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Identification of the ubiquitin interfacial residues in a ubiquitin-E2 covalent complex

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Abstract

One of the key intermediates formed during the protein ubiquitination cycle is a covalent complex between ubiquitin (Ub) and the conjugation enzyme, UBC1. In order to probe the interface between these two proteins we have formed the covalent complex *in situ* (in the NMR tube) using Ub, the catalytic domain of UBC1, UBC1Δ450, an activation enzyme, E1, and Mg^{2+}-ATP. The size of the Ub-UBC1Δ450 complex (25 kDa) and its relatively short lifetime (∼ 4 h) makes assignment of the backbone resonances in the covalent species difficult. In order to monitor the formation and identify the interface in the complex we have used fast ^{1}H-^{15}N HSQC spectra to monitor the decay of ^{1}H-^{15}N correlations as a function of time until the complex formed reached about 90%. The residual peak intensities were used to probe the surface of interaction between Ub and UBC1Δ450 and provided a clear surface of interaction on Ub.

Abbreviations: Ub, ubiquitin; Ub(K48R), yeast ubiquitin having a K48R substitution; UBC1Δ450, catalytic domain of yeast UBC1 having a K93R substitution.

Introduction

A key area of study in the field of structural biology is the analysis of protein-protein and protein-ligand interactions. NMR spectroscopic techniques have proven to be very useful for identifying key residues that regulate the association and activity of a protein complex. One approach to doing this is to determine the high-resolution three-dimensional structure of the complex. Alternatively, a common and more rapid way to monitor the effects of a protein's association with another molecule is the 'SAR by NMR' method (Shuker et al., 1996). In this analysis, changes in chemical shift in the ^{1}H-^{15}N HSQC spectrum are measured as a function of added ligand and residues are classified based on the magnitude of these changes. Since chemical shift is very sensitive to chemical environment, it is an excellent indicator for the identification of potential ligand binding sites. This method is particularly useful for monitoring the binding of a relatively small molecule to a larger protein and has been successfully used to map protein-ligand contacts for protein-drug (Shuker et al., 1996) and protein-peptide complexes (Barber et al., 1999), especially when the dissociation rate is in the fast-exchange regime on the NMR chemical shift time-scale. Typically only a few residues on the protein shift upon ligand binding and, in the fast-exchange limit, titration studies allow one to follow movement of each peak with increased ligand addition. Thus assignment of shifted peaks in the ^{1}H-^{15}N HSQC spectrum of the complex can be accomplished with relative ease. Additionally, when the binding molecule is small, there is no significant increase in molecular weight of the protein

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being studied. Therefore, the effect of forming the complex on the correlation time (τ_c) of the protein is negligible and, under favourable kinetic conditions ($k_{off} \gg |\delta_{complex} - \delta_{free}|$), the ^{1}H-^{15}N HSQC spectra before and after complex formation have essentially the same linewidths. However, for many systems this method can be more difficult to apply effectively. For example, a larger protein-protein complex typically has a much broader interface than that observed with a smaller ligand. Complexation may double or triple the molecular weight compared to that of the individual protein resulting in a corresponding increase in τ_c and individual linewidths. Further, the ability to follow the movement of resonances as a function of the binding molecule may be made difficult should binding not occur in the fast-exchange limit. Taken together, these factors suggest that the interpretation of larger protein-protein interactions is complex when trying to map the interface.

One particular example of a larger protein complex is that formed between ubiquitin (Ub) and the catalytic domain of UBC1 (UBC1Δ450), a conjugating enzyme of the ubiquitin-dependent proteolytic pathway (Seufert et al., 1990). Ubiquitin, which is one of the most highly conserved proteins in eukaryotes, plays a well-established role in targeting proteins to the proteasome for degradation via its covalent linkage to the target protein in the form of multi-ubiquitin chains (reviewed in Hochstrasser et al., 1996). We have used NMR spectroscopy to investigate the mechanism of ubiquitin chain formation by identifying the protein-protein interactions in intermediates that are formed in a reconstituted system composed of Ub, UBC1Δ450, and E1. The initial phase has been the analysis of the Ub-UBC1Δ450 thiolester intermediate, which forms via a covalent bond between the C-terminal carboxyl group of Ub (G76) and the thiol group of the active-site cysteine residue on UBC1 (C89). As one of the first essential complexes formed upon Ub activation by E1, the thiolester complex is most likely a key determinant of activity in the subsequent steps of multi-ubiquitin chain formation. In the Ub-UBC1 thiolester complex, however, one cannot assign resonances of the ^{1}H-^{15}N HSQC spectrum by taking advantage of titration studies to monitor peak movement because the complex formation is not an equilibrium process. Also, because the complex has a lifetime of about 3–4 h, use of multi-dimensional NMR experiments for re-assignment of the ^{1}H-^{15}N HSQC spectrum is not practical. However, identification of the interactions between Ub and UBC1 is still important in order to understand a structural rationale for their roles in ubiquitination. In this work, we have compared the change in chemical shift and peak intensity methods to identify the residues in Ub that are effected by association with UBC1Δ450 to form the 25 kDa covalent complex. This work is based on our previous assignment of UBC1Δ450 (Hamilton et al., 2000) and assignment of yeast Ub (Ub) at neutral pH, which has not been previously reported.

Methods

Protein expression and purification

Uniformly ^{15}N-labelled and ^{15}N,^{13}C-labelled Ub(K48R) proteins were over-expressed in the BL21DE3pLysS *E. coli* strain from a pET3a-based plasmid which was constructed as previously described (Hodgins et al., 1996). Cell growths were performed using minimal media supplemented with either 1 g/l $^{15}NH_4Cl$ or 1 g/l $^{15}NH_4Cl$ and 2 g/l ^{13}C-glucose (Neidhart et al., 1974; Muchmore et al., 1989). Unless otherwise stated, all buffers used for Ub purification contained 1 mM EDTA and 1 mM DTT and were maintained at pH 7.5. Cell lysates were prepared by freezing harvested cell pellets at −80 °C, thawing them in the presence of 50 mM Tris containing 1 mM PMSF and protease inhibitors, then clarifying by sonication and centrifugation at 100 000*g*. Proteins were purified by anion exchange chromatography (Mono-Q Sepharose) in 50 mM Tris followed by gel filtration (Superdex-75) in 40 mM HEPES and 150 mM NaCl, to greater than 95% purity. Samples for NMR studies were concentrated to 0.8–0.9 mM, then dialysed into 40 mM HEPES and 50 mM NaCl and ^2H_2O was added to a 10% (v/v) concentration.

The catalytic domain of UBC1 having a K93R substitution (UBC1Δ450) was over-expressed and purified as previously described (Hamilton et al., 2000), except that growths were performed in LB liquid media and were grown for 5–6 h after induction with 0.4 mM IPTG.

A 6His-tagged yeast E1 derivative was over-expressed in the *Saccharomyces cerevisiae* strain JD77.1A, which was a gift from Dr Seth Sadin. Cultures were grown in YPD media at 30 °C and E1 expression was induced with 0.1 mM $CuSO_4$. Unless otherwise stated, all buffers used for E1 purification contained 0.5 M NaCl and were maintained at pH 7.5. Harvested cells were washed with ice cold water then

20 mM phosphate and 10 mM imidazole, and lysed by freezing in liquid nitrogen and grinding with an electric grinder. The resultant cell paste was re-suspended in the same buffer and clarified by centrifugation at 100 000 *g*. The supernatant was loaded onto a Ni^{2+}-agarose column equilibrated in 20 mM phosphate and 10 mM imidazole, the column was washed with 20 mM phosphate and 50 mM imidazole and the His-tagged E1 was eluted with 20 mM phosphate and 400 mM imidazole. All E1-containing fractions were combined and dialysed into 50 mM HEPES, 1 mM EDTA, 300 mM NaCl and 1 mM DTT at pH 7.5.

NMR spectroscopy

All NMR experiments were performed at 30 °C using a Varian Unity 500 MHz NMR spectrometer equipped with a pulse field gradient, triple-resonance probe. NMR data was processed using the NMRDraw, Pipp and Stapp programs on a Silicon Graphics Indigo 2 workstation (Garrett et al., 1991; Delaglio et al., 1995).

^{1}H, ^{13}C, and ^{15}N assignments for yeast Ub(K48R) were performed by acquiring the sensitivity-enhanced HNCACB, ^{15}N-edited NOESY (150 ms mixing time), and ^{15}N-edited TOCSY (51 ms mixing time) experiments (Muhandiram and Kay, 1994; Zhang et al., 1994).

^{1}H-^{15}N HSQC experiments were performed according to the methods of Kay et al. (1992). The thiolester reaction was performed at 30 °C using 0.8 mM concentrations of ^{15}N-Ub(K48R) and unlabelled UBC1Δ450 with 10 μM E1, 10 mM ATP, and 5 mM $MgCl_2$ in 40 mM HEPES, 450 mM NaCl, 1 mM EDTA, pH 7.5. Studies were performed by acquiring a ^{1}H-^{15}N HSQC spectrum of ^{15}N Ub(K48R), then repeated in the presence of an equimolar amount of unlabelled UBC1Δ450. The initial NMR spectrum for the time course reaction was acquired approximately 4 min after an appropriate reduction in sample volume, addition of E1 enzyme and ATP, and thorough mixing of the sample. The spectral windows and number of complex data points used for the time-course analysis of thiolester formation were 6500 Hz, 512 (^{1}H) and 1500 Hz, 32 (^{15}N), with each acquisition taking 1.5 min, and were collected continuously for 80 min. Higher resolution spectra in the ^{15}N dimension, with 64 or 128 complex data points, were then acquired at approximately 80-min and 90-min reaction times, respectively. Identification of Ub-UBC1Δ450 thiolester as the sole product in the reaction conditions used for the NMR spectroscopy studies was confirmed by gel filtration chromatography, and the product was found to be stable for approximately 4 h at 30 °C.

Results and discussion

Using an *in vitro* conjugation system composed of purified yeast proteins (E1, Ub and UBC1Δ450), previous studies have demonstrated the ability to reconstitute multi-ubiquitin chain formation in the common Lys48 linkage, which is conjugated to Lys93 of UBC1 (Hodgins et al., 1996). In this work we have used a Ub(K48R) mutant, to remove the possibility of multi-ubiquitination chain formation, and a K93R substitution in UBC1Δ450, to limit the reaction to a covalent mono-ubiquitinated Ub-UBC1Δ450 thiolester, formed between C89 of UBC1Δ450 and G76 of Ub(K48R).

Assignment of Ub(K48R)

The analysis of the site(s) of interaction on Ub(K48R) when covalently bound with the catalytic domain of UBC1 (UBC1Δ450) in the thiolester complex required assignment of the ^{1}H-^{15}N HSQC spectrum of this protein. Previously reported NMR spectroscopy studies of Ub have been done with human ubiquitin, which differs from yeast Ub(K48R) by four amino acids (P19S, E24D, A28S, K48R). These differences resulted in chemical shift changes for several nearby residues making a direct comparison of chemical shift difficult. Furthermore, previous assignments were performed at pH 4.7, nearly 3 pH units lower than the current work, and resulted in significant pH-dependent chemical shift differences (for example, Wang et al., 1995). Nevertheless, the assignment of Ub(K48R) was straightforward using the three-dimensional sensitivity-enhanced HNCACB, ^{15}N-edited NOESY and ^{15}N-edited TOCSY experiments. The assigned ^{1}H-^{15}N HSQC spectrum for Ub(K48R) at pH 7.5 is represented in Figure 1. Backbone $^{1}H^{N}$, ^{15}N, $^{13}C\alpha$, $^{1}H\alpha$, as well as $^{13}C\beta$ and $^{1}H\beta$ assignments were made for all residues except M1, T9, S19, P37, P38, A46, R74, and G75 at pH 7.5. Residues M1, T9, S19, A46, R74, and G75 do not appear in the HSQC spectrum at pH 7.5 due to rapid exchange of the amide proton with the solvent. ^{1}H, ^{15}N and ^{13}C chemical shift assignment data for Ub(K48R) has been deposited in the BioMagRes databank under accession number BMRB 4769.

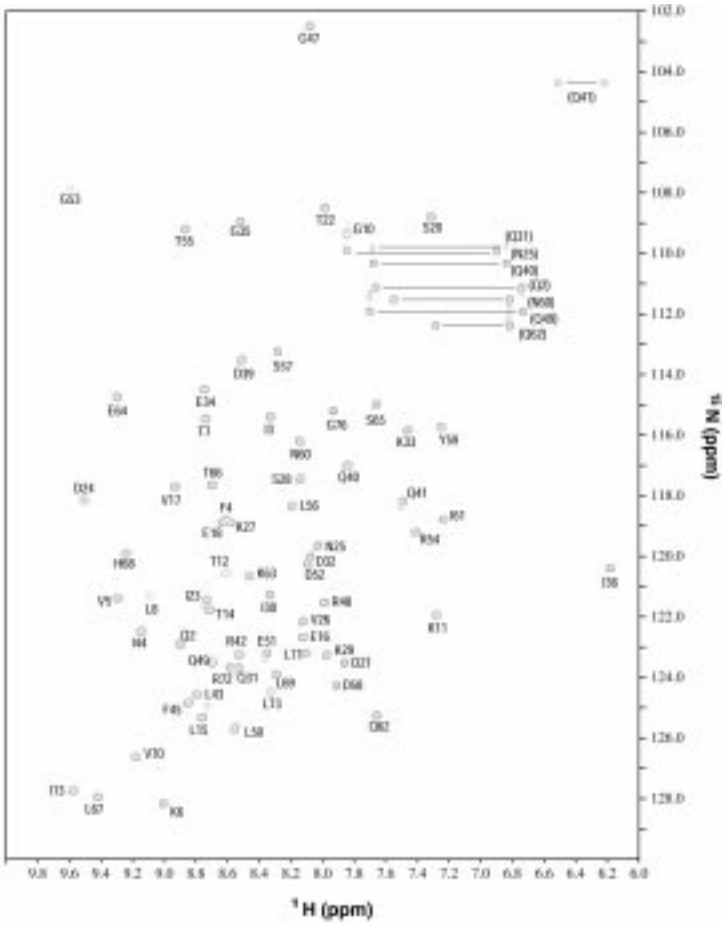

Figure 1. ^{1}H-^{15}N HSQC spectrum of Ub(K48R) in 40 mM HEPES, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, pH 7.5. Backbone amide cross peaks are labelled accordingly. Side-chain amide groups are labelled in parentheses.

Identification of interacting residues on Ub(K48R)

In studying the Ub-UBC1Δ450 thiolester, we chose to monitor formation of the thiolester intermediate of protein ubiquitination *in situ*. That is, to acquire ^{1}H-^{15}N HSQC spectra preceding complex formation, then rapidly throughout the reaction process and finally of the thiolester product. This required not only the UBC1Δ450 and Ub proteins but also the activating enzyme E1, ATP and $MgCl_2$ in order to catalyse the formation of the Ub-UBC1Δ450 thiolester. In the absence of these other co-factors, no interaction is observed between UBC1Δ450 and Ub. Figure 2 is a plot of representative residues of Ub(K48R), depicting changes in peak intensities as a function of reaction time and formation of the covalent thiolester. Of the 68 assigned residues in the ^{1}H-^{15}N HSQC spectrum of Ub(K48R), peak intensities for 60 residues could be measured from the spectra. As can be seen from this plot, the influence of thiolester formation on final peak intensity is not the same for all residues in Ub after a reaction time of 90 min. For example, the C-terminal residue, G76, where the thiolester is formed has a final peak intensity of about 0.1. Another residue such as E18, which is more remote from this region, has a peak intensity of approximately 0.33 in the thiolester complex. During the time course some variability was noted for most resonances in Ub(K48R). Nevertheless, each resonance followed a pseudo first order exponential decay (Figure 2) having an average $k_{obs} = 0.022 \pm 0.008$ min^{-1}. After 90 min all resonances maintained a constant peak intensity. The residual peak intensity of G76 (0.1) and measurement of the Ub-UBC1Δ450 complex by size exclusion chromatography indicated that the extent of the reaction was approximately 90%.

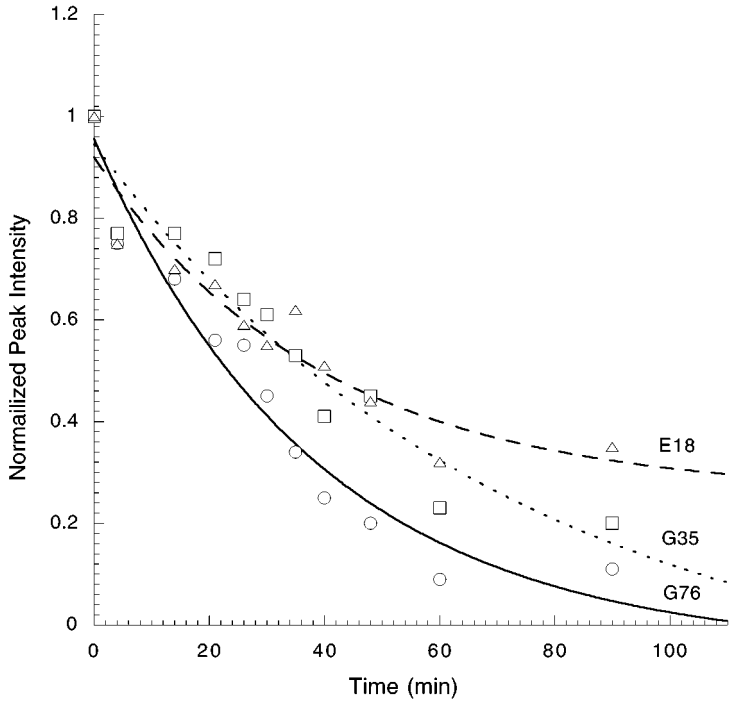

Figure 2. Time course measurement of peak intensities from ^{1}H-^{15}N HSQC spectra acquired over a 90-min reaction time period. Representative residues are shown to illustrate the three categories of final intensity ($\leq$ 0.1, 0.11–0.16, $\geq$ 0.17). Intensities plotted were normalized to the 0 time point values, which represent the peak intensities for the sample containing ^{15}N-Ub(K48R) and UBC1, without E1 and ATP. Each curve is shown fitted to a single exponential having a rate constant $k_{obs} = 0.022 \pm 0.008$ min^{-1}.

The results of this time course and the observation of differing final peak intensities between residues prompted the use of change in peak intensity upon thiolester formation as an indication of residues involved in the interaction between Ub and UBC1Δ450. This avoided relying on complete assignment of the thiolester spectrum and the potential problems due to small changes in experimental conditions, which were out of our control, in doing the reaction *in situ*. For example, ^{1}H-^{15}N HSQC spectra of Ub(K48R) were acquired at a series of pH conditions between 4.7 and 7.5 in an attempt to assign the Ub(K48R) species based on previously reported data on the human homologue. This data clearly indicated extreme chemical shift sensitivity for several peaks in the ^{1}H-^{15}N HSQC spectrum for Ub(K48R). Using the highly resolved ^{1}H-^{15}N HSQC spectra that were acquired before and after thiolester formation, intensities of the backbone amide cross peaks were measured. Normalized peak intensities after the reaction were obtained by comparison to a ^{1}H-^{15}N HSQC spectrum of ^{15}N-Ub(K48R) and UBC1Δ450, without E1, as shown in Figure 3. In this type of analysis, the reduction in peak intensity ratio from unity for all residues upon complex formation is expected since Ub(K48R) effectively undergoes a threefold increase in molecular weight; from 8.5 kDa to 25 kDa. Assuming that the complex behaves as a globular species, this would lead to a theoretical threefold increase in the individual linewidths in the spectra and, therefore, all peak intensity ratios would be expected to have an approximate value of 0.33. Residues that are at the protein-protein interface or especially those which moved from a flexible environment to the interface might be expected to be significantly lower than this. This may be especially true for residues near the extreme C-terminus of ubiquitin (L73-G76), which show order parameters significantly lower than 0.8 (Schneider et al., 1992). Using this method of analysis, residues were classified according to their peak intensity ratio values. Since the thiolester reaction proceeds to approximately a 90% yield, peaks with an intensity ratio of 0.1 or less are either those residues which have peaks that have changed significantly in chemical shift or have had a more dramatic increase in linewidth while chemical shift remains essentially unchanged. These residues are, therefore, classified

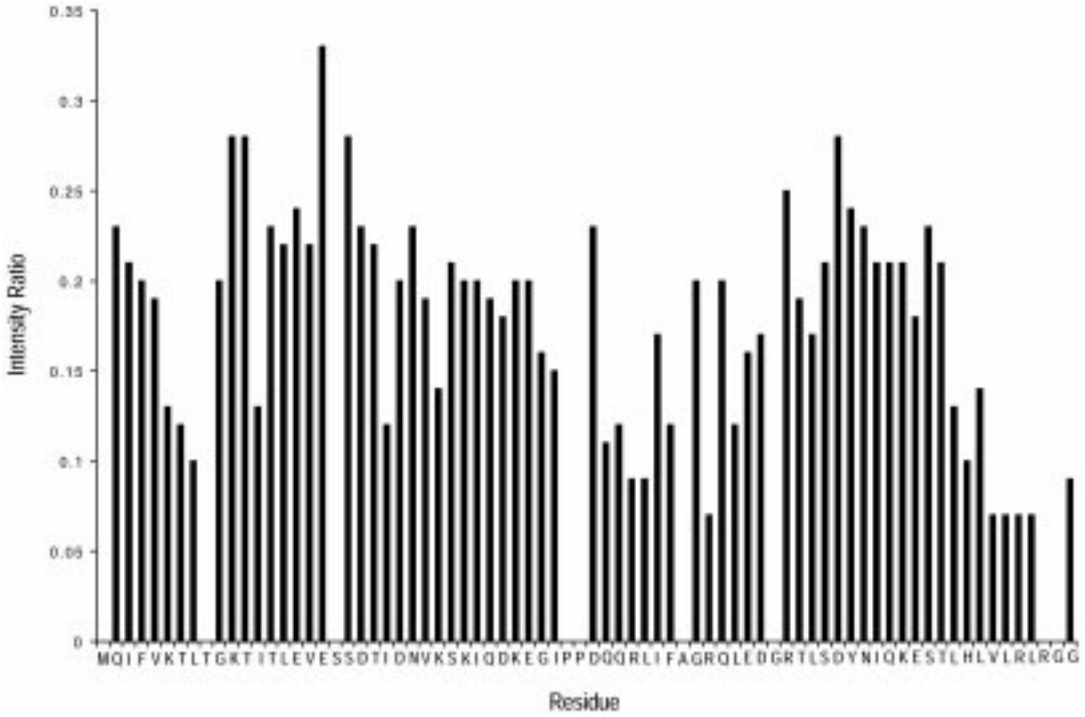

Figure 3. Histogram representing the normalized peak intensity ratios measured for assigned residues of Ub(K48R). Ratios were calculated using the peak intensity measurement of high-resolution spectra before and after thiolester formation (at 90 min). Residues which do not have a bar are those which are absent in the ^{1}H-^{15}N HSQC spectra.

as those which are most effected – presumably due to direct interactions or being very close to the site of interaction(s) in the complex. A second class are those which have decreased by greater than twofold from the theoretical ratio (0.33), that are between 0.1 and 0.16, and are presumed to be near the interface of the Ub(K48R)–UBC1Δ450 complex. All other characterised residues are placed into a third class, with peak intensity ratios greater than 0.16. This classification of residues is represented as a Connelly surface diagram of Ub(K48R) in Figure 4 (panels a and b). This depiction demonstrates that the most effected area on Ub is the C-terminal 'tail' extension (V70-G76), as well as R42, L43 and R48 of β-sheets 4 and 5, respectively, with intermediate effects on residues which surround this area. Interestingly, the residues in the C-terminal 'tail' of Ub have been shown to be flexible in the uncomplexed form of the protein (Schneider et al., 1992). Therefore, the extreme line broadening of these resonances likely corresponds to a decrease in flexibility of this region that accompanies interaction with UBCΔ450. Together, these residues form a cohesive surface in Ub(K48R) that presents a plausible interacting region for UBC1Δ450.

Peak intensity ratios vs. changes in chemical shift

One of the most common methods used for analysing ^{1}H-^{15}N HSQC spectra to identify residues that are involved in protein-protein or protein-ligand interactions is to measure the change in chemical shift of each of the backbone amide cross peaks in the spectra upon complex formation (Shuker et al., 1996). The correlation is made between extent of chemical shift change and participation in the interaction – i.e., residues that change the most are those most likely to participate directly in the interaction. For comparative purposes we also applied this method to our analysis of Ub(K48R) interactions with UBC1Δ450 in the thiolester complex. Since the thiolester complex is not stable for more than a few hours, reassignment of the ^{15}N-Ub spectrum when it is in complex with UBC1Δ450 could not be performed experimentally. Thus, for those peaks which had changed in chemical shift from the original, the nearest new peak in the thiolester ^{1}H-^{15}N HSQC spectrum to the original was assigned to that residue. In principle, this would represent only a minimum change in chemical shift since the actual peak could be farther away and could

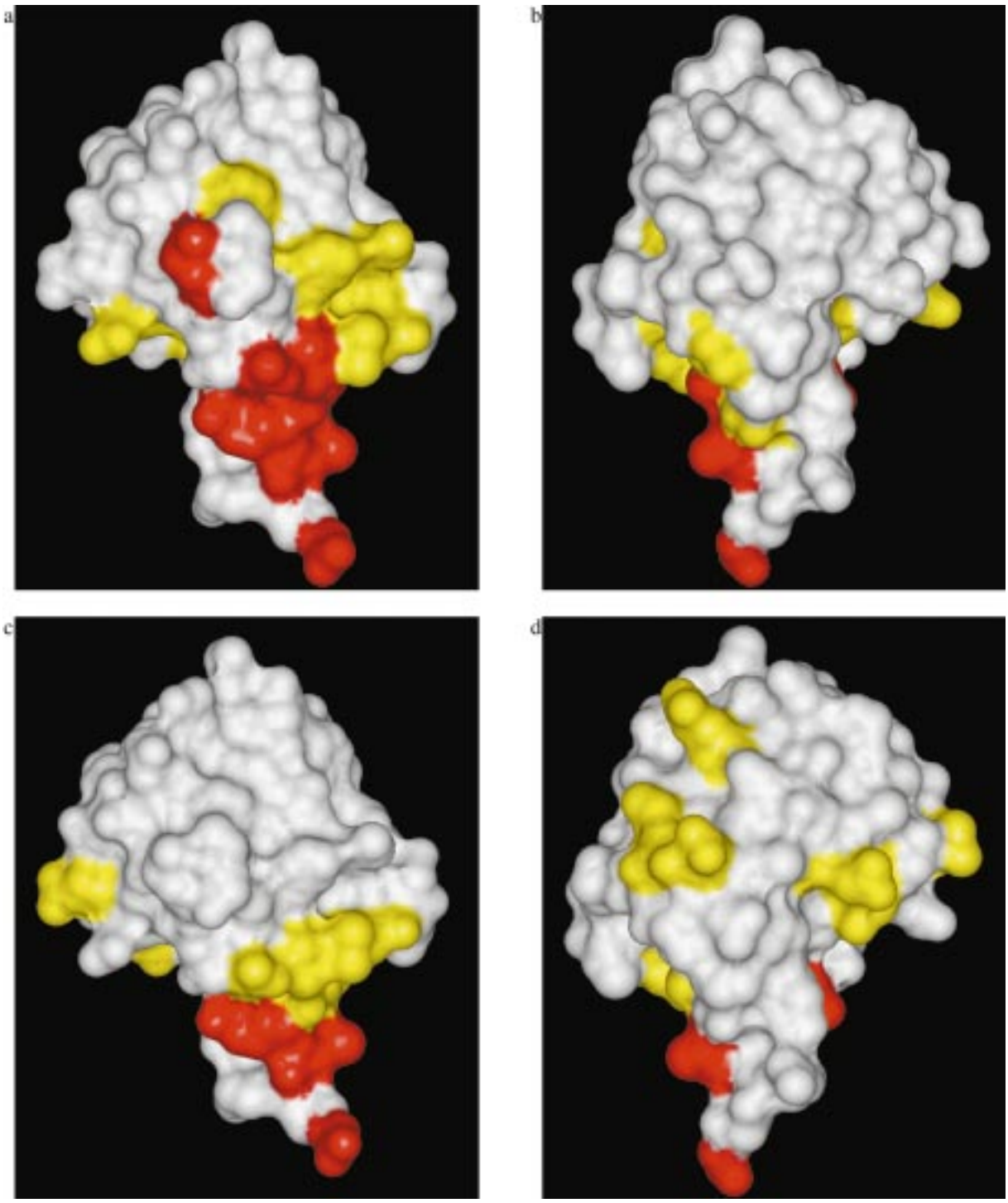

Figure 4. Residues effected by thiolester formation in Ub(K48R). In each case a Connelly surface of ubiquitin is shown, based on X-ray crystallographic coordinates (Vijay-Kumar et al., 1987) and generated using InsightII (MSI). (a, b) Residues in Ub(K48R) identified by peak intensity ratio analysis. Residues coloured red had a peak intensity ratio $\leq$ 0.1 (R42, L43, R48, V70-G76) while yellow residues had peak intensity ratios between 0.11–0.16 (K6, T7, L8, I13, K27, G35, I36, Q40, Q41, F45, L50, E51, L67, H68, L69). (c,d) Residues in Ub(K48R) identified by chemical shift change analysis. Residues coloured red had a shift change $\geq$ 0.1 ppm (R72, L73, G76), while yellow residues had a shift change of 0.05–0.09 (L8, E16, D24, D32, K33, R42, L43, D52, R54, L69, V70, L71) according to $\Delta\delta|^{1}\mathrm{H}| + 0.2(\Delta\delta|^{15}\mathrm{N}|)$ (Shuker et al., 1996). Panels a and c depict the same orientation of the molecule while (b) and (d) show the opposite side of the same molecule.

Table 1. ^{15}N-Ub(K48R) chemical shift changes in thiolester complex

Δ ppm*	Residues on Ub
>0.1	R72, L73, G76
0.05–0.09	L8, E16, D24, D32, K33, R42, L43, D52, R54, L69, V70, L71

*Calculated according to $\Delta\delta|^1H| + 0.2 \times \Delta\delta|^{15}N|$ (Shuker et al., 1996).

not be determined directly by assignment. Measurements were made for all backbone amide cross peaks before and after thiolester formation and changes in chemical shift values were calculated according to the method of Shuker et al. (1996). Residues were classified into (i) those that undergo significant chemical shift changes, (ii) those that undergo an intermediate chemical shift change and (iii) those with very little or no chemical shift change. Data for residues in categories (i) and (ii) is summarised in Table 1 and presented in Figure 4 (panels c and d) as a Connelly surface diagram. The most effected residues were R72, L73, and G76 of the C-terminal 'tail' on Ub. Also effected are the intermediary residues E16, (β sheet1), D24, D32, K33 (helix 1), L69, V70, L71 (β sheet 3), R42, L43 (β sheet 4), L8 (turn 1) and D52, R54 (turn 5). In comparing the residues effected by thiolester formation that were identified by both the chemical shift and peak intensity ratio methods, many additional residues were identified in the analysis by chemical shift change.

Due to the experimental protocol chosen to study thiolester formation, with sequential additions to the original sample, slight changes in experimental conditions such as pH and ionic strength were unavoidable. The finding that many residues of Ub(K48R) are highly sensitive to experimental conditions such as pH, could influence the interpretation of the analysis by the chemical shift method, especially for *in situ* protein complexes similar to Ub-UBC1Δ450. In fact, several effected residues that were identified by the chemical shift analysis were some of the residues that were highly sensitive to changes in pH (E16, D24, D32, K33, D52, L69, and L73). Perhaps as a result of this Figure 4 (panels c and d) indicates an extensive distribution of 'effected' residues on the surface of Ub, and does not provide as clear an indication of the site(s) of interaction as that determined by peak intensity measurements (Figures 4a and 4b). In addition, R48, one of the largest affected residues in terms of peak intensity change is not evident.

Conclusions

The ^{1}H-^{15}N HSQC experiment is one of the most sensitive NMR techniques for studying proteins and, provided that good spectral resolution is achieved, the ideal method for analysing protein-protein interactions. An essential component to this type of analysis is complete assignment of the ^{1}H-^{15}N HSQC spectrum of the protein under study. Although the human ubiquitin protein has been previously assigned, the inherent sensitivity of this technique necessitated *de novo* assignment of the Ub(K48R) protein used for these studies by three-dimensional NMR experiments. The four amino acid difference between human and Ub(K48R) not only resulted in four completely new peaks in its spectrum, but several residues surrounding these amino acids underwent a significant change in chemical shift. Also, the large difference in pH conditions from previous work (pH 4.7 vs. 7.5) resulted in significant changes in peak chemical shift.

In order to identify residues in Ub(K48R) that are involved in the association with the catalytic domain of UBC1 in the thiolester complex, we chose to monitor peak intensity changes upon reaction. By using this method, we were able to build an image of the interacting face on Ub without concern for reassigning the ^{1}H-^{15}N HSQC of the complex or the extreme sensitivity of chemical shift to small changes in experimental conditions that are unavoidable when synthesising the thiolester *in situ*. By comparison, performing the analysis by measuring changes in peak chemical shift upon complex formation resulted in a more confusing prediction, with several identified residues being scattered over the surface of Ub. It is interesting to note that two other studies with ubiquitin-related proteins have used measurement of chemical shift change to define the interacting surfaces (Miura et al., 1999; Liu et al., 1999). However, in at least one case it was not possible to use this surface to dock the Ub and E2 proteins (Miura et al., 1999) to see how the two surfaces might interact. In contrast, we have recently used our peak intensity data to dock the Ub and UBC1Δ450 proteins and provide the first model of a Ub-E2 covalent complex (K.S.H., personal communication). This may indicate that for larger protein complexes measurement of changes in peak intensity provides a viable and facile method to map an inter-

acting surface and may provide a clearer picture of the interacting surfaces between two proteins.

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