 M) were examined in situ by inclusion of *S. cerevisiae* E1 (0.3 M), ATP (5 mM), and MgCl$_2$ (5 mM) as described previously [27]. Addition of hMms2 (330 M) to this sample allowed for the examination of the hMms2/15N-hUbc13~Ub species. Studies described elsewhere [27] have shown that thiolester formation is rapid (minutes) whereas the formation of Ub-conjugate on hUbc13 is slow (hours). Furthermore, the onset of conjugate formation can be clearly identified based on the accumulation of new peaks emanating from the mixed population of Ub species. The 2D $^1$H-$^{15}$N-HSQC NMR experiments were therefore performed between 10 and 120 minutes after the addition of E1 in order to minimize the impact of possible side-reactions. UbK63R was employed as the Ub species in order to eliminate the possibility of chain formation by the hUbc13/hMms2 heterodimer, and hence eliminate further complication of the spectra [27]. The 2D $^1$H-$^{15}$N-HSQC NMR reference spectrum used when calculating changes caused by Ub in thiolester complexes were either (i) $^{15}$N-hUbc13 alone to examine the changes caused in hUbc13 by itself or (ii) $^{15}$N-hUbc13/hMms2 in order to probe the changes in chemical shift in hUbc13 in the context of the heterodimer.

Non-covalent interactions between $^{15}$N-hUbc13 and Ub were detected by including unlabeled UbK63R into NMR samples in the presence or absence of unlabeled hUbc13 under conditions identical to thiolester formation with the exception that E1, ATP, and MgCl$_2$ were omitted. A 2D $^1$H-$^{15}$N-HSQC NMR spectrum was then acquired for each sample. However, no changes in $^{15}$N-hUbc13 cross peaks were observed in either case, and therefore no further analysis was performed.
Molecular modeling

Molecular modeling of the surfaces of interaction was accomplished using the BiGGER soft-docking algorithm [30,31] using the unbound structures of Ub (target) [32] and the hUbc13/hMms2 heterodimer (probe) [26]. The BiGGER algorithm systematically searches the complete 6-dimensional binding spaces of both target and probe, and then evaluates these solutions in terms of a global scoring function consisting of geometric complementarity, electrostatic interactions, desolvation energy, and the pairwise propensities of amino acid side chains to interact across molecular interfaces. Docking parameters in this initial search included a 15 degree angular step, 5000 maximum solutions, and 300 minimum atomic contacts. The top 5000 solutions based on global score were then filtered using the NMR chemical shift perturbation data in the following manner. First, surface-exposed residues on hMms2 and Ub, respectively, which produced significant $\Delta \delta_{\text{total}}$ values upon non-covalent interaction were determined, and the number of atomic contacts between these two groups within a 5 Å distance cutoff in each of the top 5000 solutions as determined by global score was evaluated. The top solution based on these criteria was then accepted as the “correct” orientation, and subsequently underwent minimization using the INSIGHTII suite of programs. The thiolester bound Ub placement upon the heterodimer was then determined in an identical manner using $\Delta \delta_{\text{total}}$ values.

Results

When engaged in catalysis, the hUbc13/hMms2 heterodimer necessarily exists as part of a tetramer that is composed of the heterodimer in association with two Ub molecules. One Ub molecule is linked as a thiolester to the active site of hUbc13 (the donor) while the other Ub
molecule interacts non-covalently with hMms2 (the acceptor). While a high-resolution crystallographic structure for the heterodimer has been determined [26], a crystallographic structure for the Ub-bound tetramer is unlikely. This conclusion is based both on the instability of the hUbc13-Ub thiolester bond [33] and the relatively weak interaction that exists between the acceptor Ub and hMms2 (K_d ~ 100 M^{-1}). Based on these considerations, we have pursued an alternative NMR-based approach to determine the structure of the hUbc13/hMms2-Ub2 tetramer. The tetramer has three major protein-protein interfaces: 1) the hMms2-hUbc13 interface, 2) the hMms2-Ub (acceptor) interface and, 3) the hUbc13 Ub (donor) interface. In this and previous studies [27], we have used \(^1\)H-\(^{15}\)N HSQC NMR spectroscopy to observe the chemical shift perturbations that are induced in upon interaction to define the footprint that each protein makes with its partner.

The method that we have chosen here relies upon the comparison of \(^1\)H-\(^{15}\)N HSQC NMR spectra for each protein component in an unbound form and bound to its partner. To simplify the analysis, only one component of the complex is \(^{15}\)N-labeled in any given experiment. Backbone amide \(^1\)H_N and \(^{15}\)N chemical shifts are sensitive to a variety of factors including hydrogen bonding, electrostatic interactions, and aromatic ring current effects to name a few. Therefore changes to chemical shifts that can result from differences in chemical environment upon complex formation can be used to identify residues that are either directly involved at the binding interface or correspond to long-range structural changes.

A necessary precursor to chemical shift mapping is the complete assignment of backbone amide \(^1\)H_N-\(^{15}\)N cross peaks in the \(^1\)H-\(^{15}\)N HSQC NMR spectra for a given component of the complex. Recently, we have completed the full backbone chemical shift assignments for both

\(^1\) Manuscript in Preparation
hUbc13 and hMms2 (available upon request). Each protein exhibits well dispersed and resolved
$^1$H-$^{15}$N HSQC NMR spectra at 600 MHz, as is shown for hMms2 (Fig. 1). Furthermore, the
spectra retain these qualities fairly well upon formation of higher order complexes of up to 42.5
kDa, though the signal-to-noise ratio is reduced as expected, due to increased linewidths.
Chemical shift assignments in the 2D $^1$H-$^{15}$N-HSQC NMR spectra were made relative to the
appropriate reference spectrum, assuming that the closest shifted cross peak represented the
correct one. This approach was required due primarily to the lability of complexes containing
thiolester linkages.

**Mapping the heterodimer interface.**

To map the interface between hUbc13 and hMms2, two heterodimer complexes were
prepared *in situ*: one containing $^{15}$N-hUbc13 with unlabeled hMms2, and the other containing
$^{15}$N-hMms2 with unlabeled hUbc13. The hUbc13/hMms2 heterodimerization (34 kDa) proceeds
efficiently upon equimolar addition of each protein, and results in the formation of a stable
complex that remains associated during high-resolution size-exclusion chromatography [27].
Residues whose backbone amide $^1$H and $^{15}$N chemical shifts exhibited a perturbation upon
complex formation were identified, and quantified in terms of the total change in chemical shift,
$\Delta \delta_{\text{total}}$. The major $\Delta \delta_{\text{total}}$ upon heterodimerization for either $^{15}$N-hMms2 or $^{15}$N-hUbc13 are
associated with residues found at the heterodimer interface (Fig. 2B, 3B), indicating the
similarity of this interface in both the crystalline and solution phases.

Residues resulting in the greatest effect on $\Delta \delta_{\text{total}}$ for interactions within the heterodimer
or between the heterodimer and Ub (see below) have been summarized in Figure 4 according to
sequence and secondary structure. Also shown are the chemical shift indices (CSI) for each
residue contained in hMms2 and hUbc13, which provide a measure of the deviation between the observed chemical shifts and their random coil values, and are indicative of the type of secondary structure [28,29]. A comparison between secondary structural elements for hMms2 and hUbc13, determined by X-ray crystallography to those determined from the CSI demonstrate a close correlation between types of secondary structure determined in the solution and crystalline states.

The non-covalent interaction between hMms2 and Ub.

Both hMms2 and hUbc13 have each been observed to exist in a monomeric state and as the heterodimer [23,27], whereas homodimerization has not been observed (see Experimental Procedures), and therefore an examination of the interaction between Ub and the hMms2 subunit is of interest. The chemical shift perturbations that result from the interaction of $^{15}$N-hMms2 subunit with unlabeled acceptor Ub are shown in Figure 2A. The greatest effects on $\Delta\delta_{total}$ upon interaction with Ub are observed at the N-terminal portion of hMms2. Specifically, the affected residues are located in helix $\alpha$1 (E20, G22, K24), sections of strand $\beta$1 (V31, S32, L35), strand $\beta$2 (T47, G48, M49), strand $\beta$3 (Y63, L65), helix $\alpha$2 (L119) as well as the loop joining helix $\alpha$1 to strand $\beta$1 (V26, T30). Intermediate effects on $\Delta\delta_{total}$ are found close in sequence to the greatest changes and include the c-terminal portion of $\alpha$1 (Q23), sections of $\beta$1 (W33), $\beta$2 (W46), L2 prior to $\beta$3 (N60, R61), $\beta$3 (V67, G70) and the loop joining $\alpha$1 to $\beta$1 (G25, G27, G29). Intermediate changes are also found in $\alpha$2 (Q120, L125, E130) and the c-terminus (G140, Q141).

As expected, many of the residues in hMms2 that exhibit the greatest backbone amide chemical shift perturbations are located on the surface of the protein, and contain surface
exposed side chains that may be involved in non-covalent interactions with Ub (Fig. 5A). These residues cluster onto one face of hMms2, forming three distinct patches. Interestingly, no significant changes in chemical shift were observed for residues on the opposite surface of hMms2. The first patch is perpendicular to the hUbc13/hMms2 interface, and is composed of residues at the c-terminal end of α1 and the loop that joins α1 to β1 (E20, E21, G22, Q23, K24, G25, V26, G27, G29, V31), portions of β1 (S32, W33, L35), β2 (T47, G48, M49), and β3 (R61, Y63, L65). The second patch is found at the c-terminal portion of hMms2 (E139, G140, M141). Notably, the total surface area of both these hMms2 patches corresponds well with the complementary patch on Ub that has previously been demonstrated to interact with hMms2 [27]. Additionally, the combined electrostatic surface potential of the hMms2 patches is complementary to that found on Ub (Fig. 5C). Interestingly, the third patch involves hMms2 residues that would normally interact with hUbc13 in the heterodimer, and include V7, K8, (greatest Δδ\textsubscript{total} and other n-terminal amino acids of hMms2 (intermediate Δδ\textsubscript{total}).

Our previous findings indicated that the Ub contact surface with hMms2 remained largely the same when alone or in complex with hUbc13 [27]. When we next examined the \textsuperscript{15}N-hMms2–Ub interaction as a heterodimer with hUbc13 we similarly found that the hMms2 residues that undergo change on Ub binding closely parallel those of the individual subunit with some notable exceptions (Fig. 2C). As with hMms2 alone, many of the major Δδ\textsubscript{total} are found near the c-terminus of α1 (E20, Q23), the loop that joins it to β1 (V26, G29, T30), β1 (V31), β2 (G48, M49), and β3 (R61, Y63, L65). Residues with intermediate values of Δδ\textsubscript{total} are also similar, including α1 (L19, E21), the loop joining α1 to β1 (G25), β1 (W33, G34), β2 (T47, G52), β3 (N60, I62, V67), α2 (S114, I115, V117, Q120, L125, E130), and the c-terminus (Q141). The backbone amide \textsuperscript{1}H\textsubscript{\textalpha}–\textsuperscript{15}N HSQC NMR cross peaks for three residues [L1 (D37)
and β2 (R45, I50)] either experienced large changes in chemical shift, rendering identification difficult, or their intensities were severely diminished due to line-broadening as a result of complex formation.

In contrast to the hMms2 subunit alone, none of the n-terminal residues situated at the heterodimer interface undergo significant change upon Ub binding, whereas significant change is detected within L1 (D38, D40, M41, R45). Notably, the region surrounding the vestigial active site of hMms2 does not appear to play a role in Ub binding. This result clearly distinguishes the hMms2-Ub interaction from other previously reported E2-Ub interactions. The changes in the surface characteristics of the hMms2 component of the heterodimer upon Ub binding are shown in Fig. 5B.

**The interaction between hUbc13 and thiolester-linked Ub.**

The major changes to the $^{15}$N-hUbc13 subunit that result from thiolester formation with Ub are found in and around the active-site (C87) (Fig. 3A). These include: the active-site cysteine itself, L4 (N79, L83, R85) to the n-terminal side of C87, the 3-10 helix c-terminal to C87 (D89, I90), the loop preceding helix α3 (L111, N116, D118, D119), and helix α3 (D124, V125, E127, K130). Intermediate perturbations of $\Delta\delta_{\text{total}}$ are found around and inter-digitated with the major changes described above. These include: L4 (M72, I75, Y76, H77), near the active site (L88), the 3-10 helix (K92, W95, S96, A98), the loop preceding α3 (S113, A114), and α3 (A126, T131).

Heterodimerization of $^{15}$N-hUbc13 with hMms2 results in somewhat fewer $\Delta\delta_{\text{total}}$ upon thiolester formation when compared with the thiolester formed with $^{15}$N-hUbc13 alone (Fig. 3C when compared to 3A). It is noted, however, that a number of cross peaks in the $^1$H-$^1$N HSQC
NMR spectra of the heterodimer thiolester remain unassigned due to line broadening or large changes in chemical shift upon complex formation. The major and intermediate changes to $\Delta \delta_{\text{total}}$ occur within secondary structural regions including L4 (K74, I75, Y76, H79, L83, G84, R85), the active-site (C87), the 3-10 helix (L88, D89, I90, L91, D93), the loop preceding $\alpha$3 helix (N116, D118, L121, A122, D124) and the $\alpha$3 helix (V125, A126, W129, K130, T131).

Surfaces involved in the interaction between hUbc13 and its thiolester-linked Ub were determined by mapping the major $\Delta \delta_{\text{total}}$ for the $^{15}$N-hUbc13 subunit alone or in complex with hMms2s onto a surface projection of the hUbc13 crystal structure (Fig. 5D, 5E). In the absence of hMms2 (Fig. 5D), the greatest effect is found around the active site (C87) where the majority of affected residues have solvent exposed side chains [L4: (R70, L83, R85, I86, C87, D89). $\alpha$2: (L106, Q109, A110, L111). $\alpha$3 and preceding loop: (N116, D118, D119, D124, A126, E127, K130)].

From Figure 5E, it is apparent that hUbc13 exhibits a similar Ub dependent pattern of backbone amide chemical shift changes when present with hMms2. Significantly, all of the solvent exposed residues important in thiolester formation present themselves on only one face of the hUbc13 molecule regardless of dimerization state. We conclude from these results that the hUbc13~Ub thiolester interaction is largely unaffected by the presence or absence of hMms2. These results are consistent with our previous NMR experiments demonstrating that both the c-terminal tail and a slightly basic surface on Ub form contacts with hUbc13 within the hUbc13~Ub thiolester regardless of the presence of hMms2 [27].

**Modeling the tetramer**
The soft-docking algorithm BiGGER [30,31] was employed to generate models for the Ub₂-hUbc13-hMms2 tetramer based on geometric complementarity, electrostatic interactions, desolvation energy, and the pair wise propensities of amino acid side chains to interact across interfaces. Surface residues from the heterodimer (results presented herein) and Ub [27], that exhibited the greatest change to $\Delta \delta_{\text{total}}$ upon complex formation, were incorporated as constraints into the BiGGER docking program (see Experimental section). The c-terminus of the donor Ub was not covalently linked to the active site of hUbc13. The top ten structures based on these criteria were subsequently averaged, and the resulting structure was subjected to energy minimization using the INSIGHTII suite of programs. The final structure of the model is shown in Figure 6.

The non-covalent interaction between acceptor Ub and the heterodimer involves hydrophobic contacts between Ub and hMms2. The surface exposed residues of the $\beta$-sheet of the acceptor Ub, and the loops connecting strands within the sheet, constitute the contact interface with hMms2, while hMms2 residues that contact the acceptor Ub are found in $\alpha_1$, $\beta_1$, $\beta_2$, and the loops connecting these secondary structural elements. The hMms2 surface involved in the interaction is located opposite to the surface containing the vestigial active site. The donor Ub makes contacts with hUbc13 through C-terminal residues 70-76, as well as some residues in $\beta_1$ and $\beta_3$. The hUbc13 residues that form contacts with the C-terminus of donor Ub are found within the active site, the loops preceding it, and residues in $\alpha_2$.

**Discussion**

Together, the NMR chemical shift perturbation results have been interpreted to produce a model of the tetramer using a molecular docking strategy that is tailored to this NMR-based approach.
The accepting Ub molecule sits on a concave face of hMms2, a distinctive feature of both E2s and UEVs, with its c-terminal tail far removed from the vestigial active site of hMms2. In combination with hUbc13, the concave face of hMms2 narrows to form a channel or funnel as it approaches the active site of hUbc13. The side-chain of K63 for the acceptor Ub lies within this channel, placing the ε-nitrogen within 3 Å of the sulphur atom contained within the active site cysteine of hUbc13. The interaction between the accepting Ub and the heterodimer buries 2792 Å² of surface area, a rather large value in light of our observation that the interaction between the two is weak (Kₐ ~100 M⁻¹). The model likely overestimates the buried surface area of the acceptor Ub because the imposed chemical shift restraints force the contact regions to be maximized and may include residues that are affected indirectly through induced structural changes in the proteins.

There are two features of the accepting Ub-heterodimer interface that bear directly on its biochemical function. First, the c-terminal tail of the acceptor is neither constrained nor sterically hindered, raising the likelihood that it can serve as the poly-Ub chain anchor in either the free form or when attached to an appropriate protein target. Second, K48 of the acceptor is buried within the protein-protein interface, thereby excluding this residue as a potential site for chain assembly of the canonical type.

The donor Ub interacts exclusively with a hydrophobic concave surface that narrows to an acidic cleft on hUbc13 and culminating with the active site cysteine (Fig. 5F). The tail of the donor Ub lies within the active site cleft of the E2 placing the c-terminal carboxyl carbon of G76, the active site sulphur and the ε-nitrogen of K63 for the acceptor Ub molecule within 3.5 Å of each other.
In terms of the position and orientation of the components, the model presented here agrees moderately well with that proposed by VanDemark et al. [25] for the *S. cerevisiae* complex. It differs significantly however, from the model proposed by Pornillos et al. [36] who examined the non-covalent interaction between the human Tsg101 UEV domain and Ub by a similar approach to the one used here. The structural differences between the Ub-hMms2 interaction and Ub-Tsg101 interaction results from the presence of an extended β-hairpin that links strands 1 and 2 in Tsg101 that sequester Ub. The fact that this motif is absent in hMms2 illustrates that UEVs have evolved different strategies for Ub binding.

Our high confidence in this model stems both from the NMR-constrained docking approach used here. The docking algorithm BiIGGER is particularly well suited for these analyses because of its ability to use NMR chemical shift perturbation results as information to filter suitable models [30,31]. The BiIGGER docking algorithm requires no information that constrains the orientation of the docking partners, and therefore represents a fairly unbiased approach for using NMR data to model the tetramer interactions. The validation of this approach lies in the predicted positions of the three atoms involved in linking the c-terminus of the donor Ub molecule to K63 of the accepting Ub molecule: 1) the cysteine sulfur atom of the hUbc13 active site, 2) The G76 carboxyl group of the Ub donor molecule, and 3) the K63 ε- nitrogen of the accepting Ub molecule. Each of these atoms are positioned within 3.5 Å of each other (Fig. 6).

The model presented here also agrees well with the findings of a previous mutagenesis study that used the *S. cerevisiae* hUbc13/hMms2 heterodimer [25]. A Ubc13 substitution (A110R) located on the surface of α3 near the center of the predicted interaction between Ubc13 and the donor Ub, resulted in a 4-fold reduction in the rate of isopeptide bond formation. A
Ubc13 substitution (D81A) situated nearby the predicted position of K63 of the accepting Ub resulted in a diminished affinity of the acceptor Ub for the heterodimer in vitro. A Ub substitution (I44A) located in the NMR-derived surface for the acceptor but not donor, results in reduced binding of Ub to the acceptor site on Mms2, whereas the interaction with Ubc13 remains unaffected. Conversely, an Mms2 substitution (E12R) situated near the heterodimer interface but not predicted by the model to play a role in acceptor Ub binding, does not weaken the interaction of the acceptor Ub with the heterodimer in vitro [25].

The structure of the hUbc13~Ub thiolester presented here holds features in common with the models for the Ubc1~Ub thiolester from S. cerevisiae [34] and the human Ubc2b~Ub serine ester [33], each derived by similar NMR-based approaches. All three E2s employ a common thiolester-binding motif (L4 around the active site, regions of α2, and the loop that joins α2 to α3) that constrains the c-terminal tail similarly amongst models. In contrast, the folded domain of Ub is positioned slightly differently on the each of the three E2s (Fig. 4). These differences are likely explained by properties associated with catalysis. The tail of the Ub donor must be bound to the E2 strongly enough to secure its alignment during isopeptide bond formation with the target, yet weakly enough to assure efficient transfer and subsequent turnover of the E2. E2 interactions with the rest of the Ub globular domain are therefore likely to be even weaker and can be imagined to vary significantly by differences of a few key surface residues from one E2 to the next.

An examination of high-resolution E2 structures has revealed that the active site is part of an unstructured loop [37-43]. Our previous and present findings suggest that the interaction of hMms2 with hUbc13 alters the activity of hUbc13 by altering the conformation of the hUbc13 active site. We have previously shown that when hMms2 binds to hUbc13, both the rate of Ub
thiolester formation with hUbc13 (reduced 2-fold in the presence of hMms2) and the stability of
the resulting thiolester are measurably affected in vitro [27]. This observation raises the
intriguing possibility that the interaction of an E2 with other proteins could order the loop in a
particular conformation, thereby modulating its catalytic activity.

An examination of the chemical shift perturbation data reveals that there is
communication between the acceptor and donor Ub binding sites. This is reflected by a change in
chemical environment at residues that are known to play a key role in the active-site loop. For
instance, residues in the active-site cleft of hUbc13 (L83, G84, R85, L88, I90), show significant
values of $\Delta\delta_{\text{total}}$ upon dimerization with hMms2. Three of these residues (L83, G84, and R85)
are directly involved in the heterodimer interface, whereas two of these residues (L88 and I90)
are remote from the interface. In addition, Ub thiolester formation within the heterodimer results
in a significant shift of $\Delta\delta_{\text{total}}$ for the interfacial residues L83 and R85. This observation suggests
that the communication between the heterodimer interface and the active site is in fact occurring,
that is, altering the interface alters the active site and vice versa. These results appear to be in
contrast with those previously reported for S. cerevisiae Ubc13/Mms2, for which there appears
to be little communication between the dimer interface and the active site. An RMSD of 0.8
for superimposition of all backbone C$\alpha$ atoms between free and Mms2-bound Ubc13 was
reported, with the active site cleft little changed [25]. However, as chemical shift changes cannot
be directly converted into 3D structural changes, further analyses will be required to establish the
extent of similarities and differences between the human and S. cerevisiae protein complexes.

The arrangement of the four molecules within the tetramer poses no obvious steric
problem for the interaction of hUbc13 with its functionally specific E3, Traf6. The interface
between hUbc13 and Traf6 can be predicted on the basis of the X-ray crystallographic structure
for the E2-E3 complex UbcH7-c-Cbl [44]. Both c-Cbl and Traf6 contain E2-binding RING-finger domains that share significant sequence identity. Traf6 likely sits on an 11-residue patch of hUbc13, with six residues identical to those employed by UbcH7 in its interaction with c-Cbl (Fig. 6). Notably, none of these residues are involved in forming contacts between Ub and hUbc13.

Despite its small size and highly conserved fold, the E2 core domain family is apparently the centerpiece for several distinct biochemical functions that hinge on isopeptide bond formation. These functions include both target ubiquitination and the synthesis of multi-Ub chains that differ from one another in configuration. As a consequence of unknown evolutionary pressure, these proteins have apparently modeled and remodeled their surfaces with great economy and creativity. The functional repertoire of protein ubiquitination has been expanded by the ability of these proteins to interact with common or related partners in fundamentally different ways. This point is underscored in part by the present work. The E2 core fold has evolved at least three relevant and fundamentally different modes of Ub binding. Furthermore, the juxtaposition of two of these modes, through the interaction of a catalytically active fold with an inactive fold, provides the structural basis for K63 multi-Ub chain synthesis.

Acknowledgments

We thank Susan Smith for secretarial assistance, Linda Saltibus for technical assistance and all of the members of the Ellison and Spyracopoulos labs as well as Pascal Mercier and Prof. Brian Sykes for their valuable input and assistance. We also thank Prof. Lewis E. Kay for pulse sequences, Deryck Webb for spectrometer maintenance, and Yanni Batsiolas and Robert Boyko for computer expertise. This work was made possible by research grants from the NCIC
(M.J.E.) and AHFMR (L.S.). S.M. is a Natural Sciences and Engineering Research Council of Canada scholar. W.X. is funded by CIHR (MOP-53240). L.S. is an AMFMR medical scholar. T.M. is a Canadian Institutes of Health Research scholar.
References


Figure Legends

FIG. 1 **Superposition of $^1$H-$^{15}$N HSQC NMR spectra of $^{15}$N-labeled hMms2, free and in complex with Ub.** $^1$H-$^{15}$N HSQC NMR spectra resulting from either $^{15}$N-hMms2 (black), or $^{15}$N-hMms2 and Ub (red) are overlaid, and a number of representative backbone cross-peaks which were affected by complex formation are labeled.

FIG. 2 **Binding-induced NMR Chemical Shift Perturbation Analysis of hMms2 with Ub.** Comparison of backbone amide $^1$H and $^{15}$N chemical shift of hMms2 in the absence or presence of (A) Ub and (B) hUbc13, or (C) the comparison between $^{15}$N-hMms2/hUbc13 heterodimer and this heterodimer in the presence of Ub. The total change in chemical shift, $\Delta \delta_{\text{total}}$, was calculated for hMms2 interacting with various binding partners and plotted as a function of primary amino acid sequence. Dashed lines represent the average change in $\Delta \delta_{\text{total}}$ and one standard deviation unit above this average. Residues whose change in chemical shift could not be identified are indicated with an asterisk (*).

FIG. 3 **Binding-induced NMR Chemical Shift Perturbation Analysis of hUbc13 with Ub.** Comparison of backbone amide $^1$H and $^{15}$N chemical shift of hUbc13 in the absence or presence of (A) thiolester-linked Ub and (B) hMms2, or (C) the comparison between hMms2/$^{15}$N-hUbc13 heterodimer and this heterodimer in the presence of thiolester-linked Ub. The total change in chemical shift, $\Delta \delta_{\text{total}}$, was calculated for hUbc13 under each of the conditions and plotted as a function of primary amino acid sequence. Dashed lines represent the average change in $\Delta \delta_{\text{total}}$ as well as one standard deviation above this average. Residues whose change in chemical shift could not be identified are indicated with an asterisk (*).

FIG. 4 **Sequence Alignments of the Important Interfacial Residues in hUbc13 and hMms2 With *S. cerevisiae* Ubc1 as Determined by $^1$H-$^{15}$N HSQC NMR Chemical Shift Perturbation.** Residues experiencing the greatest $\Delta \delta_{\text{total}}$ upon formation of hMms2/hUbc13 are colored in yellow and blue, respectively, and are compared to interfacial residues in the crystal structure (boxed) [26]. hMms2 residues experiencing the most significant $\Delta \delta_{\text{total}}$ upon formation of non-covalent interaction with Ub are labeled in red, as are residues in hUbc13 upon formation of the thiolester adduct with Ub. For comparison, residues deemed responsible for the interaction between Ubc1 and Ub in the thiolester complex are also colored red [34]. Secondary structural elements are shown above (for E2s) and below (for hMms2) the sequence alignments, as are the average chemical shift index values as determined from $C_\alpha$, $C_\beta$, and $H_a$ chemical shifts (up arrow=+1, down arrow=-1, no arrow=0) as obtained from the program NMRview program using the Wishart peptide database [45], pH 7.5 and 303K.

FIG. 5 **Connolly Surfaces of the Binding Interfaces on hMms2 or hUbc13 Upon Interaction with Ub.** The surface of hMms2 is presented either (A) alone, or (B) in the context of hMms2/hUbc13 heterodimer (hUbc13, yellow). The surface of hUbc13 is presented either (D) alone, or (E) in the context of hMms2/hUbc13 heterodimer (hMms2, blue). Residues affected by non-covalent interaction with Ub are colored with a linear gradient from white ($\Delta \delta_{\text{total}}=0$) to dark red ($\Delta \delta_{\text{total}}\geq \Delta \delta_{\text{total}(av)}+1s$) as determined by $^1$H-$^{15}$N HSQC NMR chemical shift perturbation analysis (Fig. 2 and 3). Residues, whose $\Delta \delta_{\text{total}}$ could not be determined unambiguously due to
broadening or extreme changes in chemical shift are colored orange. The active-site cysteine (C87) of hUbc13 is colored green as a point of reference. Electrostatic surface potential of the hMms2/hUbc13 heterodimer (C) and (F) shown in the same orientation as (B) and (E) respectively. The relative electrostatic potentials are displayed as a linear gradient, from acidic (-10, red), to neutral (0, white), to basic (+10, blue) as determined by the program GRASP [50].

FIG. 6 NMR-Derived Model of the Tetrameric Ub-conjugating Enzyme Complex. (A) The surfaces of interaction between either acceptor (top) or donor (bottom) Ub molecules (red, ribbon) and the hUbc13(yellow)/hMms2(blue) heterodimer are presented. Of specific interest is the active-site C87 of hUbc13 (green), K63 of the acceptor Ub (purple), and G76 of the donor Ub (purple). Residues hypothesized to represent the RING binding domain are colored in white. The NMR-derived model of the tetrameric complex was determined using the BiGGER docking algorithm [30,31] and the INSIGHTII suite of programs as described in the Materials and Methods section of this manuscript. (B) Close-up of the model of the region surrounding C87 of hUbc13.
Supplementary Information: FIG. 1  **Surfaces of Interaction in the hMms2/hUbc13 Heterodimer.** Ribbon presentation of the hMms2/hUbc13 backbone in which residues affected by heterodimer formation are colored using a linear gradient from white ($\Delta \delta_{\text{total}} = 0$) to dark red ($\Delta \delta_{\text{total}} = (\Delta \delta_{(\text{av})+1})$) as determined by $^1$H-$^15$N HSQC NMR chemical shift perturbation (Fig. 2 and 3). Residues whose $\Delta \delta_{\text{total}}$ could not be determined unambiguously due to broadening or extreme changes in chemical shift are colored orange. The active-site cysteine of hUbc13 (C87) is colored in green as a point of reference. (A) Interaction surface of $^15$N-hMms2 (top) with unlabeled hUbc13 (yellow, bottom). (B) Interaction surface of $^15$N-hUbc13 (bottom) with unlabeled hMms2 (blue, top).
Supplementary Information: FIG. 2  **Determination of the Dissociation Constant for the Interaction Between hMms2 and Ub.** Shown are the binding curves from the averaged normalized changes in chemical shift from ten individual $^{15}$N-hMms2 backbone amide crosspeaks during the course of titration with Ub. $^1$H-$^{15}$N-HSQC NMR spectra of $^{15}$N-hMms2 were acquired upon addition of Ub to molar ratios of 0.33, 0.66, 0.99, 1.34, 1.68, 2.04, 2.39, 2.74, and 3.09 relative to hMms2. Curve fitting and $K_d$ determination were accomplished using the xcrvfit program, with the curve fitting script available upon request at www.pence.ca/software.
An NMR based model of the ubiquitin-bound human ubiquitin conjugation complex
Mms2/Ubc13: The structural basis for lysine 63 chain catalysis
Sean McKenna, Trevor Moraes, Landon Pastushok, Christopher Ptak, Wei Xiao, Leo Spyracopoulos and Michael J. Ellison

*J. Biol. Chem.* published online February 4, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212353200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2003/03/21/M212353200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2003/02/04/jbc.M212353200.citation.full.html#ref-list-1